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Dissecting Cellulase Hypersecretion in *Neurospora crassa*

By

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ABSTRACT

Dissecting Cellulase Hypersecretion in *Neurospora crassa*

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Fungi are the major decomposers of lignocellulosic material in the natural world, and our ability to harness the enzymes that fungi use for degrading plant biomass in their environment have revolutionized the food, fuel, textile and pharmaceutical industries. These enzymes are used to alter naturally occurring polymers from plant materials into everyday products like corn starch based sweeteners, renewable cellulosic biofuels, better performing clothing detergents, or pharmaceuticals. What makes many of these industries feasible is the high capacity of secretion that is inherent to many filamentous fungal organisms. While yeast strains are able to secrete up to 3 g/L of human serum albumin, filamentous hosts have been shown to secrete upwards of 100g/L of cellulase proteins into the supernatant. Thus, many of these industries are fundamentally interested in generating a high producing strain of their protein of interest, and generating a highly productive filamentous fungal host could provide a potential source for these secreted proteins.

Unfortunately, the efficient production of well folded and functional protein becomes the major cost limitation of many of these industries. While the essential components of the secretory pathway in fungi are well studied, much less is publicly known about how to engineer a filamentous fungal host for more efficient protein production. There are many reasons for this: (1) known hypersecreting strains have poor genetic amenability, (2) these strains have very few established tools for determining causal alleles and (3), the intellectual property culture that revolves around many of these industrial strains prevents the dissemination of much of this knowledge.

In order to address our knowledge gap in filamentous fungal hyper-production, we perform five tasks in this work, (1) we establish *Neurospora crassa* as a filamentous host for protein production through an iterative mutagenesis process using a quantitative screen to select for the highest cellulase hypersecreters, (2) we use bulk segregant DNA pooling and next-gen sequencing to determine the causative locus, (3) we identify the functional knockout of a conserved flippase as one of the reasons for transcriptionally-independent hypersecretion phenotype, (4), we characterize the flippase mutant for mechanistic alterations to the secretion

pathway that may be contributing to hypersecretion, and (5) we determine functional signal peptides for the initial step of secretion of a heterologously produced protein.

Finally, we propose further lines of inquiry using the established tools for studying cellulase secretion and regulation in a model filamentous fungal host. This may lead to the identification of additional conserved alleles for future engineering studies of protein expression. This work takes both a discovery-based and a directed approach to study fungal hypersecretion with the ultimate goal of understanding the biology of protein secretion in a filamentous fungus. This may uncover new strategies to rationally engineer fungal organisms for more efficient protein production that may significantly benefit society by the development carbon neutral fuels and more sustainable industrial processes.

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CHAPTER 1: INTRODUCTION

1.1 Context and Motivation

Lignocellulose is an abundant waste product of many agricultural and processing industries that can be utilized for the production of renewable, carbon-neutral cellulosic fuel substitutes for petroleum based fuels. One of the major bottlenecks in the economic feasibility of cellulosic fuels is the cost efficiency of converting complex carbohydrate polymers found in lignocellulose into soluble sugars for fermentation. Market studies have shown that 25 cents of every dollar of cellulosic bioethanol goes to the production of the enzymes that are used to degrade lignocellulose (Humbird, Davis et al. 2011). Saprophytic filamentous fungi are capable of degrading the carbohydrate polymers in the plant cell wall, including cellulose, hemicellulose, and pectin, through the secretion of glycosyl hydrolases and other carbohydrate active enzymes (CAZymes).

The current industrial standard of cellulase production comes from a mutagenized strain of the soft mold, *Trichoderma reesei*. Originally isolated from the Solomon Islands off of canvas tents in the 1950's, *T. reesei* was later realized to be a prolific source of cellulases in the 1970's through mutagenesis studies (Mandels and Reese 1957, Mandels, Weber et al. 1971) Only in the last couple decades, however, has the mechanism of lignocellulolytic gene regulation been revealed. While there are a number of pathways involved in CAZyme regulation, we focus on four broad cellular mechanisms: direct transcriptional regulation, upstream regulation by nutrient sensing pathways, regulatory feedback from the secretory pathway, (Fig 1.1) and downstream secretion of enzymes. The expression of genes encoding CAZymes important for plant biomass deconstruction is directly regulated by transcription factors that activate transcription when cells are exposed to carbohydrates found in plant cell walls. General nutrient sensing pathways that prioritize the use of preferred carbon sources act upstream of the direct activation of genes encoding CAZymes to inhibit the energy intensive production of plant biomass degrading enzymes. Feedback from downstream factors, such as the fungal secretory system, also acts to regulate the production/secretion of these enzymes.

1.2 Direct transcriptional regulation of genes encoding lignocellulolytic enzymes

Saprophytic filamentous fungi have evolved to degrade lignocellulose through the expression and secretion of plant cell wall degrading enzymes. In order to utilize the available nutrients in the most efficient manner, filamentous fungi only express these CAZymes when plant cell wall material is available in the absence of preferred carbon sources. The transcriptional induction of plant cell wall degrading enzymes is tightly controlled by transcription factors that are specifically activated by the presence of plant cell wall material

1.2.1 Primary transcription factor

In fungi, the zinc binuclear cluster class of transcription factors is often involved in the regulation of nutrient sensing pathways. The most prominently studied member of this class is the transcriptional regulator of the galactose utilization pathway in *Saccharomyces cerevisiae*, Gal4 (MacPherson 2006). Like the *S. cerevisiae* galactose utilization pathway, primary transcription of genes encoding the major lignocellulolytic enzymes in many species of filamentous fungi is promoted by a zinc binuclear cluster transcription factor. In the industrially important species, *T. reesei* and *Aspergillus niger*, the major transcriptional regulator of cellulose and hemicellulose degrading enzymes is XYR1/XlnR (van Peij, Visser et al. 1998, van Peij, Gielkens et al. 1998, Rauscher, Wurleitner et al. 2006). However, in *Neurospora crassa* and *Aspergillus nidulans*, primary transcription of genes encoding cellulases and hemicellulases has been decoupled. In *N. crassa*, the transcription factor CLR-2 drives expression of genes required for cellulose degradation, and the orthologous protein, ClrB, modulates the expression of cellulolytic enzymes in *A. nidulans* (Coradetti, Craig et al. 2012, Coradetti, Xiong et al. 2013) Hemicellulose degradation is regulated by XLR-1 and XlnR, the *N. crassa* and *A. nidulans* orthologues of XYR1, respectively (Sun, Tian et al. 2012, Klaubauf, Narang et al. 2014) (Tamayo, Villanueva et al. 2008). In species such as *Penicillium oxalicum*, cellulase and hemicellulase gene regulation appears only partially decoupled, with roles for both ClrB and XlnR in cellulase regulation (Li, Yao et al. 2015)(Fig 1.1 and Table 1.1).

Pectin is a far more heterogeneous component of lignocellulosic biomass than either cellulose or hemicellulose, and this may account for the lack of a single transcription factor regulating pectin utilization (Mohnen 2008). Several conserved zinc binuclear cluster transcription factors required for the regulation of genes encoding enzymes important for the transport and utilization of the component saccharides have been identified. In *Botrytis cinerea* and *A. niger*, the utilization of galacturonic acid, the most abundant pectin component, is regulated by GaaR (Alazi, Niu et al. 2016, Zhang, Lubbers et al. 2016), while in *A. niger* and *A. nidulans*, the utilization of rhamnose and arabinose, two minority pectin components, is regulated by RhaR and AraR, respectively (Battaglia, Hansen et al. 2011, Battaglia, Visser et al. 2011, Gruben, Zhou et al. 2014, Pardo and Orejas 2014) (Fig 1.1 and Table 1.1). The identification of additional transcription factors may be necessary to fully elucidate the regulation of pectin utilization.

In *T. reesei*, over-expression of XYR1 increases the transcription of downstream targets, but does not activate the full hemicellulolytic response (Mach-Aigner, Pucher et al. 2008). Thus, XYR1/XlnR/XLR-1 requires activation/de-repression prior to the induction of (hemi)cellulase gene transcription. Activation/de-repression can occur through a single, conserved amino acid substitution in XYR1/XLR-1 that is sufficient for constitutive expression of the major cellulases and hemicellulases in *T. reesei* and for constitutive expression of hemicellulase genes in *N. crassa*. The conserved amino acid lies inside a putative domain of unknown function found in Gal4-like zinc binuclear cluster transcription factors (Derntl, Gudynaite-Savitch et al. 2013, Craig, Coradetti et al. 2015). How this region regulates function of XYR1/XLR-1 is not clear, nor is the mechanism of activation/de-repression of XYR1/XLR-1 understood.

Aberrant expression of *N. crassa* CLR-2 through a constitutive promoter drives a nearly complete cellulase transcriptional response, even during growth on a normally repressive carbon source, indicating that CLR-2 does not require post-translational activation (Coradetti, Xiong et al. 2013). This is not the case for other CLR-2 orthologs, however, since constitutive expression

of *A. nidulans* and *P. oxalicum* ClrB does not result in constitutive activation of cellulolytic genes (Coradetti, Xiong et al. 2013, Li, Yao et al. 2015). In *N. crassa*, regulation of *clr-2* transcription is governed by a second zinc binuclear cluster transcription factor, CLR-1

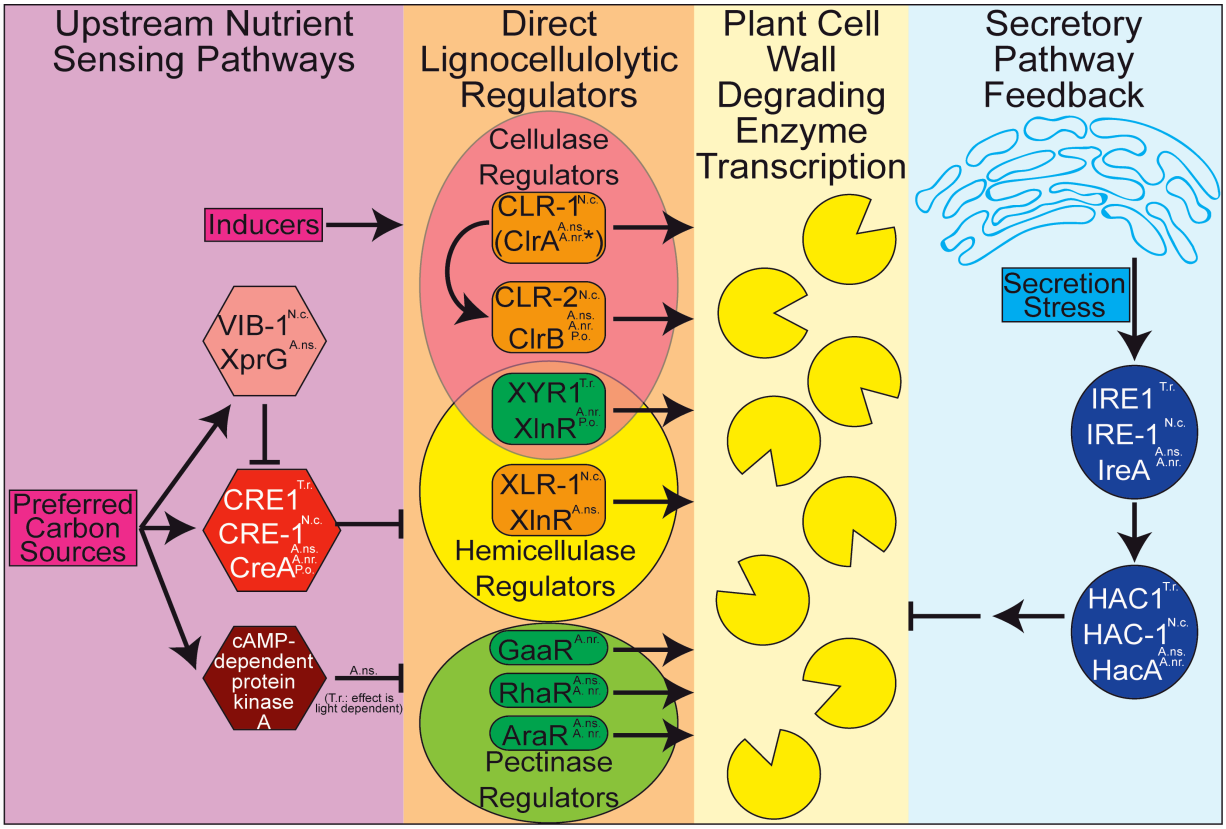

Figure 1. 1 Filamentous fungal transcription regulation
Transcriptional control of lignocellulolytic CAZymes occurs through direct regulators, upstream nutrient sensing pathways, and downstream secretory pathway feedback. Upstream nutrient sensing pathways: Cells prioritize the utilization of preferred carbon sources over plant biomass through the transcription factor CRE1/CRE-1/CreA, which is regulated in part by VIB-1/XprG. When activated, CRE1/CRE-1/CreA represses transcription of both direct lignocellulolytic regulators and plant cell wall degrading enzymes. The cAMP-dependent protein kinase A (PKA) also plays a role in nutrient sensing, although its effect is species dependent. Inducers derived from the carbohydrates in plant cell walls activate lignocellulolytic regulators. Direct lignocellulolytic regulators: In *T. reesei*, *A. niger*, and *P. oxalicum*, XYR1/XlnR activates transcription of both cellulase and hemicellulase genes, while in *N. crassa* and *A. nidulans*, XLR-1/XlnR only regulates transcription of hemicellulase genes. Cellulase gene transcription in *N. crassa*, *A. nidulans*, and *A. niger* is regulated by CLR-1/ClrA and CLR-2/ClrB. In *N. crassa*, CLR-1 activates transcription of some genes necessary for cellulose utilization as well as the main transcriptional activator of cellulase transcription, CLR-2. The CLR-2 homolog, ClrB is involved in cellulase transcription in *A. nidulans*, *A. niger*, and *P. oxalicum*. ClrA appears to play a small role in the regulation of cellulase transcription in *A. nidulans* and *A. niger*, although it does not control ClrB transcription. Pectin utilization is regulated by several transcription factors, which each regulate the utilization of individual pectin components: GaaR regulates galacturonic acid utilization, RhaR regulates rhamnose utilization, and AraR regulates arabinose utilization. Secretory pathway feedback: Secretion stress activates IRE1/IRE-1/IreA, which cleaves a non-canonical intron from the transcription factor *hac1/hac-1/hacA* allowing for its activation. HAC1/HAC-1/HacA activates the unfolded protein response, which may cause downregulation of the transcription of plant cell wall degrading enzymes. (*A.nr.* = *Aspergillus niger*, *A.ns.* = *Aspergillus nidulans*, *N.c.* = *Neurospora crassa*, *P.o.* = *Penicillium oxalicum*, *T.r.* = *Trichoderma reesei*).

(Coradetti, Craig et al. 2012). The direct transcriptional targets of CLR-1 also include genes necessary for the breakdown of cellulose and import of soluble products (Craig, Coradetti et al. 2015). Together, CLR-1 and CLR-2 transcriptionally activate genes that make up the full cellulolytic response. However, unlike *clr-2*, constitutive expression of *clr-1* does not cause expression of target genes, although CLR-1 binds cis-regulatory elements even under noninducing conditions (Coradetti, Craig et al. 2012, Craig, Coradetti et al. 2015).

These data indicate that CLR-1 also requires activation/derepression. Elucidation of the molecular mechanism by which CLR-1 and XYR1/XlnR/XLR-1 are activated/de-repressed will be an important step in understanding transcriptional regulation of lignocellulolytic genes. Although XYR1/XlnR/XLR-1 regulate different target genes in different fungi, the binding motifs of XYR1/XlnR in *T. reesei* and *A. niger* show conservation with those of *N. crassa* XLR-1. These three species have a conserved 5'-GGCTWWW-3' consensus sequence in the cis regulatory elements of the target genes (van Peij, Visser et al. 1998, Zeilinger, Mach et al. 1998, Furukawa, Shida et al. 2009, Craig, Coradetti et al. 2015). The promiscuity of regulons amongst homologous transcription factors is further reflected as *N. crassa* CLR-2 has a consensus sequence nearly identical with its closest yeast paralogue, Gal4, but without a clear function in the *N. crassa* galactose utilization pathway (Bram and Kornberg 1985, Giniger, Varnum et al. 1985, Craig, Coradetti et al. 2015). The evolution of CLR-2/ ClrB and XYR1/XlnR/XLR-1 as the major transcription factors of CAZymes is poorly understood and may represent a functional shift in environmental niches from an ancestral lifestyle.

1.2.2 Accessory transcription factors

Two accessory transcription factors in *T. reesei*, ACE1 and ACE2, were originally identified from a yeast one-hybrid screen that promoted activation of a selectable marker tethered to the promoter of the cellulase gene, cbh1 (Saloheimo, Aro et al. 2000). While both ACE1 and ACE2 promote transcription of genes driven by the cbh1 promoter in *S. cerevisiae*, the two transcription factors appear to have opposing roles in the regulation of cellulase transcription in vivo. *T. reesei* cells lacking ace2 had no change in the expression of cellulases on sophorose (a gratuitous inducer of cellulases in *T. reesei* (Mandels, Parrish et al. 1962, Sternberg and Mandels 1979), but decreased cellulase expression after growth on cellulose (Aro, Saloheimo et al. 2001). Conversely, cells lacking ace1 showed an increase in the transcription of the major cellulases, implying a negative regulatory role for ACE1 (Aro, Ilmen et al. 2003). A third *T. reesei* accessory transcription factor, ACE3, which plays a positive role in CAZyme regulation, was identified by mining transcriptome data for transcription factors that appear to be co-regulated with genes involved with lignocellulose utilization.

Several other transcription factors, including the *T. reesei clr-2* homolog, also appear to be co-regulated with genes encoding CAZymes or sugar transporters and could be targets for future investigation (Hakkinen, Valkonen et al. 2014). Studies on the promoter region of the *T. reesei* cellulase gene *cbh2* identified an 11 base pair region necessary for transcriptional activation, termed the *cbh2* activating element, or CAE. Characterization of this region showed a multi transcription factor binding arrangement that led to the identification of a potential role for the HAP2/3/5 complex in cellulase regulation, as well as possible binding sites for XYR1 and ACE2 (Zeilinger, Mach et al. 1998, Zeilinger, Ebner et al. 2001). In *S. cerevisiae*, the HAP complex

functions in glucose-repressible transcriptional activation (Guarente, Lalonde et al. 1984). A study investigating nucleosome positioning at the *cbh2* regulatory region also implicated the shifting of nucleosome complexes in the promotion of *cbh2* transcription (Zeilinger, Schmoll et al. 2003).

There is some evidence for a role for accessory transcription factors in other fungal species. The *A. niger clr-1* and *clr-2* homologs, *clrA* and *clrB*, have been implicated in lignocellulase gene regulation (Raulo, Kokolski et al. 2016), and a basic leucine zipper transcription factor, ClrC, modulates cellulase expression in *P. oxalicum* (Lei, Liu et al. 2016). Homologs of *ace1* are present in the genomes of *N. crassa*, *A. nidulans*, *A. niger*, and *P. oxalicum*, although these species lack clear homologs of *ace2* and only *P. oxalicum* has a close *ace3* homolog. However, a role for homologs of *ace1/ace2/ace3* in regulating CAZyme gene transcription has not been established in these other species. Similarly, while clear homologs of *P. oxalicum* ClrC exist in *A. nidulans* and *A. niger*, these homologs have not been implicated in the lignocellulolytic response. In *N. crassa*, chromatin immunoprecipitation high throughput sequencing (ChIP-seq) of sequences bound by CLR-1, CLR-2, and XLR-1 identified several uncharacterized transcription factors, potentially involved in modulating the enzyme response to plant cell wall carbohydrates (Craig, Coradetti et al. 2015).

1.3 Upstream nutrient sensing pathways regulate lignocellulase transcription

While direct transcriptional induction of the genes involved in plant cell wall degradation is the first step of enzyme production, there are many competing signals that control whether these enzymes will be produced. One of these mechanisms, known as carbon catabolite repression (CCR), involves the down regulation of genes necessary for consumption of a less preferred carbon source when a more preferred carbon source is present.

1.3.1 CreA/CRE1/CRE-1-mediated carbon catabolite repression

In filamentous fungi, CCR is regulated by the zinc binuclear cluster transcription factor, CreA/CRE1/CRE-1. CreA was first identified in *A. nidulans* (Bailey and Arst 1975, Arst Jr and Bailey 1977, Arst, Tollervey et al. 1990) and later homologs were identified in many other filamentous fungi, including *N. crassa* and *T. reesei* (Ilmen, Thrane et al. 1996, de la Serna, Ng et al. 1999). In *T. reesei*, a *cre1* truncation mutant was identified after selection for CCR mutants in an effort to generate cellulase hyper-secreting strains (Ilmen, Thrane et al. 1996). The regulation of CreA/CRE1/CRE-1 appears to be at least partially dependent on intracellular localization. Under repressing conditions, CreA/CRE1/CRE-1 is found in the nucleus where it can regulate the expression of target genes, and when under de-repressing conditions, CreA/CRE1/CRE-1 exits the nucleus (Brown, de Gouvea et al. 2013, Lichius, Seidl-Seiboth et al. 2014, Cupertino, Virgilio et al. 2015). However, the genetic regulation of CreA/CRE1/CRE-1 appears to be fairly divergent amongst the filamentous fungi. In *A. nidulans*, the AMP-activated kinase, SnfA, is necessary for nuclear export of CreA (Brown, de Gouvea et al. 2013), but in *T. reesei*, while phosphorylation of CRE1 is necessary for de-repression of target genes, this phosphorylation is not dependent on the SnfA homolog (Cziferszky, Mach et al. 2002, Cziferszky, Seiboth et al. 2003).

Although CCR has primarily been thought of as glucose related repression, in organisms capable of consuming a wide variety of carbon sources of varying complexity, like saprophytic filamentous fungi, CCR has expanded to include a range of preferred carbon sources. During the utilization of plant cell wall carbohydrates, CCR can be activated not only by exogenously added sugars, but also by breakdown products resulting from plant cell wall degradation. For example, in *A. nidulans*, xylose is an inducer of xylanase genes, but this response to xylose can be severely curtailed by the concurrent presence of glucose in the media (Piñaga, Fernández-Espinar et al. 1994, Orejas, MacCabe et al. 1999, Orejas, MacCabe et al. 2001). Cells containing mutations in CreA have substantial transcription of xylanase genes even in the presence of glucose. These mutations in CreA also vastly increase the amount of xylanases produced in the presence of xylose as a sole carbon source. Presumably, xylose acts not only as an inducer of xylanase gene expression through the transcriptional activator XlnR, but also as a repressor of xylanase gene expression through the transcriptional repressor CreA (Piñaga, Fernández-Espinar et al. 1994, Orejas, MacCabe et al. 1999, Orejas, MacCabe et al. 2001, Tamayo, Villanueva et al. 2008). In *N. crassa*, deletion of *cre-1* causes an increase in cellulase production in strains grown on crystalline cellulose, presumably due to repression caused by the glucose, cellobiose, and other cellulose breakdown molecules released by the secreted cellulases (Sun and Glass 2011).

Studies on the transcriptional response to CCR have elucidated that the mechanism of CreA/CRE1/CRE-1 repression works through regulation of both the genes necessary for degradation and consumption of plant cell wall material as well as the transcription factors which control their expression (Fig 1.1 and Table 1.1). In *A. nidulans*, CreA regulates xylanase genes as well as expression of their transcriptional regulator, *xlnR* (Orejas, MacCabe et al. 1999, Orejas, MacCabe et al. 2001, Tamayo, Villanueva et al. 2008). In *N. crassa* and *P. oxalicum*, regulation of cellulolytic genes as well as the transcription factor *clr-2/clrB* is thought to be under CCR (Sun and Glass 2011, Coradetti, Craig et al. 2012, Xiong, Sun et al. 2014, Li, Yao et al. 2015). Like in *N. crassa* and *A. nidulans*, in *T. reesei*, CRE1regulates genes necessary for utilization of plant biomass as well as the transcription factors which regulate their expression. Interestingly, expression of the primary regulator of cellulase genes, *xyr1*, appears to be repressed by CRE1when glucose is available, while also requiring CRE1 for full expression under some inducing conditions (Portnoy, Margeot et al. 2011, Antonieto, dos Santos Castro et al. 2014). As transcriptomics and proteomics become an increasingly commonplace tool in biological investigations, additional mechanisms of CreA/CRE1/CRE-1-mediated CCR as well as the specific response of cells to soluble carbohydrates released from plant biomass will likely be revealed.

1.3.2 Other factors involved in carbon catabolite repression

Although CreA/CRE1/CRE-1 is an important player in CCR, other proteins also regulate CCR and the expression of genes encoding plant cell wall degrading enzymes (Fig 1). The cAMP dependent protein kinase A (PKA) is a highly conserved protein kinase involved in the sensing of sugar and the regulation of metabolism, which often plays an opposing role to the AMP kinase, SnfA/SNF1 (Thompson-Jaeger, Francois et al. 1991, Santangelo 2006, Barrett, Orlova et al. 2012). In *T. reesei*, a homolog of a PKA catalytic subunit, *pkac1*, regulates cellulase gene expression, most likely through the regulation of upstream transcription factors, such as *xyr1*, and may also mediate interactions between cellulase production and the circadian clock

(Schuster, Tisch et al. 2012). In *A. nidulans*, a PKA catalytic subunit, PkaA affects cellulase production and CCR. When grown on crystalline cellulose, cells lacking *pkaA* express glycosyl hydrolases, as well as their upstream regulators *xlnR* and *clrB*, at higher levels earlier in the cellulose response. Cells lacking *pkaA* are partially glucose-blind, allowing cellulase production under conditions where CCR is active in wild type cells (de Assis, Ries et al. 2015).

Table 1. 1: Orthologs of transcription factors of discussed filamentous fungi

Regulated by	*N. crassa* OR74A	*A. nidulans* FGSC A4	*A. niger* CBS 513.88	*T. reesei* QM6a	*P. oxalicum* 114-2	Role
Cellulose	*ace1*	*sltA*	An16g02040	***ace1***	*aceA*	Negative regulator of
	NCU09333	AN2919		**Trire2_75418**	PDE_01988	cellulase transcription
	N/A	N/A	N/A	***ace2***	N/A	Positive regulator of
				Trire2_78445		cellulase transcription
	N/A	N/A	N/A	***ace3***	PDE_09536	
				Trire2_77513		
	clr-1	***clrA***	***clrA***	N/A	PDE_04046	
	NCU07705	AN5808	An05g00020			
	clr-2	***clrB***	***clrB***	Trire2_26163	***clrB***	
	NCU08042	AN3369	An12g01870		**PDE_05999**	
	NCU04058	AN4324	An04g00480	Trire2_65315	***clrC***	
					PDE_09023	
Cellulose and hemicellulose			***xlnR***[a]	***xyr1***[a]	***xlnR***[a]	Positive regulator of
			An15g05810	**Trire2_122208**	**PDE_07674**	cellulase and hemicellulase transcription
Hemicellulose	***xlr-1***[b]	***xlnR***[b]				Positive regulator of
	NCU06971	AN7610				hemicellulase transcription
Pectin	NCU09033	***rhaR***	***rhaR***	Trire2_79871	PDE_00828	Positive regulator of
		AN5673	An13g00910			rhamnose utilization
	NCU04295	AN10548	***gaaR***	Trire2_67339	PDE_06088	Positive regulator of
			An04g00780			galacturonic acid utilization
	N/A	***araR***	***araR***	N/A	PDE_04461	Positive regulator of
		AN0388	An04g08600			arabinose utilization
Preferred carbon source	***cre-1***	***creA***	***creA***	***cre1***	***creA***	Negative regulator of
	NCU08807	AN6195	An02g03830	**Trire2_120117**	**PDE_03168**	lignocellulase transcription
ER stress	***hac-1***	***hacA***	***hacA***	***hac1***	PDE_01436	Positive regulator of
	NCU01856	AN9397	An01g00160	**Trire2_46902**		unfolded protein response

Phenotypic evidence exists for genes in bold.
a Orthologs regulated by cellulose and hemicellulose.
b Orthologs regulated by hemicellulose.

Another actor in the glucose response, which may be regulated by PKA, is a transcription factor involved in the regulation of extracellular proteases in response to carbon and nitrogen starvation, *vib-1/xprG* (Katz, Flynn et al. 1996, Dementhon, Iyer et al. 2006, Katz, Gray et al. 2006, de Assis, Ries et al. 2015). In *N. crassa*, VIB-1 is necessary for full cellulase and hemicellulase induction and appears to be an upstream regulator of genes involved in CCR, including cre-1 and the *N. crassa* homologs of two genes with potential roles in the ubiquitination pathway, *creB* and *creD* (Hynes and Kelly 1977, Kelly and Hynes 1977, Lockington and Kelly 2001, Boase and Kelly 2004, Xiong, Sun et al. 2014). VIB-1 also regulates the expression of col-26, the *N. crassa* homolog of *T. reesei bglR*, a zinc binuclear cluster transcription factor that positively regulates glycosyl hydrolase expression (Nitta, Furukawa et al. 2012, Xiong, Sun et al. 2014).

From the above studies, it is clear that there are unidentified genes/proteins involved in CCR, and the mechanism of action of many identified genes, such as *col-26/bglR*, require additional

elucidation. Although there are several highly conserved nutrient-sensing pathways that have been implicated in the lignocellulosic response, their role in the regulation of plant cell wall degrading enzyme transcription is still unclear. For example, the TOR complex is involved in nutrient sensing in eukaryotes and has been shown to be highly phosphorylated under cellulolytic conditions in *N. crassa* (Wullschleger, Loewith et al. 2006) (Xiong, Coradetti et al. 2014). Putative ubiquitination and deubiquitination enzymes, such as CreB/CRE-2 and CreD, have been implicated in CCR and glycosyl hydrolase production potentially through ubiquitination of CreA itself, however, additional protein targets and the precise mechanism of action of the genes involved has yet to be identified (Lockington and Kelly 2001, Lockington and Kelly 2002, Boase and Kelly 2004, Denton and Kelly 2011, Colabardini, Humanes et al. 2012, Xiong, Sun et al. 2014, Ries, Beattie et al. 2016). The elucidation of the role of these and other highly conserved actors like the AMP-activated kinase, SnfA/SNF1, in the repression of plant cell wall degrading enzymes could help illuminate the regulation of transcription under cellulolytic conditions as well as nutrient-sensing pathways in eukaryotic species.

1.4 Secretion of Cellulases

The secretory system has been well-studied in *S. cerevisiae* and many components have clear orthologues in filamentous fungi. Cellulases, while absent from *S. cerevisiae*, presumably undergo a similar process of import into the ER, modification and sorting to become proper functioning proteins in the external environment. Post-transcriptional regulation of cellulase secretion is less well understood in filamentous fungi, as the mechanisms for protein secretion is highly complex and undergo many steps of protein modification and sorting en route to the external environment.

1.4.1 ER import

Protein secretion begins at the stage of protein transport into the ER. Cellulase proteins that are being imported must cross the biological membrane of the ER. The current understanding of protein import starts with the direct translation of protein across the ER membrane into the lumen for proper folding (Ast, Cohen et al. 2013, Aviram and Schuldiner 2014). Protein import is encoded within the protein itself by the N-terminal peptide sequence. All cellulases that are secreted by eukaryotic organisms have a canonical secretion sequence that is encoded by the N-terminus of the peptide. Secretion of cellulases requires the delivery of the nascent peptides to the ER and for the signal sequence to be cleaved.

Cellulase mRNA is transcribed and translation is initiated in the cytosol of the cell. Once the ribosome translates the signal sequence and 30-35 amino acids further(Pechmann, Chartron et al. 2014), the signal sequence, comprising of either a signal peptide or a transmembrane domain extends out of the ribosome exit tunnel. The signal sequence forms a hydrophobic helix that is recognized by the signal recognition particle (SRP)(Walter and Blobel 1981), a protein complex made up of 6 proteins and a hinge RNA backbone (Walter and Blobel 1982). Once the SRP is recognized and bound to the signal peptide helix, the SRP changes conformation to bind to an allosteric inhibition site on the ribosome to halt translation (Walter and Blobel 1981, Chartron, Hunt et al. 2016). The SRP-ribosomal mRNA complex is then delivered to the ER membrane from where the SRP54 subunit of the SRP complexes with the SRP receptor alpha protein

(Rapiejko and Gilmore 1994) to escort the nascent signal peptide into the protein translocon tunnel, Sec61 (Gilmore and Blobel 1983, Deshaies and Schekman 1987, Beckmann, Spahn et al. 2001).

Once the signal particle of the cellulase protein is delivered to the Sec61 translocon tunnel, the SRP dissociates from the nascent complex and protein translation continues (Wild, Rosendal et al. 2004). The force of ribosomal exit is enough motive force to translocate the protein across the Sec61 translocon, and chaperones on the lumen of the ER bind to the nascent protein across the biological barrier. The signal sequence, however does not fully translocate and is bound by the Sec61 tunnel where the transmembrane protein, signal peptidase, recognizes the sequence for cleavage, and cleaves the signal sequences from the rest of the protein allowing for proper folding and modification (Dalbey and Wickner 1985, Bohni, Deshaies et al. 1988).

Signal recognition particles are not required for import of all nascent proteins into the ER (Ast, Cohen et al. 2013, Aviram and Schuldiner 2014, Chartron, Hunt et al. 2016). The ability of proteins to be first fully translated before import has shed light on the complexity of protein import. The Sec61 translocon complex is able to interact with the Sec62, Sec63, Sec66, Sec72 complex to form a SRP-independent translocon that imports proteins into the ER without the force of translation (Aviram and Schuldiner 2014). In the case of SRP-independent translocation, the molecular chaperones on the luminal side of the translocon drives the import as a molecular ratchet. As the binding of the nascent protein is pulled into the ER, Sec63, a DnaJ domain containing protein, recognizes the import of peptides and facilitates Hsp70 binding to the imported protein to guide folding to its native state (Brodsky and Schekman 1993, Brodsky, Goeckeler et al. 1995).

1.4.2 Cleavage and N-terminal modifications

The cleavage site of the signal peptide has been well determined by studies of secreted proteins. As the N-terminal helix of the signal peptide is translocated, cleavage sites of the signal sequence typically have a helix-breaking residue (proline or glycine) that prevents the helix packing of the rest of the signal peptide, which allows for presentation of the cleavage site to the signal peptidase (Bohni, Deshaies et al. 1988, Nielsen, Engelbrecht et al. 1997). The signal peptidase, an ER localized serine protease, recognizes a consensus sequence of small residues on the translocating protein and cleaves off the signal peptide (Auclair, Bhanu et al. 2012),which is subsequently degraded (Weihofen, Binns et al. 2002).

Once the signal peptide is cleaved, many cellulases such as the major cellobiohydrolase protein, GH7-1 and the major endoglucanase, GH5-1 contain glutamines as the first residue post cleavage (Dana, Dotson-Fagerstrom et al. 2014). While in many well-studied secreted proteins, the N-terminal residue is not altered, the glutamine has a distinctive modification where the N-terminal glutamine is cyclized (pyroglutamate) (Dana, Dotson-Fagerstrom et al. 2014). In the GH7 and GH5 families of CAZymes, the cyclized glutamine provides stability to the protein by preventing protease attack of the N-terminus (Wu, Dana et al. 2017). In the GH7 family, *T. reesei* Cel7a has been shown through structural studies to pack the N-terminal cyclized glutamate into a hydrophobic pocket of the enzyme. The ability of the N-terminal pyroglutamate to pack into the

enzyme has been hypothesized to promote increased thermostability and activity of the enzyme (Dana, Dotson-Fagerstrom et al. 2014, Wu, Dana et al. 2017).

1.4.3 ER luminal folding

As the nascent peptide is transported across the ER membrane, molecular chaperones in the ER lumen help to properly fold the protein by preventing aggregation of the hydrophobic residues. The folding capacity of the ER is crucial to the health of the organism as mechanisms to regulate the amount of protein folding capacity are essential for cellulase protein secretion (Vogel, Misra et al. 1990, Wu, Ng et al. 2014, Fan, Ma et al. 2015). As the proteins are imported, they are bound first by the HSP40 domain of Sec63. The HSP40 subunit of the heat shock protein complex that provides specificity for proteins that need to be refolded (Brodsky and Schekman 1993, Brodsky, Goeckeler et al. 1995). Once the HSP40 recognizes the unfolded protein substrate, an ATP-HSP70 chaperone subunit is recruited to the unfolded substrate. HSP40 activates the HSP70 ATPase activity, resulting in hydrolysis of ATP to ADP (Schmid, Baici et al. 1994). The high affinity ADP-HSP70 complex binds to the nascent protein until the ADP in HSP70 is exchanged for ATP by a Nucleic Exchange Factor, GrpE, resulting in release of the protein substrate. The chaperones prevent the aggregation of unfolded substrates, and continue progressive binding and release cycles until the protein is properly folded into its native state (Young 2010).

In *S. cerevisiae* the major HSP70 in the ER lumen is Kar2p (Rose, Misra et al. 1989); the predicted ortholog is NCU04265 in *N. crassa*. HSP40s in the ER lumen take on many forms, including transmembrane HSP40, Sec63p (Brodsky and Schekman 1993), and the major soluble luminal HSP40, Scj1p (Blumberg and Silver 1991). Protein folding capacity of the ER lumen is critical to the amount of protein that can be adequately processed and sorted through the ER into vesicles bound for the cis-Golgi network. Significant dysfunction occurs when the folding capacity of ER is surpassed. This phenomenon often occurs under cellulose induction conditions as the protein load is greatly increased for proteins to be correctly folded and secreted (Fan, Ma et al. 2015). In filamentous fungi, as protein secretion is increased, protein secretion capacity is also increased through induction of the unfolded protein response, or UPR.

1.4.4 The unfolded protein response

The UPR is an evolutionarily conserved phenomenon activated when unfolded proteins accumulate in the ER. This ER stress response was initially observed in mammalian cells expressing a mutant form of a virus that was unable to properly fold, and later found to act in the secretory pathway in fungal cells (Kozutsumi, Segal et al. 1988, Normington, Kohno et al. 1989, Rose, Misra et al. 1989). Although there are some major differences between the metazoan and fungal UPR, the basic mechanism is highly conserved amongst fungi (Hollien 2013) and has been most well characterized in *S. cerevisiae*. In *S. cerevisiae*, accumulation of unfolded proteins in the ER activates Ire1, an ER-membrane spanning protein with endonuclease function.

When activated, Ire1 cleaves a non-canonical intron from the mRNA of the basic leucine-zipper transcription factor, Hac1, releasing it from transcriptional inhibition (Sidrauski and Walter 1997). Efficiently translated Hac1 activates the transcription of a broad set of genes that allow

for enhanced ER folding capacity and more efficient protein trafficking (Mori, Kawahara et al. 1996, Kaufman 1999). A similar role for Ire1 and Hac1 orthologs in the UPR has been reported for a number of filamentous fungal species (Saloheimo, Valkonen et al. 2003, Mulder, Saloheimo et al. 2004, Sims, Gent et al. 2005, Arvas, Pakula et al. 2006, Guillemette, van Peij et al. 2007).

The UPR may also play a role in balancing the need for increased protein secretion with ER processing capacity during the lignocellulolytic response (Fig 1.1 and Table 1.1). In *N. crassa*, expression of *ire-1*, *hac-1*, and genes encoding other proteins associated with the UPR is upregulated in response to crystalline cellulose (Benz, Chau et al. 2014). Deletion of either *ire-1* or *hac-1* results in a reduction in cellulase secretion, although cellulase transcription is unaffected (Fan, Ma et al. 2015, Montenegro-Montero, Goity et al. 2015). In a *T. reesei* strain selected for increased cellulase secretion, the UPR is activated earlier in the lignocellulolytic response than in wild type cells, although it is not clear how this affects glycosyl hydrolase production (Wang, Zhang et al. 2014). Further investigation into how the UPR affects cellulase quality control and flux through the secretary pathway during cellulase secretion will be necessary to fully understand its role during the lignocellulolytic response.

1.4.5 Other ER stress responses

Two other protein quality control mechanisms that may function during the lignocellulolytic response and may be under the control of the UPR are ER-associated Degradation (ERAD) and transcriptional repression under secretion stress (Travers, Patil et al. 2000, Carvalho, Jorgensen et al. 2012, Fan, Ma et al. 2015). Proteins that are not correctly folded even after an extended stay in the ER are targeted for proteasomal degradation via the ERAD pathway (Lippincott-Schwartz, Bonifacino et al. 1988, Sommer and Jentsch 1993). Although the role of ERAD during the lignocellulolytic response has not been investigated in detail, it may play a role, since mutations that may inhibit accurate folding of CBH1 in *T. reesei* cause the upregulation of genes involved in ERAD as well as co-localization of the mutant CBH1 with the proteasome (Kautto, Grinyer et al. 2013). Transcriptional repression under secretion stress is a poorly understood phenomenon where filamentous fungi down regulate transcription of lignocellulose degrading enzymes when exposed to chemical stresses that target the secretory system or during expression of constitutively active HacA/HAC1/HAC-1 (Pakula, Laxell et al. 2003, Al-Sheikh, Watson et al. 2004, Carvalho, Jorgensen et al. 2012). Although there have been several studies which have identified promoter regions that may be necessary for this phenomenon, the transcription factors and other genes involved in mediating this process are still unknown (Pakula, Laxell et al. 2003, Zhou, Wang et al. 2015). Future investigation will be necessary to determine the genetic actors involved in this feedback mechanism as well as a concrete role for transcriptional repression under secretion stress during the lignocellulolytic response.

1.4.6 ER modifications

Once proteins are fully translocated into the ER, folding and reinforcement of protein folds begins through signal peptide cleavage, glycosylation, disulfide bridges, and other post translational modifications of proteins. The extent of protein modification of cellulases are a large part of why engineering of cellulases is particularly difficult. With 8-12 disulfide bridges,

at least 2 N-linked glycosylation sites and 12 O-linked glycosylation sites, a significant amount of post-translational processing is performed on the cellulases that are secreted into the environment (Boer et al., 2000) (Tang, Bubner et al. 2016).

Within the ER, cellulases are processed by protein disulfide isomerases (PDI) to form disulfide bonds for reinforcement of tertiary structure (Wilkinson and Gilbert 2004). Enzymes are initially imported into the ER with reduced cysteine residues. Upon exposure to PDI, the cysteines are oxidized and form covalent, disulfide bridges that stabilize the enzymatic reaction centers and proper tertiary structure of the protein (Wilkinson and Gilbert 2004). *T. reesei* CBH1 contains 12 disulfide bridges per protein (Bhikhabhai and Pettersson 1984), and *N. crassa* CBH-1 contains 24 cysteine residues with predicted orthologous function. These disulfide bridges contribute to the extraordinary stability of the cellulase proteins and, in part, explain their ability to be active in supernatants under extreme temperatures (Kim and Ishikawa 2013, Tanghe, Danneels et al. 2017).

Post-translational modifications of enzymes in the ER also include the initial establishment of glycosylation of surface asparagines, which are recognized by conserved motifs, N-X-S/T (Deshpande, Wilkins et al. 2008). N-linked glycosylation begins with a conserved branched glycosylation chain of GlcNac2Man9Glu3 is transferred en bloc from the dolichol glycosylation donor lipid to the asparagine residue of the N-X-S/T motif (Deshpande, Wilkins et al. 2008). In the ER, the glucose molecules are progressively cleaved by glucosidases as the protein is folded and progressively matured. If the glucose residues remain on the glycosylation chain, the quality control proteins, calnexin and calreticulin, bind to the glucose residues and retain the unfolded protein within the ER (Ellgaard and Helenius 2003).

While the initial glycosylation events occur in the ER, the glycosylation chains are further modified in the Golgi as the cellulases mature and are exported (Adney, Jeoh et al. 2009). Beyond N-linked glycosylation, cellulases have a large extent of O-linked glycosylation that is necessary for proper enzyme maturation (Deshpande, Wilkins et al. 2008). The function of O-linked glycosylation is less well established in filamentous fungi, but has been hypothesized to help extend the cellulose-binding domain for proper processing along the cellulose chain (Goto 2007). Like N-glycosylation, O-glycosylation initiates in the ER as a single mannose molecule, and then is further extended into di-tri- and tetra-hexoses in the Golgi (Tang, Bubner et al. 2016).

1.4.7 Downstream secretion

Once cellulases mature in the ER lumen through folding, disulfide bridge formation, and attachment of initiating glycosylation chains, they are brought to ER exit sites (ERES), which then package the luminal proteins into COPII coated vesicles for transit to the Golgi (Kurokawa, Okamoto et al. 2014). At ER exit sites, the COPII coat is responsible for deforming the membrane that buds off from the ER to traffic proteins and other cargos to the cis-Golgi (Gomez-Navarro and Miller 2016). However, proteins that remain soluble in the lumen cannot directly bind to the COPII coat, therefore, sorting proteins and adaptors present as transmembrane proteins are responsible for concentrating the cargo proteins and engaging the COPII proteins at the ERES (Dancourt and Barlowe 2010). The protein complex responsible for

bridging the cargo sorting proteins is the Sec23/Sec24 complex that binds specific membrane spanning proteins and cargo receptors.

The two isoforms of Sec24 in *N. crassa* potentially expand the specificity of cargo proteins. *N. crassa* has a single cargo receptor for each of the major families of p24 protein cargo receptors, ERP-1, ERP-2, EMP-24, and ERV-25, and contains the multi-spanning membrane sorting receptors, ERV-29, ERV-14 and ERV-26. Recent work performed in *N. crassa* has implicated specific roles for cargo adaptors for trafficking cellulases, where the p24 adapter, ERV-25, is important for CBH-1 and ERV-29 cargo adaptor is important for CBH-2 (Starr, unpublished results).

After the COPII-coated vesicle buds off the ERES, the coat proteins are shed and vesicles are transported to the Golgi, where proteins are further glycosylated, proteolytically processed, and sorted to final target membranes. Within progressive maturation in the Golgi, proteins are further modified to their final forms. This includes maturation of the glycosylation chains, which involves mannases to trim back the mannose residues on the N-linked glycan chains, and transfer of GlcNac residues to obtain its mature form (Tang, Bubner et al. 2016). When heterologously expressed in yeast, the major cellulase Cel7a/CBH-1 is properly glycosylated up to the time it reaches the Golgi. However, *S. cerevisiae* contains a mechanism for hyper-mannosylating the N-linked glycosyl chains (Ilmen, den Haan et al. 2011). Hyper-mannosylation in *S. cerevisiae* prevents many of these heterologous expressed proteins from functioning optimally, which results in poor yields from consolidated bioprocessing (van Zyl, Lynd et al. 2007).

After maturing in Golgi, adaptor proteins making up the Ap1, Ap2 and Ap3 complex attach to cargo proteins for sorting from the trans-Golgi network to endosomes, plasma membrane to endosome, and Golgi to lysosomes, respectively (Gomez-Navarro and Miller 2016). Superfolding GFP marked GH5-1 in *N. crassa* has been shown to accumulate in the endosomes when a strain containing a temperature-sensitive mutation in *apl-4*, encoding the major gamma-adaptin subunit for the AP-1 complex, is grown at restrictive temperatures. These observations suggest that cellulase trafficking is in part dependent on the AP-1 complex for proper secretion (Scheurg and Starr, unpublished results).

At this point, cellulase delivery to the exterior is not particularly well studied, although secretory vesicles in other fungi have been well elucidated while being transported to the Spitzenkörper for secretion at the hyphal tip. In *Aspergillus nidulans*, secretory vesicles labeled with RabE-GFP, the *A. nidulans* homologue of human Rab11, traverse along microtubules that span the length of the hyphae (Penalva, Zhang et al. 2017). The motor proteins necessary for RabE delivery to the tip is dependent most notably on KinA, the orthologue of human kinesin Kin1. KinA is able to provide delivery along the microtubules and upon arrival at the hyphal tip, myosin motor protein, MyoE, assists with delivery of the secretory vesicles to the Spitzenkörper. These two proteins in coordination with one another help to traverse the length of the hyphae, and to load secretory vesicles to the vesicle organizing center of the Spitzenkörper (Penalva, Zhang et al. 2017).

1.4.8 The Spitzenkörper

The Spitzenkörper is a dynamic, densely packed vesicle supply depot structured with cytoskeletal elements anchored at the growing tip of the hyphae (Steinberg 2007). It is a strong indicator of directionality and efficient polar growth. The Spitzenkörper uses actin microfibrils to generate a stratified structure of vesicles (Sanchez-Leon, Verdin et al. 2011). The core contains micro-vesicles, dense vesicles 30-40 nm in size that have been linked to chitin synthase activity and associated with previously found chitosomes, while the outer ring of vesicles is made up of macro-vesicles, spheroid bodies 70-100 nm in length (Bartnicki-Garcia 2006). Studies on functional stratification of the Spitzenkörper have confirmed the presence of chitin synthase (CHS) localization to the Spitzenkörper core, while the outer Spitzenkörper ring contains a distinct set of proteins including GS-1, a glucan synthase regulator (Verdin, Bartnicki-Garcia et al. 2009). The origins of the vesicles of the Spitzenkörper has been shown be post-Golgi vesicles of the secretory pathway (Sanchez-Leon, Bowman et al. 2015), as the role of the exocyst complex is essential for the anchoring of the Spitzenkörper vesicles for growth (Riquelme, Bredeweg et al. 2014). These observations implicate the sorting of vesicle cargo earlier in the secretion pathway as distinct vesicle populations reside in the Spitzenkörper core and ring.

1.5 Conclusions

Understanding the regulation and secretion of the lignocellulosic response by filamentous fungi will be necessary as we strive to make lignocellulosic biofuels a viable component of our renewable energy repertoire. When filamentous fungi sense the presence of plant cell wall carbohydrates, cells activate transcription of enzymes necessary for complex carbohydrate degradation primarily via zinc binuclear cluster transcription factors. These CAZymes are also under the transcriptional control of signaling pathways that are involved in nutrient sensing or in sensing the energy state of the cell. Regulation of enzyme production continues post-translationally through phenomena including, but likely not limited to, the UPR, ERAD, transcriptional repression under secretion stress, and protein sorting.

Although the identity of many of the primary transcription factors that activate CAZymes is known, constitutive expression of these transcription factors is not sufficient for a full lignocellulolytic response, indicating that post-translational activation or de-repression of these transcription factors is necessary (Mach-Aigner, Pucher et al. 2008, Tamayo, Villanueva et al. 2008, Coradetti, Craig et al. 2012, Derntl, Gudynaite-Savitch et al. 2013). It is obvious that insoluble substrates, like cellulose itself, are competent to induce the cellulolytic or hemicellulolytic response, but it is likely that soluble sugars constitute the intracellular activation signal. The β-1,4-glucose dimer, cellobiose, and a transglycosylated form of this sugar, sophorose, are sufficient to elicit a cellulolytic response in *N. crassa* cells lacking the three major β-glucosidases and in *T. reesei*, respectively (Mandels, Parrish et al. 1962, Sternberg and Mandels 1979, Znameroski, Coradetti et al. 2012). However, these molecules do not appear to be universal inducers, suggesting that each species may respond to slightly different signals (Gielkens, Dekkers et al. 1999, Suzuki, Igarashi et al. 2010, Znameroski, Coradetti et al. 2012). Importantly, we do not know whether the inducer molecule directly activates primary transcription factors or whether additional, unidentified genes/proteins are involved. Transcriptional activation or de-repression by nutrient sensing pathways is also necessary for full induction of the lignocellulolytic response.

The effect of CCR is the best studied of these nutrient sensing pathways in filamentous fungi, but while the transcriptional effects of its most prominent player, CreA/CRE1/CRE-1, have been fairly well studied, there are clearly other proteins, such as VIB-1/XprG and COL-26/BglR, whose mechanisms of action require further study (Nitta, Furukawa et al. 2012, Xiong, Sun et al. 2014, de Assis, Ries et al. 2015). Additionally, there are other highly conserved pathways that sense the nutritional state of the cell, such as PKA and TOR complex activation, that have been implicated in the lignocellulosic response, but whose role in plant cell wall degrading enzyme production needs further clarification (Xiong, Coradetti et al. 2014, de Assis, Ries et al. 2015).

While it is well established that the lignocellulolytic response is under tight transcriptional control, the volume of proteins sent through the secretory pathway when filamentous fungi degrade plant cell wall material appears to induce significant stress on the secretory system. This may be why activation of the UPR and ERAD is associated with CAZyme secretion (Fan, Ma et al. 2015, Tanaka, Shintani et al. 2015). Filamentous fungi also respond transcriptionally to secretion stress. Future studies into the mechanism of how cells detect ER stress and the signal transduction mechanisms involved in transcriptional feedback from transcriptional repression under secretion stress will contribute to our understanding of the role that the secretory pathway plays in regulation of the lignocellulolytic response. Recent work has also implicated other conserved mechanisms that affect secretion of plant cell wall CAZymes, including components of the clathrin complex and the sterol regulatory element binding protein (SREBP) pathway (Pei, Fan et al. 2015, Reilly, Qin et al. 2015, Qin, Wu et al. 2017).

Secretion of cellulases downstream of transcription needs considerable more work, as current models of secretion for cellulases have yet to be explored adequately. While the role of cargo adapter proteins has been associated with cellulase secretion, it is unclear whether the p24 and ERV-29 cargo adaptor proteins are directly advancing the maturation of cellulase proteins, or if they are indirectly mis-trafficking cellulase proteins (Starr, unpublished results). Furthermore, a comprehensive understanding of cellulase trafficking in vesicles of the secretory system needs to be pursued, as previous work has implicated that sorting and maturation of CBH-1 and CBH-2 cellulase proteins may occur through alternative processes.

Characterization of these pathways and others that affect the secretion of plant cell wall CAZymes may help us to better understand how filamentous fungi have evolved to become the dominant plant biomass degrading organisms in nature. Elucidation of nutrient sensing, carbon utilization, and secretion stress pathways can be harnessed in filamentous fungi to increase the efficiency of lignocellulosic breakdown for biofuels and specialty products to begin the long road necessary to replace fossil fuels as our go-to source of hydrocarbons.

1.6 Scope of this work

While the essential components of secretion have been well elucidated in *S. cerevisiae* and extrapolated to various systems under orthologous functions, the secretion systems in filamentous fungi are still poorly understood. As the organisms that are the most prolific degraders of cellulosic material, filamentous fungi have a high capacity for protein secretion. Cellulases, a family of proteins that are secreted out of filamentous fungi can reach titers up to

100 mg/L under basal culturing conditions, and have been engineered to secrete up to 100 g/L under highly controlled fermentation conditions.

One route of study in cellulase secretion is the heterologous expression of cellulase proteins in *S. cerevisiae* to understand cellulase trafficking. However, while there have been many attempts to generate yeast strains that are able to secrete cellulolytic enzymes for consolidated bioethanol production, unfortunately, *S. cerevisiae* are not endogenously cellulolytic and thus, have not evolved to secrete the level of cellulases that are necessary for consolidated bioprocessing (van Zyl, Lynd et al. 2007, Ilmen, den Haan et al. 2011). Thus, while there is a continued reliance on filamentous fungi for the production of cellulolytic proteins for various processing industries, the necessary routes for cellulase protein sorting and maturation remain poorly understood, and the steps for engineering a secretory mutant that is able to increase secretion of enzymes remains obscured by industrial trade secrets. Additionally, industrially-derived hypersecreting strains do not have well described sexual cycles and tools for facile genetic manipulation (Ni, Feretzaki et al. 2011).

In this work, we attempt to shed some light on understanding the necessary factors of protein hypersecretion in a filamentous fungal host. Understanding the post-translational factors that are necessary to generate a hypersecreting strain of a filamentous fungus and the mechanisms of protein import and secretion will have wide ranging effects in engineering more efficient protein secretion hosts in the food and fuel industry. We begin by developing a quantitative screen for the generation of a hypersecretory mutant, and use genomics to dissect the phenotype to a causal allele (Chapter 2). Then, we further characterize the contributions that flippases make to known mechanisms of hypersecretion and the mechanism by which the flippases may be affecting secretion through protein sorting (Chapter 3). We then look at the first step of secretion and find the mechanisms by which we can increase the amount of shuttled protein into the ER by proper recognition by the Signal Recognition Particle (SRP) for initial import into the ER (Chapter 4). In this way, we can begin to elucidate the conserved roots of cellulase hypersecretion in the model filamentous fungus, *N. crassa*. Finally, I conclude this body of work with the possible future directions and routes of inquiry that would be possible with the tools that have been established in this work (Chapter 5).

This chapter contains text and figures I have contributed to a published manuscript:
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CHAPTER 2: MUTAGENESIS AND IDENTIFICATION OF ALLELES CONTRIBUTING TO A HYPERSECRETION PHENOTYPE OF *NEUROSPORA CRASSA*

2.1 Introduction

2.1.1 Hypersecretion

Lignocellulose is an abundant waste product of many agricultural and processing industries that can be utilized for the production of renewable, carbon-neutral cellulosic fuel substitutes for petroleum based fuels (Kuhad, Gupta et al. 2011, Payne, Knott et al. 2015, Reilly, Magnuson et al. 2016). Currently, one of the major costs of the process that governs the price of cellulosic fuels is the production of the enzymes used to convert the lignocellulose into soluble sugars for fermentation. Filamentous fungi are the primary producers of such proteins used to degrade cellulosic feedstocks. These organisms have an innate capability to secrete high titers of protein up to 100g/L of cellulases under controlled fermentative conditions (Cherry and Fidantsef 2003) The current industrial standard for cellulase production comes from a mutagenized form of the soft mold, *Trichoderma reesei*. Originally isolated off canvas tents in the 1950's, *T. reesei* was realized to be a prolific source of cellulases in the 1970's through iterative mutagenic studies. (Mandels and Reese 1957, Mandels, Weber et al. 1971).

These mutagenic studies were performed through labor intensive mutagenesis where *T. reesei* spores were mutagenized and subsequently plated on cellulose medium, which were then stained for polysaccharide digestion visualized by a lack of staining using an amyloidophilic dye. While a team at Rutgers University isolated the current industrial standard, Rut-C30, a hyper-producing strain of *T. reesei* that is reported to secrete cellulases up to 30 g/L of protein under controlled batch fermentation conditions (Montenecourt and Eveleigh 1977), very few causal mutations have been elucidated from the mutagenesis efforts (Le Crom, Schackwitz et al. 2009). For the first half century after the identification of the Rut-C30 strain and its WT precursor strain, QM6a, have only been propagated asexually (Seidl, Seibel et al. 2009). A sexual phase has only recently been reported, but industrial strains still fail to mate (Seidl, Seibel et al. 2009). As a result of a poorly defined sexual cycle and a lack of other genetic tools, mutations in the Rut-C30 strain and subsequently derived industrial strains have not been sufficiently analyzed. Furthermore, *T. reesei* strains have been continuously studied in the industrial context, and little information has been made open to the public. As a result of these issues, the five decades that span the original isolation of the mutant until present day, there has been little genetic work done or openly reported to identify causal mutations for the increased cellulolytic phenotype (Peterson and Nevalainen 2012).

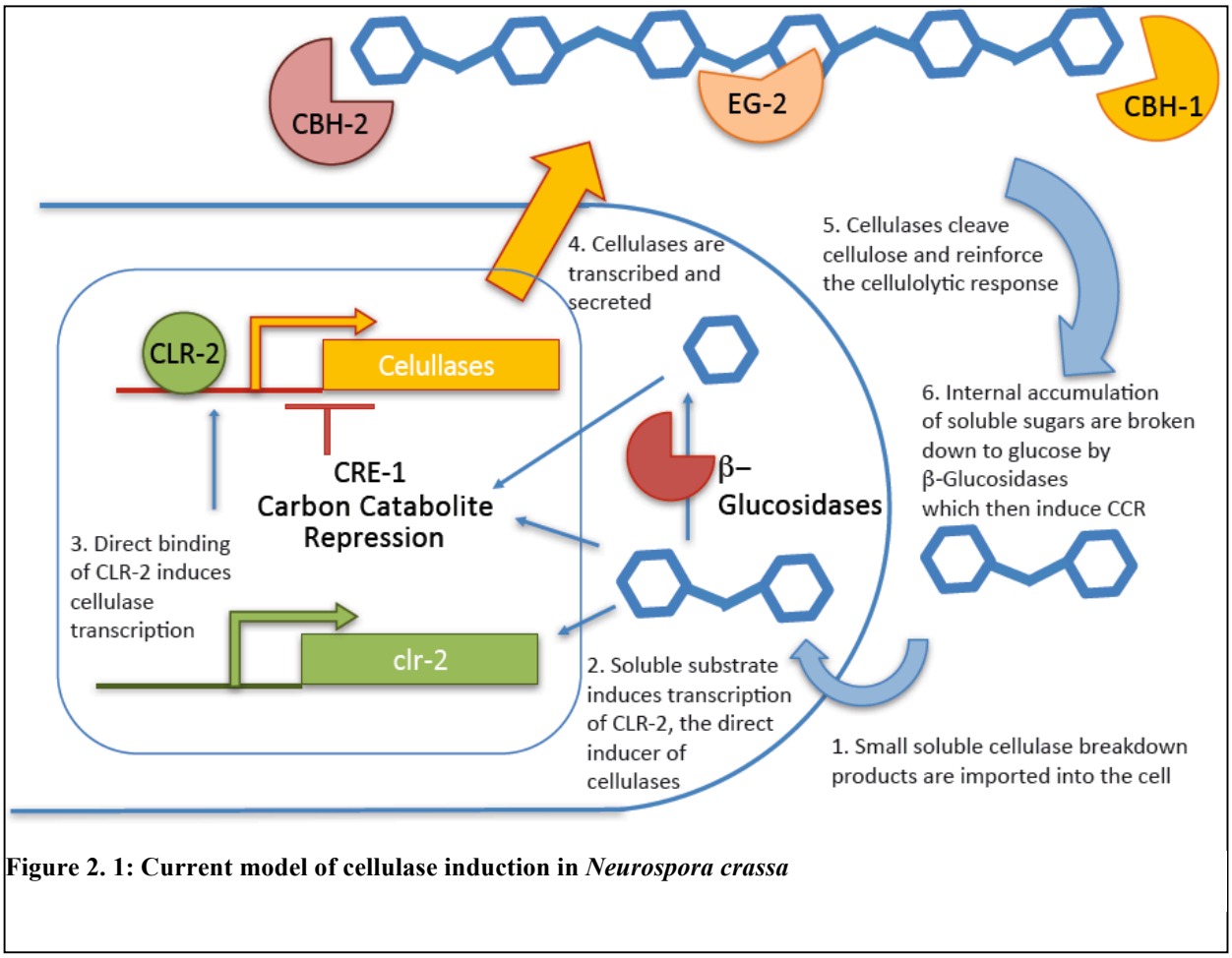

Figure 2. 1: Current model of cellulase induction in *Neurospora crassa*

This dearth of information that affects the secretion of proteins in filamentous fungi has largely prevented the engineering of new strains and species of filamentous fungi for hypersecretion. What has been identified from the original screens, however, is the near complete relief of carbon catabolite repression by glycerol (Peterson and Nevalainen 2012). Screening of the *T. reesei* mutants used 2-dexoyglucose, a non-metabolizable glucose analogue, with glycerol supplemented cellulose media forced the relief of carbon catabolite repression (Montenecourt and Eveleigh 1977). The *cre1* gene was found to have mutated alleles in both starting strains of industrial application, Rut-C30 and QM9414, and subsequent work discovered the role of second transcription factor, BglR/COL-26, in the relief of CCR (Xiong, Sun et al. 2014).

Beyond carbon catabolite repression, the major area of focus for engineering cellulase hypersecreters in *T. reesei* is through direct increases in cellulase transcription. The major regulator of lignocellulases in *T. reesei* is the transcription factor Xyr1 (Mach-Aigner, Pucher et al. 2008). Over expression of *xyr1* gives a nominal boost in cellulase transcription (Mach-Aigner, Pucher et al. 2008). Accessory transcription factors have also been studied en route to upregulating the transcription of cellulase genes (Portnoy, Margeot et al. 2011), (Aro, Ilmen et al. 2003).

The role of inducers has been critical in the development of strains that are able to secrete cellulases under non-inductive conditions. *T. reesei* is known to be a broad responder to sugar substrates, with gratuitous cellulolytic responses to lactose (Morikawa, Ohashi et al. 1995) and sophorose (Mandels, Parrish et al. 1962). The ability of *T. reesei* to mount a cellulolytic response to lactose, a β-1,4 dimer of galactose and glucose, or sophorose, an a α-1,2 dimer of glucose, still remains unclear, and testing of these sugar substrates on other filamentous fungi have shown that *T. reesei* is the exception for induction on these soluble substrates (Mandels and Reese 1957, Mandels, Parrish et al. 1962).

Despite the unknown endogenous inducer of *T. reesei* cellulase secretion, use of these soluble inducers has allowed for the isolation of *T. reesei* strains that are enhanced for cellulolytic capability using fluorescently activated cell sorting, or FACS. Previous studies have utilized a transcriptional fusion of green fluorescent protein, GFP, to the *cbh1* promoter as a proxy for the cellulolytic response in the cell (Throndset, Kim et al. 2010). By screening for strains that have a high level of GFP in germinated asexual spores (germlings), FACS sorting was able to parse out the highest GFP expressing strains that demonstrated high cellulolytic activity and heterologous protein expression (Throndset, Bower et al. 2010, Throndset, Kim et al. 2010). These studies, however, only utilized internal GFP accumulation for sorting, and did not attempt to parse out the alleles that may be causing the increased cellulolytic response.

2.1.2 Known *N. crassa* hypersecretion mutants

Around the time that *T. reesei* was identified as a cellulase production host, a closely related fungus, *Neurospora crassa* was also found to have cellulolytic capabilities (Marsh, Bollenbacher et al. 1949). As a prolific fungus that grows on dead, burnt vegetation, *N. crassa* secretes an abundance of cellulases into its extracellular environment to degrade the surrounding substrate into soluble sugars and nutrients for growth and reproduction (Tian, Beeson et al. 2009). Initial studies of *Neurospora* cellulases was performed in the late 1970's (Eberhart 1977), but only until systematic characterization of *N. crassa* grown on *Miscanthus* was reported was it finally considered a potential model for understanding cellulase secretion (Tian, Beeson et al. 2009).

In the Glass Lab, we use *N. crassa* as a genetically amendable system that is easily crossed and can homologously recombine exogenous DNA products into its genome (Ninomiya, Suzuki et al. 2004, Colot, Park et al. 2006). The sequence of the *Neurospora* genome has been determined and the compact and well-annotated 40Mb genome is publically available (Galagan, Calvo et al. 2003). As a result of these advantages, *N. crassa* is recognized as the pre-eminent model system for studies in filamentous fungi. Currently, a knock out library of 80% of the genes in the genome exists as a powerful resource for rapid mutant studies (Colot, Park et al. 2006). In its natural habitat, individual *N. crassa* isolates have been sampled as sexual progeny, and next-generation sequencing has identified a myriad of genetic recombinations resulting in the ability to perform genetic association studies on wild alleles (Ellison, Hall et al. 2011, Palma-Guerrero, Hall et al. 2013). From studies of wild type collections, *N. crassa* has become a premier model system for dissecting the genetics of communication and the genetic loci that encode for environmental niche adaptation (Ellison, Hall et al. 2011, Palma-Guerrero, Hall et al. 2013, Heller, Zhao et al. 2016).

Current cellulase secretion studies have shown that, like the *T. reesei* models of hypersecretion, increases in the direct modulators of cellulase transcription has led to the increase of cellulase secretion in the supernatant. By mis-expressing the CLR-2 transcription factor so that it is constitutively transcribed, cellulases can be secreted under non-inducing conditions. This mis-expression of CLR-2 allowed for the secretion of cellulases under normally non-inducing sucrose, glucose, or starvation conditions (Coradetti, Xiong et al. 2013). Carbon catabolite de-repression has also been implicated in increases of protein secretion, as deletion of *cre-1* resulted in increased cellulase production (Sun and Glass 2011, Xiong, Sun et al. 2014).

2.1.3 Use of cellobiose as a cellulolytic inducer

Previous work investigated the use of cellobiose as an inducer for the cellulolytic response. The hypothesis originally put forth by Mandels and Reese was that cellobiose, the most direct degradation product of cellulose, induces the cellulase regulon in a basidiomycete fungus (Mandels and Reese 1960). Cellobiose was not an adequate inducer for a cellulolytic response in all fungi tested, however (Mandels and Reese 1960). It was noted that the β-glucan transporter that imports cellobiose into the cell had a much higher affinity for cellobiose than the secreted β-glucosidase enzyme that hydrolyze the cellobiose into glucose monomers (Schmoll and Kubicek 2003). Thus, the hypothesis was that at a low enough concentration of soluble β-1,4- glucan, the cell would be able to import the soluble degradation product before the cleavage into glucose molecules, and the cell would be able to initiate the induction of cellulases.

This hypothesis was confirmed experimentally in *N. crassa* by the sensitization to cellobiose of a strain carrying deletions of the three most highly regulated β-glucosidases on cellulosic induction. *N. crassa* has 7 functionally redundant β-glucosidase genes (Wu, Kasuga et al. 2013) of which a subset are regulated by cellulose (Znameroski, Coradetti et al. 2012). Transcriptome analysis of WT *N. crassa* was analyzed for determining the highest upregulated β-glucosidases on cellulase induction, and two extracellular β-glucosidases (*gh3-3*; NCU08755 and *gh3-4*; NCU04952) and an intracellular β-glucosidase, (*gh1-1*; NCU00130) were identified. The deletion of these three genes was sufficient to sensitize cells of *N. crassa* to cellobiose (Znameroski, Coradetti et al. 2012).

When WT *N. crassa* is exposed to cellobiose as the only sugar source, the fungal cells do not secrete the enzymes necessary for the breakdown of plant cell wall material. Instead, β-glucosidases are expressed which hydrolyze the cellobiose units into glucose, which is then transported into the cell and metabolized, preventing the induction of the cellulolytic response. In the sensitized strains lacking the three most prominent β-glucosidases (Δ3βG) under cellulolytic induction, the cellobiose molecules are no longer being hydrolyzed, and cellobiose presence in the cell is able to recapitulate a full transcription of cellulases as WT cells on cellulose (Znameroski, Coradetti et al. 2012).

By utilizing a soluble inducer in *N. crassa*, we could mutagenize the Δ3βG strain, and induce a cellulolytic response using cellobiose as an inducer, upon which we could FAC sort mutants with increased cellulase production. By using the advantages of the *N. crassa* sexual cycle and the genetic tools available, we could rapidly identify genes of interest and parse out the essential

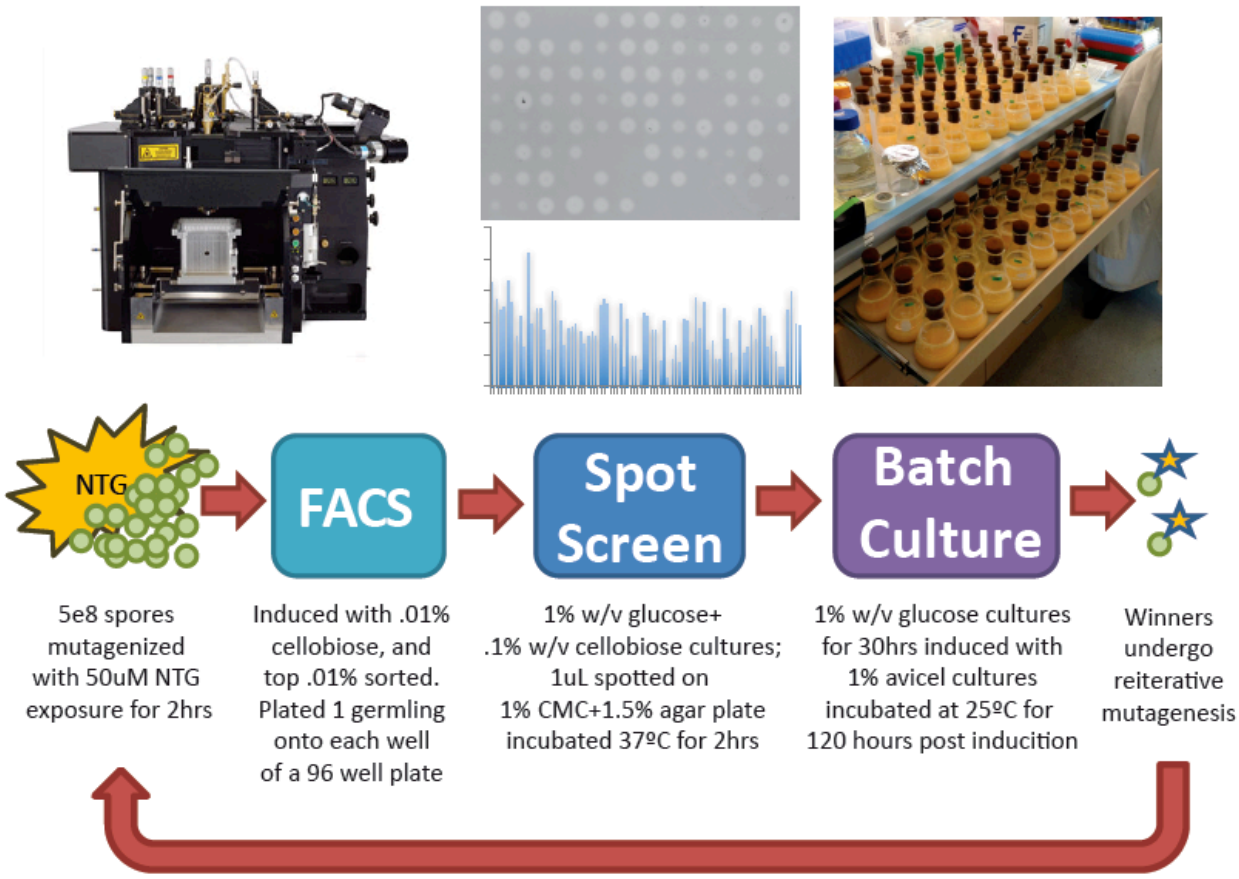

Figure 2. 2: Overview of quantitative screening for generating a cellulase hypersecreting strain of *N. crassa*

alleles that contribute to the cellulase hyper-secretion phenotype. This approach would allow us to expand on a growing set of alleles to develop as a genetic toolbox for future engineering of cellulase hypersecretion in filamentous fungi.

2.2 Results

2.2.1 Strain construction for mutagenic screen

Our goal with the screen was to update the mutagenesis that generated Rut-C30 by using FACS technology for the primary screen. This demanded that the inducing substrate be soluble and the strain would require a fluorescent tracer for the cellulolytic response. We hypothesized that by using the previously described Δ3βG strain, in conjunction with a cellulose responsive GFP, we could generate a cellobiose responsive strain that would fluorescently trace the dynamics of the cellulolytic response.

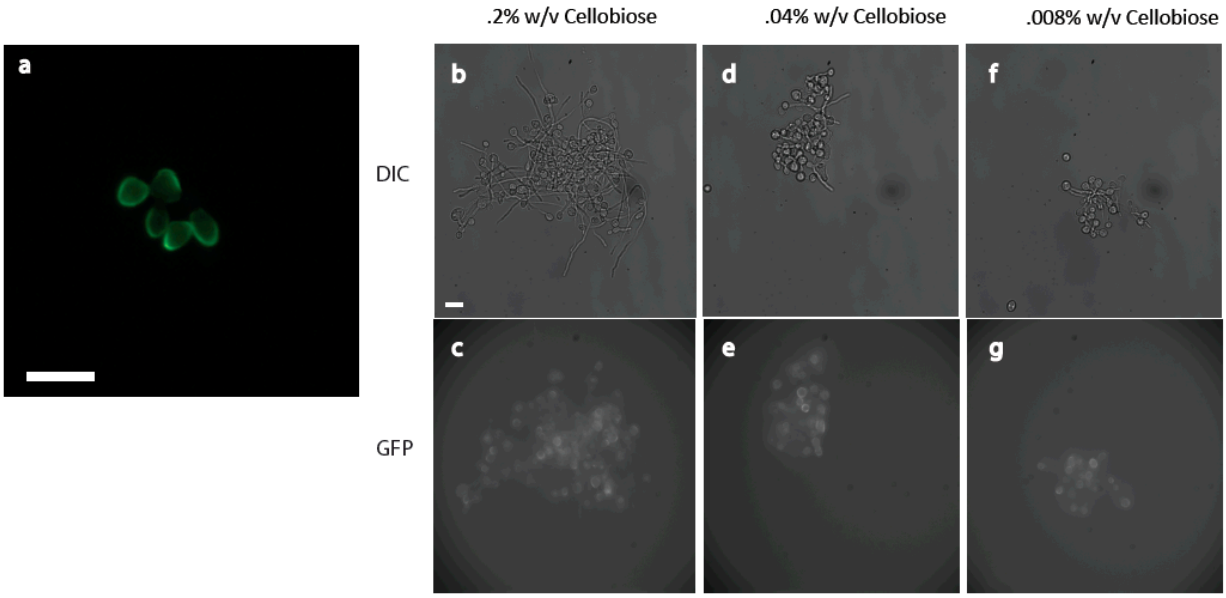

Figure 2. 3: Induction of GH5-1-GFP on cellobiose
Induced germlings of the Δ3βG; GH5-1-GFP (a). DIC and GFP fluorescence of germlings induced under variable cellobiose conditions (b-g). Hyphal growth under 0.2% (b) and 0.04% w/v (d) cellobiose has a greater germination than 0.008% w/v cellobiose (f), while maintaining similar GFP fluorescence, (c, e, g). Scale bar represents 10 μm

We mated the Δ3βG strain with a strain containing a superfolder GFP that was translationally fused to a *gh5-1* endoglucanase, NCU00762. Endoglucanase could be used as a label for the cellulolytic response because the addition of the fluorophore on the C-terminus of the endoglucanase did not alter the digestion activity of the enzyme on carboxy-methyl cellulose (CMC) (Starr, unpublished results). This was in contrast to CBH-1, the major constituent of enzymes in the cellulolytic supernatant, which has reduced activity after the addition of GFP at the C-terminal end, possibly due to the occlusion of proper cellulose binding module function to cellulose (Starr, unpublished results). In addition, as GH5-1 represented only 6% of the cellulolytic proteins in a response to cellulose (Phillips, Iavarone et al. 2011), we hypothesized that this would allow for a higher level of accumulation before potential signal saturation. We tested GH5-1-GFP as a proxy for the cellulolytic response and visualized the expression of the cellulolytic response with the soluble inducing molecule, cellobiose, within the Δ3βG background (fig 2.3a).

2.2.2 Determining the optimal conditions of cellulolytic induction

Previous work on cellulase expression upon exposure of *N. crassa* to cellobiose has shown that 0.2% cellobiose is sufficient to induce a cellulolytic transcriptional response of Δ3βG cells similar to WT cells on cellulose (Znameroski, Coradetti et al. 2012). Visualization of cellobiose-induced cells at this concentration, however, showed a high amount of germination and mycelial network formation (fig 2.3b). We wanted to restrict these aspects of *N. crassa* growth and optimize the amount of cellobiose exposure that was necessary for a full transcriptional response to cellobiose, but would keep growth to a minimum.

We then tested for optimization by visualizing the induction of the cellulolytic response by cellobiose and the growth of germlings inoculated at $1x10^6$ spores/mL of media in 0.2%, 0.04%, 0.008% cellobiose with Vogel's minimal media (VMM). We observed germination, GFP expression, and hyphal growth over time. From microscopy of germlings in these conditions, spores at the 0.2% cellobiose + VMM concentration were highly aggregated and thoroughly germinated (fig 2.3b). The accumulation of GFP in the conidial cell walls indicated a full expression response. At a 5-fold decrease in cellobiose concentration, the germination of spores was highly attenuated, but the GFP was still apparent at the same level as the 0.2% condition (fig 2.3d, e). At a subsequent 5-fold decrease in cellobiose to 0.008%, we saw more attenuation of growth while the level of GFP was still constant (fig 2.3f, g). At a subsequent decrease of cellobiose concentration to 0.001% concentration, we saw a decrease in the amount of GFP fluorescence and a low germination rate. Thus, we utilized GFP fluorescence and lowered growth phenotype at 0.008% cellobiose for our screening to maintain high germination rate, low growth and proper GFP expression for ease of FACS analysis.

2.2.3 Mutagen used

In order to introduce random mutations in the genome, we utilized n-nitro-n-nitroso-guanidine (NTG), an alkylating agent that introduces guanidine-to-adenosine or cytosine-to-thymine transitions in the genome. Using 2 populations of 10^8 cells, we treated the first population with the NTG mutagen, while using a water treatment as a negative control for the second population. After exposure to the NTG, we washed both populations with PBS, and washed 3 times in 0.01% cellobiose + Vogel's minimal media. After 8h of growth, we visualized the presence of the GH5-1-GFP surrounding the germling cell wall (fig 2.3a). FACS analysis was then performed on the population of germlings using the water treatment as the control population for the NTG-treated germlings.

2.2.4 Primary FAC isolation and sorting

Of the 10^8 cells that were originally induced for cellulolytic response post mutagenesis, $2x10^7$ cells were analyzed by the FAC sorter. 1176 strains were sorted across 14 x 96 well plates. The sorting rate of the 10^8 germlings results in a 0.006% sorting rate. Furthermore, of the 1176 strains sorted, 939 strains were viable, so despite sorting for cells that were able to induce a cellulolytic response, there was a consequential ~80% survival rate post sort. As a secondary assay that is orthologous to GH5-1-GFP fluorescence that would help to screen out possible artifacts of secretion, we utilized carboxy-methyl cellulose (CMC) degradation as a proxy of cellulolytic induction. This would also help screen out possible strains that would be defective in proper GH5-1 folding or trafficking that may have been isolated from the original FACS analysis.

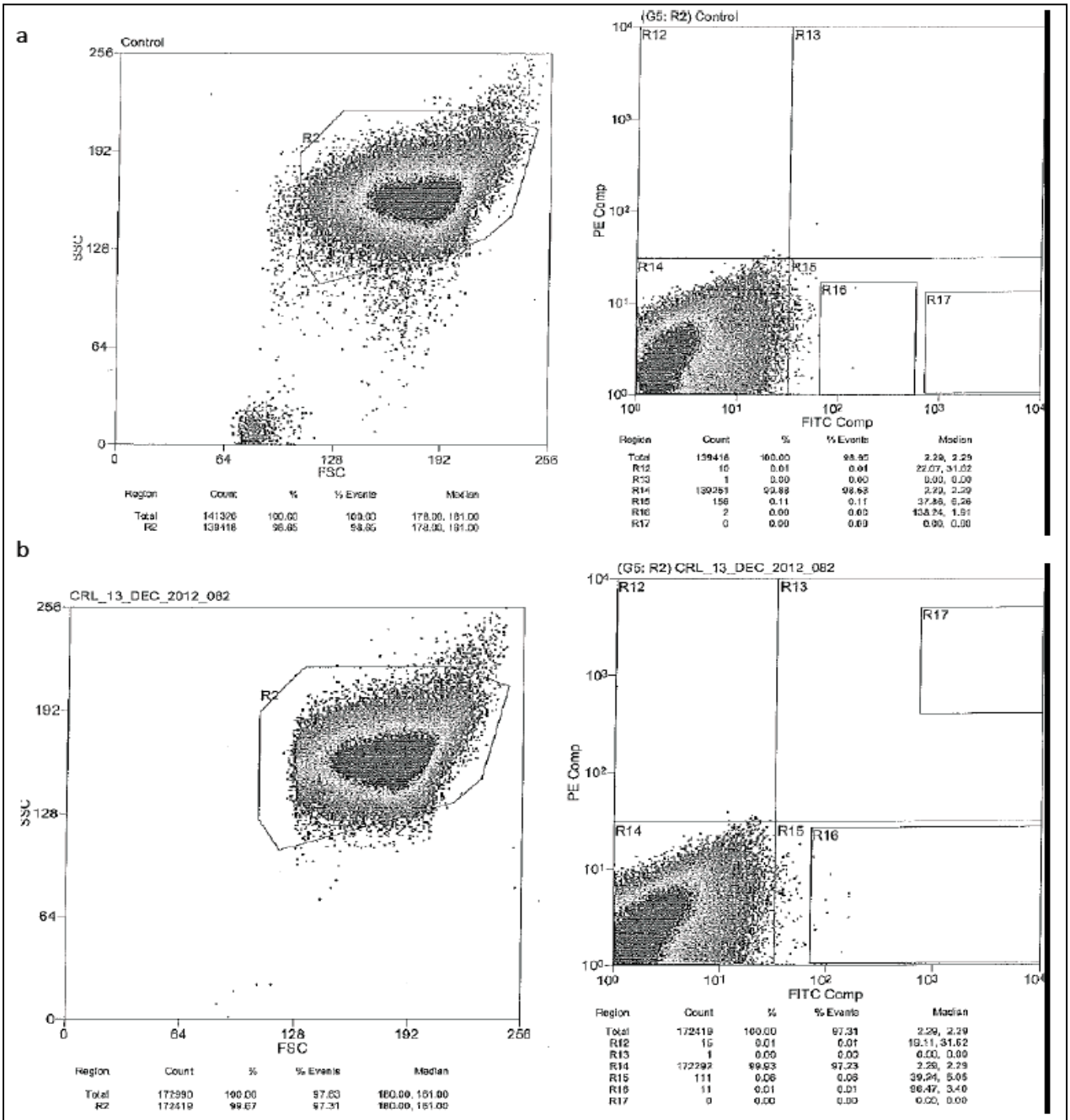

Figure 2. 4: First round FAC sorting.
FACS sorting of water treated control population (a) and mutagenized populations of germlings (b). Forward scatter (FSC) and Side scatter (SSC) plots are the same between control and mutagenized populations. Both populations were then analyzed for PE comp (internal control fluorescence) and FITC comp (GFP fluorescence). Section R16 is poorly sorting under control populations, and mutagenic populations sorts at a <.01% rate.

2.2.5 Secondary screening - functional secretion through high throughput induction

Utilizing CMC agar, we were able to assay the functional overexpression of mutant strains in a high throughput fashion. Strains were maintained in a 96 well plate format for growth and induction, and we assayed the enzyme activity of the cultures by spotting the induced supernatant culture to the CMC plate. Halo clearances were determined by 0.1% Congo Red exposure and de-staining with 1M NaCl. A higher amount of enzyme secreted into the culture supernatant resulted in a larger dispersal of CMC-degradation on the plate, which would be depicted by a subsequent increased halo clearance.

Utilizing the halo activity as a proxy for amount of cellulases secreted into the supernatant, we were able to ascertain strains that were increased in cellulase activity by computationally estimating the pixel intensity and radius of halo clearance. This semi-quantitative measure of the halo clearance allowed for a direct comparison to WT, to cellulolytic over-expression strains and to the background strain used for the mutagenesis. From the original 939 mutants sorted and grown up, we chose 68 of the top halo clearance strains for candidate follow up.

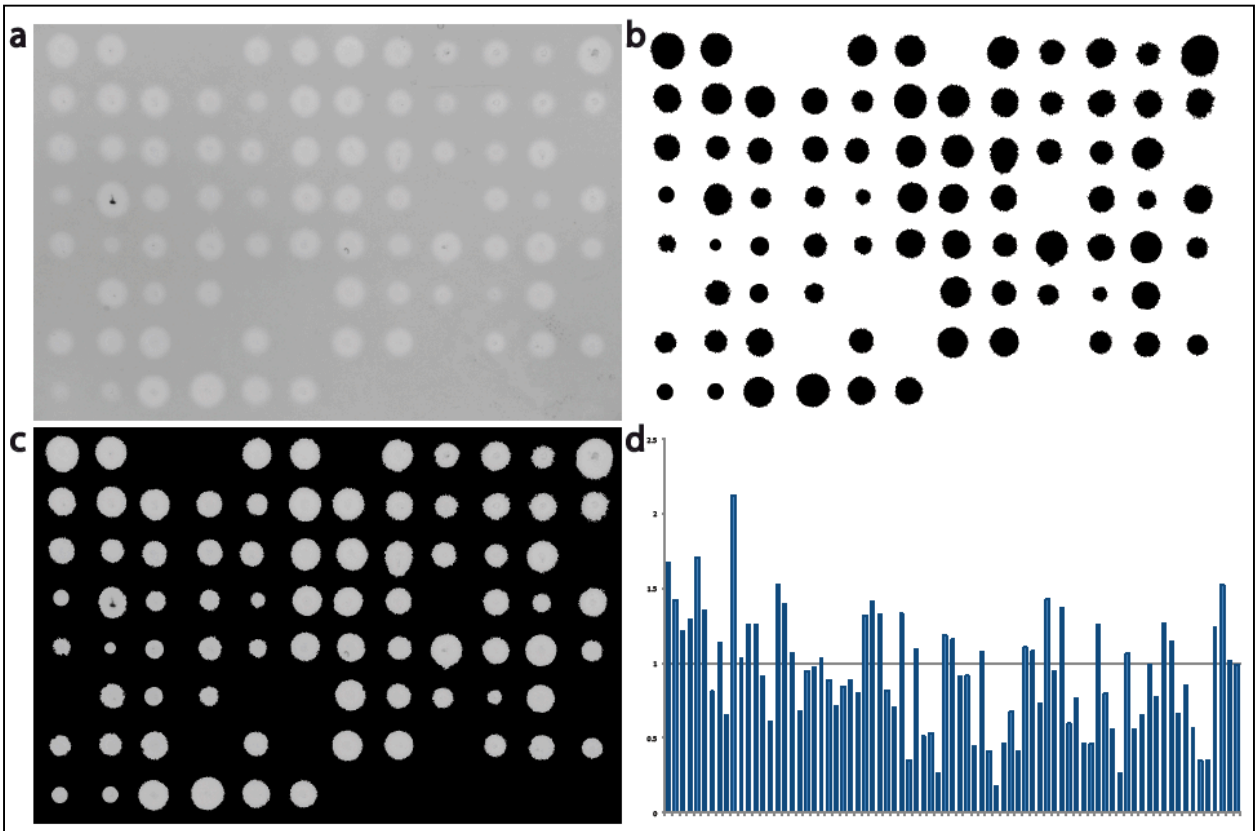

Figure 2. 5: Measurements of CMCase activity.
Original CMCase assay plate(a) shows clearance halos representing activity when stained with Congo Red solution. Masks (b) generated from halos and photo multiplication of masks and original image (c). Pixel intensity and area calculation of each halo is quantified and compared to starting strain levels (d).

2.2.6 Determination of first level hits

From the 68 candidates that were chosen for their increased halo clearance, cultures were grown on slants at 25ºC and inoculated in batch cultures using 100 mL of minimal medium in flasks. Cellulose suspensions were added to culture flasks for cellulolytic induction and incubated with shaking for 5 days at 25ºC. Time points were taken at 24h time points, and supernatants were assayed for protein content by Bradford assay, using bovine serum albumin as a ladder. Enzyme activity was assayed by incubating the supernatant with cellulose substrate for 16h, after which the amount of cellobiose and glucose released from the cellulose substrate was measured.

From the 68 isolates that we identified from the first level of screening, two strains showed increased protein secretion and cellulase activity significantly over wild type. The protein secretion/cellulase activity of strain 10F10 was increased over background by 2-fold ($p < .01$), and while strain 6G3 showed increased protein accumulation/cellulase activity by 1.5-fold (fig 2.6a).

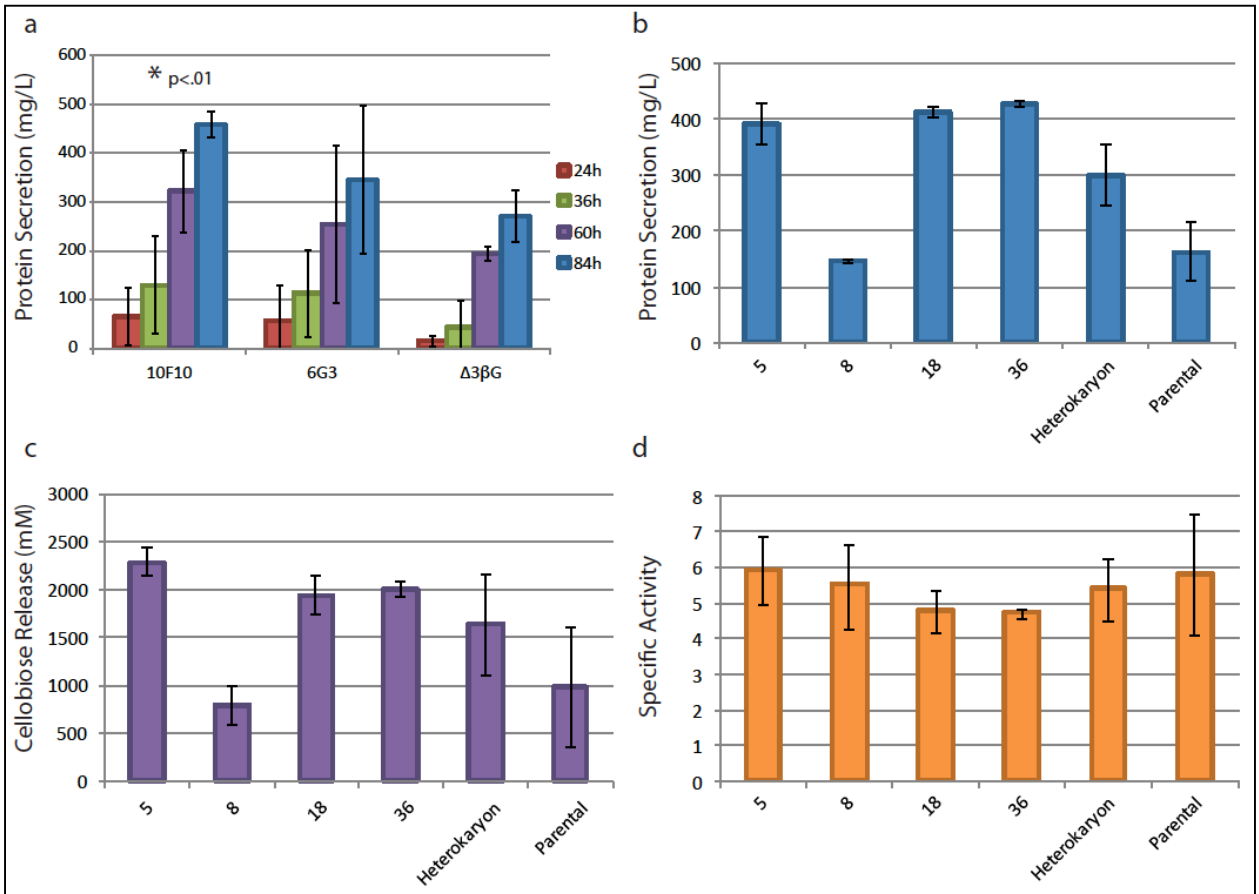

Figure 2. 6: **First round candidates and homokaryon enrichment.**
Supernatant of batch cultures of candidates 10F10 and 6G3 (a). Supernatant (b), Enzymatic activity (c) and specific activity (d) of batch culture supernatants of microspore isolates of the 10F10 heterokaryon.

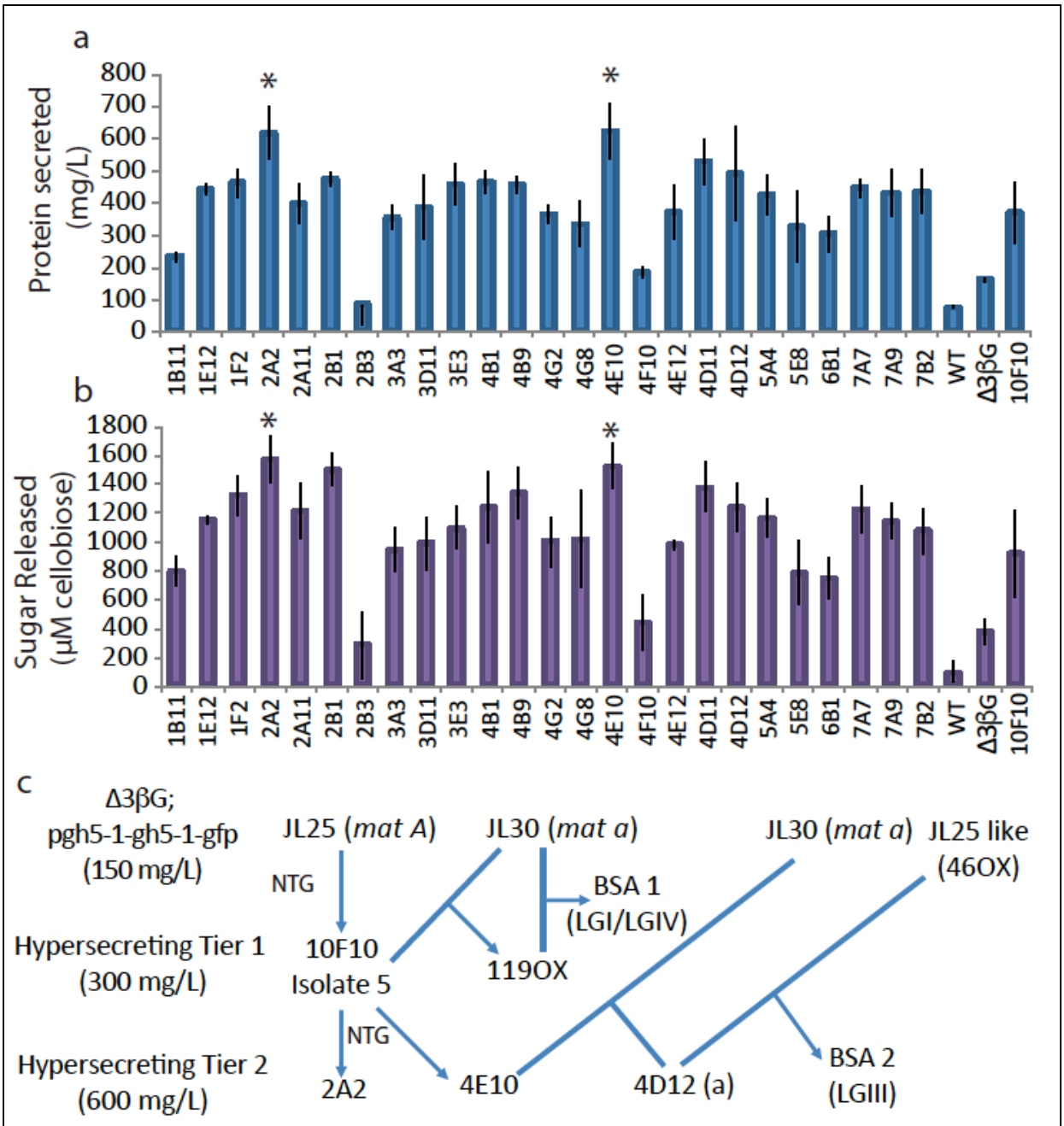

Figure 2. 7: Second level screening.
Protein secretion (a) and cellulase activity (b) of the second level hypersecreting screen. Hits 2A2 and 4E10 supernatants are 2 fold increased in protein and cellulase activity over the parental 10F10 strain. Asterisks indicate p<.01. Summary of mutagenesis and back crosses (c).

2.2.7 Homokaryotic enrichment

We assumed that the mutagenized strains that were isolated were heterokaryotic and contained mainly gain of function or dominant mutations because macroconidia, the spores we used for the mutagenesis, typically contain 2 or more nuclei per spore (Atwood 1955). In order to isolate the alleles that would be causal for the hypersecretion phenotype, we wanted to enrich for the mutant alleles without the possibility of the loss of an allele through meiosis due to meiotic or ascospore lethality. Unfortunately, we did not have markers for the component nuclei, so we enriched for homokaryons through uni-nucleate microconidial isolation (Atwood 1955). This approach would help to ensure a higher representation of strains that have the hypersecretion allele. We took the 10F10 strain and isolated 36 microconidial strains. Of the strains that we isolated from homokaryotic enrichment, we saw that the level of secretion was similar between the

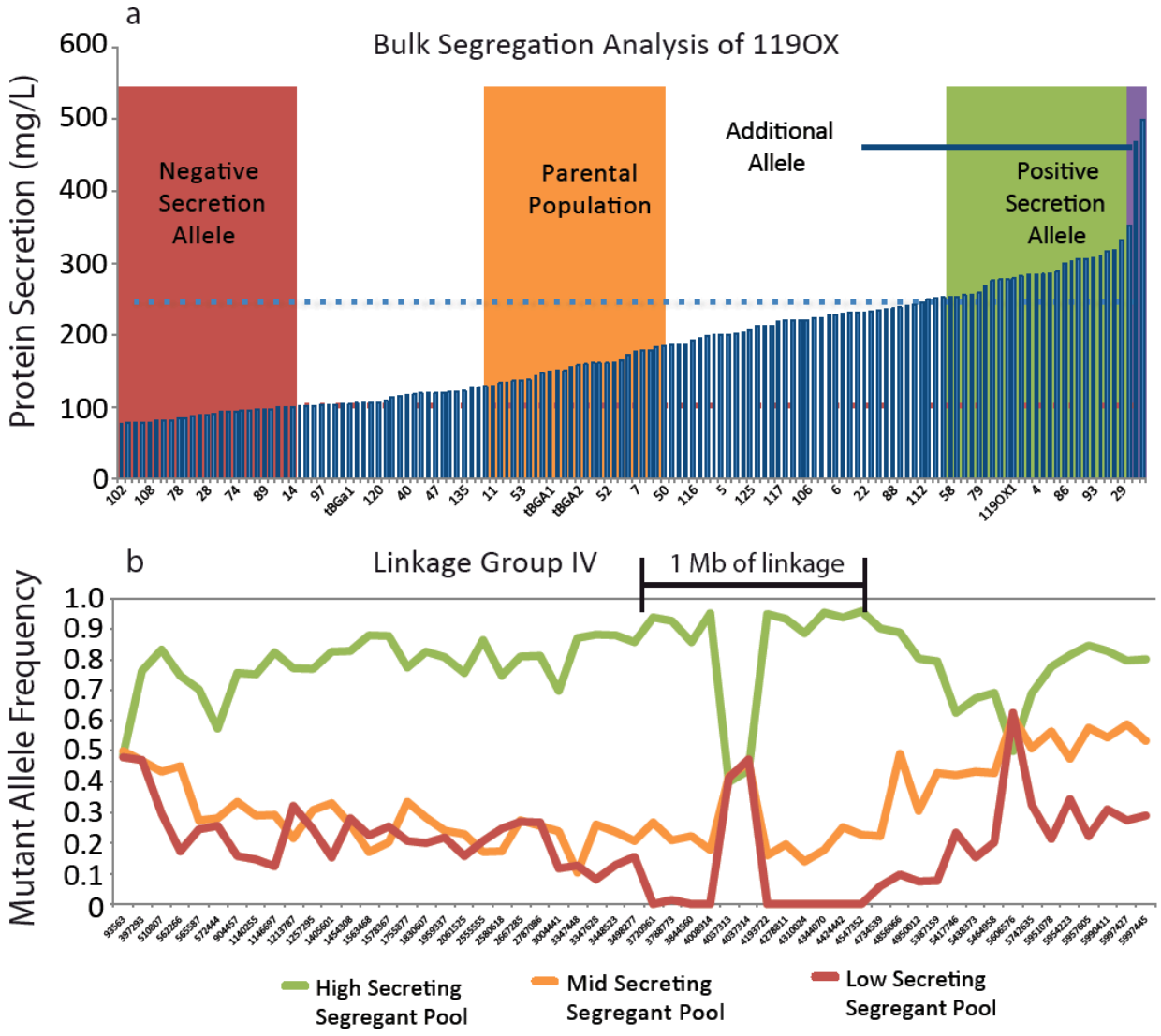

Figure 2. 8: Bulk Segregation Analysis of 10F10 progeny, 119OX.
Secretion ranked progeny (a), and genomic pools used for genomic analysis. Allele frequency distribution across Linkage Group IV implicates a high secreting allele on a 1Mb locus of LGIV (b)

hypersecreting strains, however, the specific activity of a select few isolated strains, 10F10 isolate 5 in particular, was activity, however, resulted in an alteration of supernatant specific activity. Instead of having a similar activity to the starting strain, the highest secreting isolates showed lower cellulase specific activity. Due to this result, we wanted to maintain the specific activity of the isolates and utilize 10F10 isolate 5 to continue our second round of mutagenesis (2.6 b, c, d).

2.2.8 Secondary level screening of hyper-secreters

We performed a second round of mutagenesis to find mutations that would compound the phenotype observed in the first level hypersecreting strain. 10F10 isolate 5 accumulated significantly less biomass from the same induction conditions, so we modified our mutagenesis to use a longer incubation period to accumulate more GFP and increase the resolution of GFP for FAC sorting. Thus, we utilized this slowed aspect of growth to incubate the germlings for 16h, higher than others (Fig 2.6b, c, d). Assays of cellulase
and FAC sorted the germlings into 96 well plates. $2.0x10^8$ germlings were induced on .01% cellobiose for 16 hr. Of the pool of $2.0x10^8$ germlings, cells were filtered and sorted for 1680 germlings. The ultimate sorting rate is .00084% and of the filtered germlings and 519 strains grew and conidiated, which represents a 3% survival rate. The low survival rate may be due to compounding lethality of accumulated mutations.

From this approach, two strains were isolated from the second level screening, 2A2 and 4E10. These strains showed a significant 2-fold increase in protein accumulation over the 10F10 isolate 5 strain ($p<.01$), a 4-fold increase over the starting Δ3βG strain and an 8-fold increase over WT strains (fig 2.7a, b). These hyper-producing strains are on par with the laboratory rates of protein accumulation of Rut-C30 (Montenecourt and Eveleigh 1977).

2.2.9 Backcrossing (10F10 isolate 5) and Bulk segregation analysis (BSA1)

In order to isolate the homokaryons of the hypersecretion allele, we back crossed the first level hypersecreter strain, 10F10 isolate 5 with the background strain, Δ3βG. From 136 strains that were isolated from the back cross, strain 119OX was one of the few progeny that showed a hypersecretion phenotype (fig 2.7c). Utilizing a 119OX back crossed with the original parental strain, Δ3βG, we isolated 136 strains of the secondary cross progeny for screening for bulk segregation analysis (fig 2.7c, fig 2.8a).

From the 136 strains that were analyzed in triplicate, we ranked the strains by the titer of protein accumulation in the supernatant by Bradford assay. We were surprised by the population of strains that accumulated significantly less protein than the parental, Δ3βG control, so we included this population in our sequencing analysis. We also observed that the heterokaryotic 10F10 isolate 5 strain accumulated significantly higher amounts of cellulases in the culture supernatant than even the highest secreting strain from the bulk segregation analysis. From this, we concluded that the 119OX did not contain all the alleles from the heterokaryotic hypersecreters strain.

From the backcross progeny, we bulked the 35 highest secreting strains for our high-secreting population, the 35 strains surrounding the average of the parental, Δ3βG, measurements as the mid-secretion population and the 35 lowest secreting strains as the low-secreting population (Fig 2.8a). DNA was isolated from each of the 105 strains that were identified from population analyses, pooled approximately equally within the bulked populations, and prepared for library construction, sequencing and genomic analysis.

2.2.10 Alleles analyzed on LGI and LG IV from the 10F10 back cross

From the bulk segregate analysis of the first tier hypersecreter, two linkage groups were identified as having causal alleles for the hyper-secretion phenotype. Linkage group IV contained the positive alleles for secretion. This was indicated from the BSA allele frequency that segregated the high secreting population from the mid-secreting and the low secreting populations (Fig 2.8b).

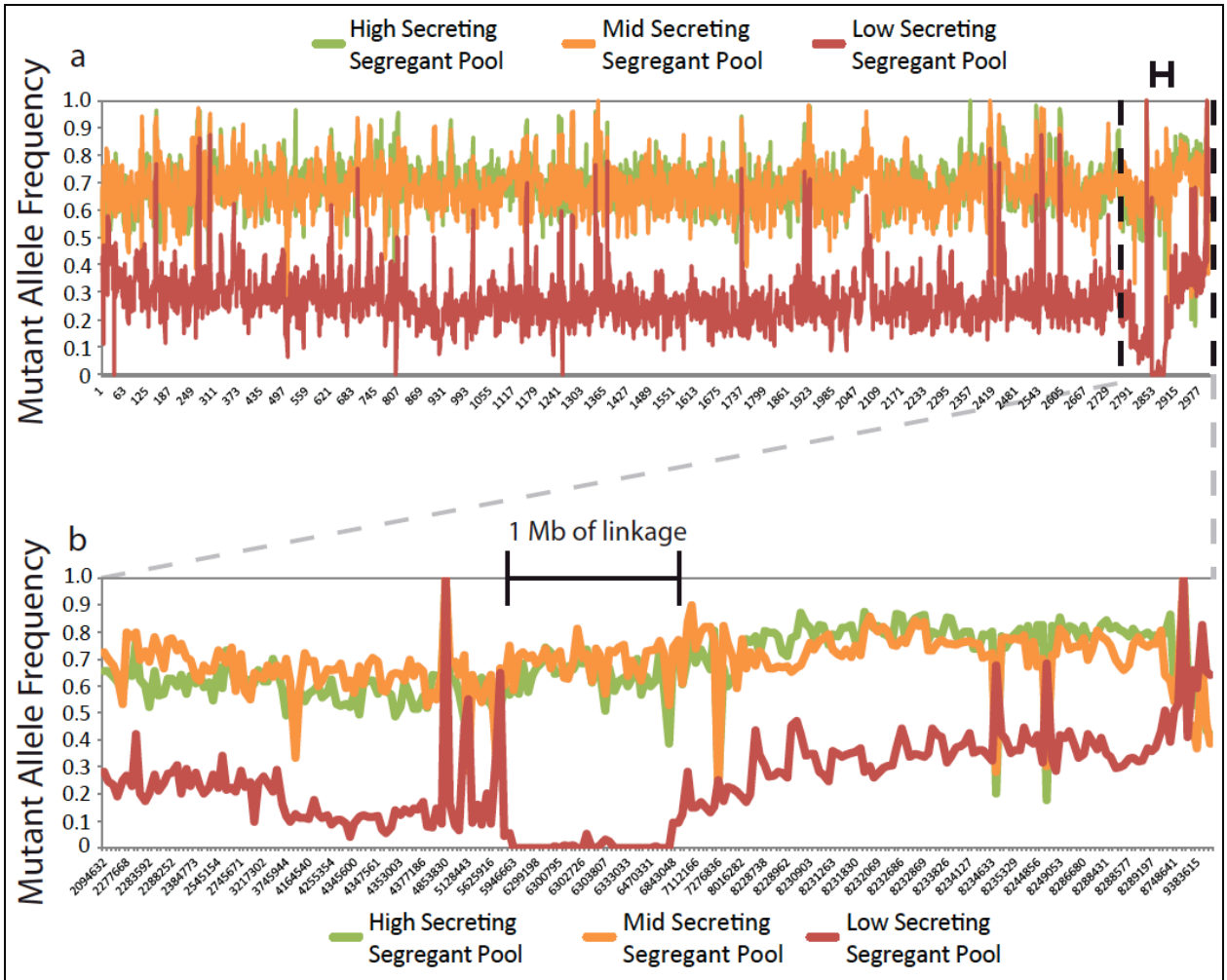

Figure 2. 9: Secondary allele of 119OX progeny.
Linkage group I had 3000 alleles spanning the 10 Mb chromosome (a). The Low Secreting Segregant had a lower than expected basal frequency, but a drop in allele frequency was seen at the far right arm of Linkage group I. Zoomed in visualization of this interval marked by arrows (b)

The region on the far-right arm of LG1 is implicated in the low secreting phenotype as the low secreting progeny group allele frequency was the most bifurcated from the mid-secreting and the high secreting progeny (2.9a, b). Thus, in order to follow up on this locus, we mapped the changed single nucleotide polymorphisms, and identified the open reading frames that were most drastically altered by non-synonymous mutations in coding sequences.

2.2.11 Screening of alleles

The regions of LG IV and LGI included a 1 Mb region of linkage to the causal allele that was most strongly correlated with the higher (LGIV) and lower (LGI) secretion phenotypes pools. We utilized single nucleotide polymorphism (SNP) information from the genomic sequencing to identify genes mutated in important genetic elements including coding sequences, promoter sequences (within 1kb of translational start site), and terminator sequence (Galagan, Calvo et al. 2003). Of the 11 genes of Linkage Group IV and the 10 genes of Linkage group I that were identified to be altered, we screened the available knock out library mutants for increased protein accumulation phenotypes (20 genes in total) (Table 2.1).

Of the strains that were screened, we identified one knock out strain, representing the serine-threonine kinase NCU07378 (*stk-12*) that showed nearly 2-fold increase in protein secretion. The serine threonine kinase was promising as a hit, as the kinases are would be able to quickly modulate expression or feedback to transcription. It is a highly conserved gene among filamentous fungi. However, when we mated the *Δstk-12* strain with a WT strain, progeny segregated in a manner that was not consistent with the hygromycin marker being the causal allele. Thus, it appeared that the *stk-12* knock out strain contained an alternative allele that did not track with hygromycin marker, but may be causal for the secretory phenotype of the Δ*stk-12* strain. This result meant that the knock out strain collection was not useful in helping to isolate the causal allele from LGIV or LGI, and that we would have to use integrations of the mutant ORFs in a WT-like genome to determine if the causal alleles were due to a subtler change as a result of SNPs rather than an ORF deletion or, alternatively, a gain-of-function mutation.

2.2.12 LG IV and LG I causal allele identifications

During our studies, we made several attempts at determining the causal loci for the first level secretory strains. The first attempt used the pCSR plasmid to integrate the mutant alleles ectopically at the CSR locus for simple cloning and verification. The second approach utilized the knock out collection to cross strains of the mutant into the his- background from which we could then ectopically integrate the mutant alleles at the *his-3* locus for analysis in the background of the knock out mutant. The third approach was to amplify the mutant alleles from the hypersecreter genome and integrate the mutated ORFs at the native locus with a marker for nourseothricin resistance either 1000bp upstream of the translational start site, or downstream of the predicted 3'UTR.

Linkage Group IV - High secretion allele				
NCU#	Function	Coding alteration	KO secretion relative to WT	Notes
NCU04442	Gal10 epimerase	A247T	50%	
NCU04415	**Hypothetical**	**D100N**	**75%**	
NCU04341	**Hypothetical**	**D66N**	**85%**	
NCU07996	**F-box, WD**	**P537A**	**75%**	
NCU11282	**DHAP-AT**	**G350S**	**n/a**	No KO available
NCU07341	**Hypothetical**	**Promoter**	**85%**	
NCU07332	**Hypothetical**	**Silent mutation**	**80%**	
NCU07308	**Fatty Acid Synthase**	**G1344D**	**80%**	Heterokaryon
NCU07277	**ACW-8**	**A159T**	**65%**	
NCU06976	**Dynein RO-1**	**L1385L**	**100%**	Heterokaryon
NCU07024	OS-2	A200V	100%	
Linkage Group I – Low secretion allele				
NCU#	Function	Coding alteration	KO secretion relative to WT	Notes
NCU03087	Hypothetical	3' UTR	100	
NCU10042	**Hypothetical**	**L137F**	**85%**	
NCU07396	**Hypothetical**	**Downstream**	**100%**	
NCU07378	**STK-12**	**S714L**	**180%**	
NCU09875	**Hypothetical**	**A373V**	**75%**	
NCU00992	**Hypothetical**	**Promoter**	**n/a**	
NCU00978	**Hypothetical**	**A655V**	**85%**	**Heterokaryon**
NCU10847	**NGF-1**	**K16K**	**0%**	
NCU00895	VTR-1	5' UTR	85%	Heterokaryon
NCU00800	Hypothetical	A657T	85%	

Table 2. 1: Hits from Linkage Group IV and Linkage Group I.
Bold genes are most highly segregating alleles from Bulk Segregation Analysis

2.2.13 pCSR integration leads to an artifact of secretion

In order to examine the ORFs that may be mutated due to the mutagenesis, we first used the pCSR vector in the mutant backgrounds so we could isolate transformants carrying the hygromycin resistance cassette of the knockout and the cyclosporin cassettes of the ectopic integration of the mutated alleles into the CSR-1 locus (Bardiya and Shiu 2007) . After cloning 12 of the genes that were altered with non-synonymous mutations, (NCU04442, NCU04415, NCU07378, NCU11282, NCU00800, NCU10847, NCU00978, NCU09875, NCU10042, NCU07308, NCU07277, NCU07996) for transformation using the pCSR locus, we screened for phenotypes on primary transformants looking for changes in protein accumulation which would indicate a causal mutation. But with every transformation, we saw at least one primary transformant that was performing >2-fold higher than WT. As a result of this screening, it quickly became apparent that the high secretion rate was much too high for a single causal allele. Instead, we hypothesized that transformation of the parental pCSR vector was actually resulting in an artifact of secretion.

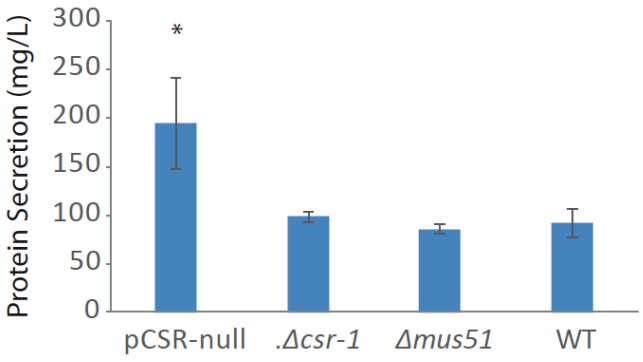

Figure 2. 10: pCSR-null integration results in hyper secretion
Asterisk represents p<.001

In order to confirm this result, we pulled out the knock out mutation of the *csr-1* gene from the KO library, and performed our own integration of the pCSR empty vector into a WT strain (fig 2.10). Indeed, as we hypothesized, the pCSR empty vector integration caused an aberrant accumulation of cellulases and protein in the supernatant. While the knock out of the *csr-1* gene was not causal for hypersecretion on its own, the resulting data indicates that cis-acting elements of the pCSR vector was causing a hypersecretion phenotype. Thus, the hits we were seeing from the ectopic integrations of the mutated ORFs were most likely false positives from the pCSR transformations, and results in an uncharacterized hyper-secretion phenotype (fig 2.10).

As a result of inconsistency of the secretion phenotype of *csr-1* integrants, we altered our methods for another approach to identify causal alleles using the ectopic integration at the *his-3* locus. In order to do this, we mated a FGSC6103 (*his-3 mat A*) or FGSC9716 (*his-3 mat a*) strain into the knockout background to isolate cross progeny that would be HygR+; *his-3*. This would mean the knock out mutation was in the progeny background along with the his- parental strain, that once transformed, would result in primary transformants that would be able to represent a single mutant ORF. The pMF272 was not adaptable to the current cloning sites, so we altered the restriction sites for an allele swap that would allow for compatible cloning between pCSR and pMF272 vectors. While cloning was efficient, the transformed progeny did not provide any clues to the necessary alleles for hypersecretion.

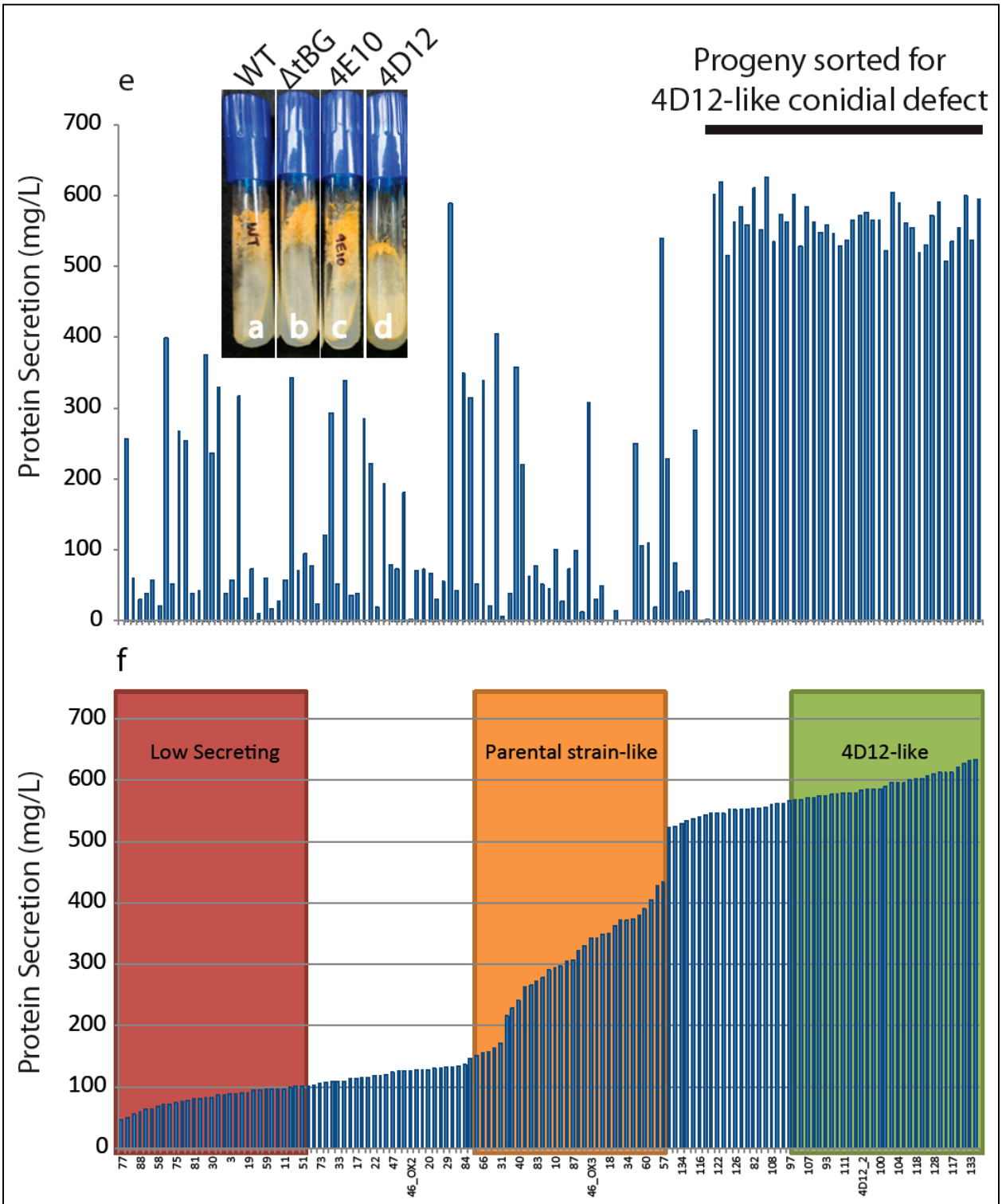

Figure 2. 11: Bulk segregant analysis of 4E10 progeny, 4D12.
Conidiation defect of 4D12 (a) is highly correlated with secretion (b). Secretion ranked progeny and bulked genome pools (c).

Finally, we began cloning of the mutant ORF into the native loci of the parental genomes. This would allow for the mutant allele to be expressed as a single allele in the strains once they are transformed and crossed. The construct amplifications were performed by generating the mutant SNP with mutant genomic sequence equal to the flanking region to the nourseothricin resistance cassette and the shorter length to either the complete 3'UTR or 1000bp upstream of the translational start site. The nourseothricin resistance construct was then added and an additional 1000 bp of genomic sequence was added to the nourseothricin resistance cassette 3' flank. This cloning, however, was difficult as many of the constructs were >8kb long and were poorly amplified in the primary PCR's. While the results of our approaches were inconclusive, the data strongly points to an allele within both genomic intervals (table 2.1).

2.2.14 Backcrossing of second tier hypersecreter, 4E10, and Bulk Segregant Analysis 2 (BSA2)

After uncovering the secondary level strains of hypersecretion, we performed back crossing and focused on alleles that would provide a phenotype as a single copy, but were not lethal in its loss. This would allow for the identification of alleles through bulk segregation analysis.

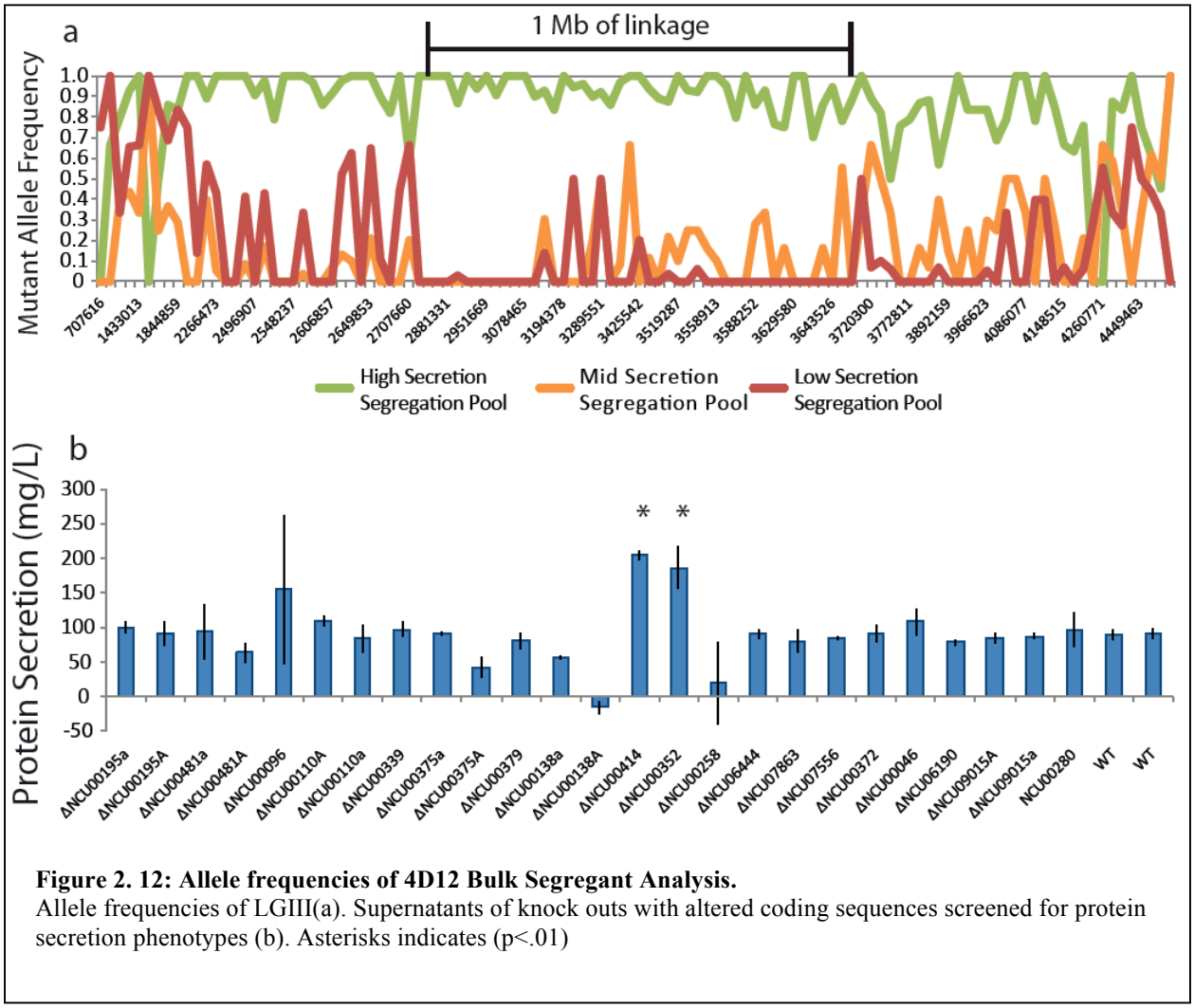

Figure 2. 12: Allele frequencies of 4D12 Bulk Segregant Analysis.
Allele frequencies of LGIII(a). Supernatants of knock outs with altered coding sequences screened for protein secretion phenotypes (b). Asterisks indicates ($p<.01$)

In order to follow the crossed mutants, we made a cross of the 4E10 second round hyper-secretion mutant with a the parental Δ3βG mutant. Utilizing our 96 well CMC-assay protocol, we isolated 600 strains that were homokaryotic by crossing. Using this assay, we discovered that the causal alleles were very lowly represented in the genome, 4 of the 600 strains isolated had a high secretion phenotype by CMC-assay, and of those screened, all of the increased secretion phenotypes also correlated with a defect in conidiation (fig 2.11a-d).

Of the strains that were most tractable was the 4D12 strain from the 4E10 backcross. The secretion rate of the 4D12 mutant was comparable to the 4E10 strain, so the mutant probably contains mutations that were causal for the increased secretion phenotype. This allowed us to make an inference that the alleles that were hit most likely did not have a lethal phenotype (fig2.11e).

2.2.15 Alleles that were analyzed from the LG3 back cross

The bulk segregant analysis of the second tier hypersecreting 4D12 back cross showed the strongest genomic interval of linkage at a 1 Mb region on Linkage Group 3 (fig 2.12a). The segregation interval included SNPs in 26 coding sequences. 19 knock outs and heterokaryotic knock out strains from the library were initially obtained to test for cellulase secretion phenotypes. Three genes were identified from the hits, NCU00096, NCU00414, NCU00352 (fig 2.12b).

The mutant of ΔNCU00096, a hypothetical small protein of 236 amino acids, showed an insignificant 1.5 fold increase in secretion over the WT strain (2.12b). A search of the NCBI database using BLASTp resulted in protein hits specific to the *Neurospora* genus, most notably a small protein in *Neurospora discreta*. Due to the low conservation of the small protein among other fungi, this was placed as low priority due to the hypothetical nature of the protein and the unclear similarities between the gene and the possibility of its role in other fungi.

Two more conserved proteins that were identified were NCU00414 and NCU00352, which encode an adenosine kinase (*ad-15*) and a phospholipid flippase (*drs-2*), respectively. The mutant of *ad-15* had a significant 2-fold increase in protein accumulation over WT. The *ad-15* mutant may result in altered AMP/ATP ratios that could alter cellulase regulation. The NCU00414 had an added benefit of showing a reduced conidiation phenotype that would at least explain the 4D12 low conidiation phenotype. However, when genotyped by PCR for the hygromycin resistance cassette marking the *Δad-15* knock out, there was a lack of a HygR+ band. These data indicated that an alternative allele in the Δ*ad-15* strain may be contributing to the hyper-secretion phenotype in the knock out strain. Since the mutation causing the secretion phenotype is unmarked by the cassette, we decided that there may be a separate contamination, or an alternative allele that may be contributing to the hyper-secretion phenotype and did not pursue the Δ*ad-15* mutant.

Lastly, we looked at the strain containing the knock out mutant of NCU00352 (*drs-2*), which encodes a phospholipid flippase that maintains lipid asymmetry on the lipid bilayer. We confirmed the correctly integrated hygromycin resistance cassette in the genome, and moving

forward, we used this candidate allele to analyze the mechanisms of hypersecretion through complementation.

2.2.16 Complementation of the Δ*drs-2* mutant with *drs-2* alleles from WT and 4D12

We hypothesized that the Δ*drs-2* knock out allele would be causal for secretion if we could backcross the allele, and see full correlation of the HygR$^+$ marker with the high secretion phenotype. Secondly, if indeed, the *drs-2* of 4D12 was causal for secretion, the *drs-2*^{4D12} allele would not be able to complement the secretion phenotype of the Δ*drs-2* knock out.

In order to test the first hypothesis, we back crossed the Δ*drs-2* knock out mutant with the WT strain and blindly phenotyped 30 progeny. We then took the same progeny and grew them on HygR$^+$ media to test whether the strains contained the HygR$^+$ cassettes. When combined, these data indicated that secretion of the progeny segregated into two classes. Fifteen progeny accumulated protein at the level of the Δ*drs-2* mutant, and the other fifteen accumulated supernatant protein levels similar to WT. When correlated with the HygR$^+$ strains, we observed a complete correlation of strains containing HygR$^+$ and a hypersecretion phenotype (fig 2.13a) (p<.001). From these results, we can infer that the hygromycin resistance cassette inserted at the *drs-2* locus is causal for the hypersecretion phenotype.

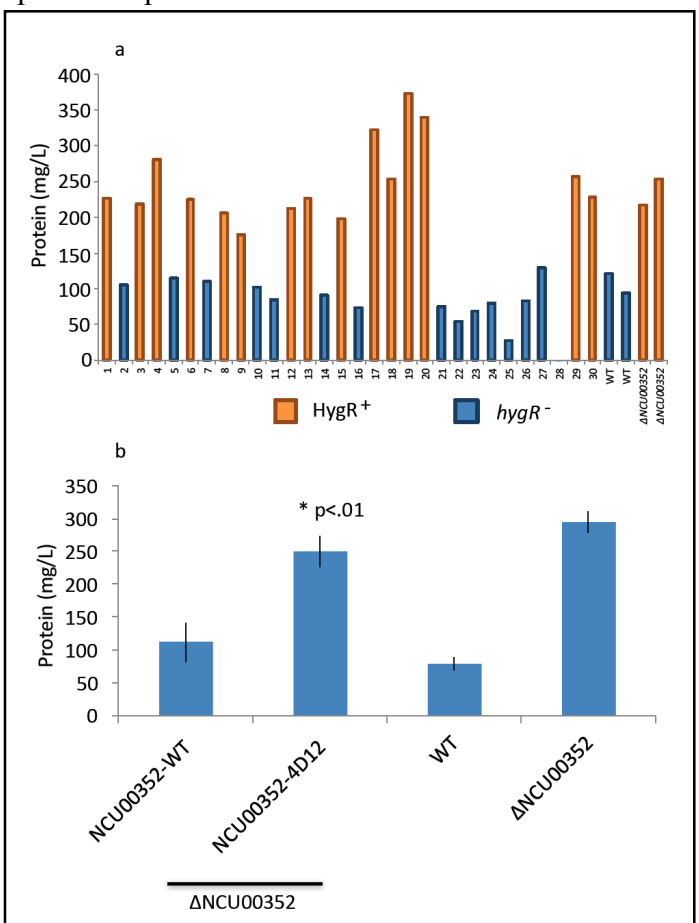

Figure 2. 13 Protein secretion complementation of Δ*NCU00352*
HygR$^+$ marked Δ*NCU00352* was back crossed and phenotyped (a). Correlation is p <.001. Complementation of WT NCU00352 rescues knockout, 4D12 allele does not rescue knockout phenotype (b).

In order to test the second hypothesis on whether the NCU00352^{4D12} allele causes a secretory phenotype, we mated the HygR$^+$ ΔNCU00352 mutant to a *his-3* strain to generate a ΔNCU00352; *his-3* strain. We then complemented the knock out mutation with the NCU00352^{WT} allele, and observed a near-complete rescue of the ΔNCU00352 hypersecretion phenotype (fig2.13b). When we then complemented the knock out strain with a NCU00352^{4D12} allele, we observed a maintenance of the protein accumulation of the ΔNCU00352 mutant, which indicated to us that the NCU00352^{4D12} allele did not complement the *drs-2* deletion. Thus, we concluded that the *drs-2* allele of 4D12 is causal for the cellulase hyper-accumulation phenotype.

2.3 Discussion

Cellulase hypersecretion has revolutionized the fields of food, fuel and textiles (Kuhad, Gupta et al. 2011, Payne, Knott et al. 2015, Reilly, Magnuson et al. 2016). The fundamental process by which many of these industries are able to stay profitable is through the efficient hypersecretion of these enzymes, however, many of the necessary components to generating a hypersecreting strain of a filamentous fungus are still unclear (Reilly, Magnuson et al. 2016). Previous efforts to understand cellulase hypersecretion have been described in the industrial model species, *T. reesei* (Le Crom, Schackwitz et al. 2009). In this work, we generated a cellulase hypersecreting mutant in the genetically amendable filamentous fungus *N. crassa*, and have dissected out candidate intervals and a causal allele for cellulase hypersecretion.

By updating the screening protocol of generating a hypersecreter to include FAC sorting as the primary isolation, we could quantitatively measure the accumulation of GH5-1-GFP in the cell periphery of germlings and quickly sort at <.01% threshold for finding extreme GFP-accumulating cells. The key to the success of the screening, however, was the generation of a sensitized Δ3βG; GH5-1-GFP strain, which was able to induce a trackable cellulolytic response that was independent of an insoluble cellulose inducer.

After mutagenesis and sorting, our secondary screening allowed for the confirmation of the cellulolytic response, which helped us to weed out potential artifacts of GFP accumulation through a functional assay of cellulase secretion. Our efforts then quickly focused down to 1-2 hypersecreters after each round of mutagenesis. The quantitative nature of the screen allowed us to reiteratively generate mutant strains that accumulated more cellulase protein, and after two rounds of mutagenesis the hypersecreting strains are currently at the level of secretion that is similar to the industrial precursor strains under batch culture conditions.

Artifacts of the FAC sorting have also been useful. In an auxiliary study, we performed a microscopy based counter-screen of the sorted mutant library for isolated strains that aberrantly accumulate intracellular GH5-1. Using this screen, we determined that the FAC sorting was powerful enough to enrich for cells mis-trafficking GH5-1 at a rate of 1% (Jun, unpublished results). These mis-trafficking mutants would most likely determine the secretory pathway of GH5-1, and should be investigated further. Pertaining to this study, these data helped to confirm the ability of the FAC sort to isolate *N. crassa* cells increased in GFP fluorescence.

Identification of the causal allele for the hypersecretion phenotype, however, has been more difficult of a task, as anticipated. The mutagenesis performed resulted in the accumulation of SNPs at random in the genome at a rate of 0.2 mutations/kb of genome. From a single backcross and sequencing, we observed 1 Mb of linkage for each causal interval, which resulted in approximately 200 SNPs that needed to be investigated in each interval. Some steps we could have taken to simplify the analysis was to perform a back cross after we found the hypersecreter, 10F10. The back cross would ensure that the strains were homokaryotic, and while we would perhaps lose an allele through potential mutants in the sexual cycle, our post-mutagenesis deconvolution of the SNPs would have been much more streamlined for identifying compounding alleles.

Our attempts at dissecting the middle tier hypersecretion mutants, 10F10/119OX, were ultimately inconclusive despite significant effort, but we observed two secretion phenotypes, a hypersecreter mutant allele on LGIV, and a low secreting mutant allele on LGI. From our analysis, we have narrowed down the region of our search to include the 12 genes on LGIV that were altered during the mutagenesis (table 2.1). The knockout of these genes, when available, did not show a significant cellulase hyper-accumulation phenotype, however, these may be due to, (1) the potential lethality of the knock out mutation. Our work flow was significantly streamlined by the use of the knock out strain collection to quickly screen mutant alleles for hypersecretion. If the genes are essential, they were not included in the library or deposited as a heterokaryon, and thus, either excluded from our knock out screening, or the gene dosage of the heterokaryon was not high enough to show a phenotype. (2) The mutant allele may be a gain-of-function mutation that is not reciprocated in the knock out mutant, which would necessitate cloning of each allele in order to reconstitute the mutant phenotype. (3) The SNP could possibly be in a trans-acting element or cis-regulatory element with an unpredicted function outside of the coding sequence, untranslated regions, and promoter. (4) The causal interval may actually contain synergistic alleles that may play a role in the hypersecretion phenotype.

On Linkage Group I, the bulk segregation analysis focused down to 10 genes that have introduced SNPs within the identified interval (fig 2.10b) (table 2.1). By using the knock out collection, we identified one strain, Δ*stk-12*, that showed a hypersecretion phenotype. However, subsequent assays on segregation of the $HygR^+$ cassette indicated that the secretion phenotype and hygromycin resistance did not fully correlate together. We thereby concluded that a mutation elsewhere in the genome was influencing the secretion phenotype without being marked by the $HygR^+$ cassette. While the deletion of *stk-12* may not contribute, our experiments at complementing the Δ*stk-12* with the WT and 119OX allele was inconclusive. Thus, the point mutation may still be the causal mutation in the hypersecretion mutant that was isolated. Future work on cloning the mutant allele into a WT background would help to identify the role of mutated NCU07378 in the 119OX secretion phenotype.

Transformation of the pCSR plasmid had an artifactual response to secretion as the Δcsr-1 knockout of the gene was not the sole responsible factor for the increase in secretion. Adding an exogenous pCSR for higher protein content was quite interesting though, as this may be a conserved mechanism to increase protein secretion in filamentous fungi. Use of the *M. thermophile* GPD promoter and terminator may be causal for the artificial secretion phenotype and future work can test if a pCSR plasmid devoid of these coding elements integrated into the genome would have similar results.

In so far, we have isolated two strains made by the knock out consortium that has contained an alternative allele for hyper-secretion. The knock out collection utilizes a strain that is defective in LIG-4 dependent non-homologous end joining (Δmus-51/Δmus-52) (Ninomiya, Suzuki et al. 2004). By mutating this DNA repair pathway, these starting strains for generating the knock outs may be sensitized to the accumulation of mutant alleles. Previous work on the collection for soft phenotypic mutants found that many strains have accumulated mutant alleles through a high frequency of soft mutations that do not co-segregate with the knock out cassette (http://www.fgsc.net/NeurosporaGenomeProject/Sfree/FreeCosegData.htm). If hypersecretion is as similarly prevalent as the soft phenotype, one route of discovering new alleles for

hypersecretion would be to screen the knock out library for marked, hypersecreting alleles and hypersecreting alternative alleles. If it is the latter, bulk segregation analysis outlined in this study may help identify the actual cause of hypersecretion in these knock out strains. Presumably the rate of mutation is lower than using a mutagen, thus the rate of causal allele identification would be enriched by using the alternative alleles. These mutations that have accumulated through the knockout collection would be extremely helpful in the discovery for new causal alleles, and may add to our current understanding of hypersecretion.

In summary, we have been successful in generating a hypersecreting mutant of N. crassa that accumulates 8-fold more protein in the culture supernatant over WT. We have also been successful in identifying a causal allele of the hypersecreter mutant, *drs-2*, that when knocked out, gives a significant hyper-accumulation of protein as compared to the WT strain. We also have two other genomic intervals that are of high importance for follow up work because the alleles are able to influence cellulase secretion, and a handful of new unidentified hypersecretion mutants that, once elucidated, may help populate a genetic toolbox for generating fungal cellulase hypersecreters in the future.

2.4 Methods

2.4.1 Media and strain maintenance

N. crassa strains were cultured on Vogel's medium (Vogel 1956) agar supplemented with 2% sucrose for 7 days at 25ºC light to obtain conidia for experimentation. Crosses were performed on Westergaard's media (Westergaard 1947). The wild-type strain (FGSC2489) and the single-gene deletion strains (Colot, Park et al. 2006) were obtained from the Fungal Genetic Stock Center (FGSC, University of Missouri, Kansas City, Missouri, USA)(McCluskey 2003). The Δ3βG strain construction was described previously (Znameroski, Coradetti et al. 2012) and the EG2-sfGFP strain was a gift of Dr. Trevor Starr (Energy Biosciences Institute).

2.4.2 Mutagenesis

The mutagenesis of *N. crassa* was performed using NTG hydrated under 50% water solubilized under a 50mM NTG of a 1000x stock and frozen in aliquots. Solutions of NTG was thawed as needed. Cultures of *N. crassa* conidia were placed in a 1x PBS buffer (pH 7.0). Conidia were washed 3 times with 1x PBS and then adjusted to $1x10^8$ cells per culture. 25mL of culture was placed in each 50mL conical and 50uM NTG placed in each conical, using one conical tube with ddH_2O as a vehicle treatment. Tubes were incubated at room temperature on a 20rpm nutator for 2h. Cells were then centrifuged at 4000rpm in a tabletop centrifuge, and washed with 1x PBS + 0.05% sodium thiosulfate. Cultures were washed 3 times with PBS + thiosulfate buffer, then washed 3 times with 1x Vogel's salts. Two volumes of 1x Vogel's salts + 0.01% cellobiose was washed into a 250mL flask and incubated for variable hours depending on the experiment.

2.4.3 FACS analysis

After incubation of the two cultures, one control and one mutagenic, the cultures are immediately placed on ice and all subsequent steps were performed at 4ºC. The cultures were centrifuged at

1000rpm on a tabletop centrifuge and resuspended in 5mL of residual medium. The culture was filtered through a 20um filter top into a 7 mL polypropylene tube and sorted using the filtered cultures at a theoretical 10^7/mL start of culture. Starting with the control cultures, we determined the conidial sorting rate through Forward scatter (FSC) as a proxy for size and Side scatter (SSC) as a proxy for viability. Once the germling populations were properly gated, they were plotted for populations of GFP. The increase in GFP populations were then thresholded for less than 0.01% sorting per $2x10^5$ germlings. Control cultures were then switched out for mutagenic cultures and sorted using the controlled thresholds. Sorted germlings were then isolated into 1x Vogel's + 2% sucrose medium and incubated in 25ºC light for 7 days. Cultures were then frozen down to -80ºC with 30% glycerol.

2.4.4 CMC-agar assay and image quantification

Using a 96 well plate for growth, we inoculated the sorted hits on 2% glucose Vogel's minimal media for 24h, upon which we then induced using a total volume of 0.1% cellobiose for 48h. At this point, the secreted enzymes should be contained in the supernatant of the medium. Utilizing 15cm Petri plates filled with 50 mL 1% low viscosity CMC- 1.5% agar, we plated 1uL of the supernatant onto the plate, and incubated the plates at 37 ºC for 2 h. After incubation, we stained the residual CMC agar with a 0.1% solution of Congo red, a hydrophilic amyloidophilic dye which adheres to the cellulosic fibrils of CMC. After staining for 1h at room temperature, we then washed the plate 3 times with 1M NaCl to destain the plates and observe the halos of clearance that appeared due to the ability of cellulases to clear the CMC. Images were obtained with a Biorad imager using a white filter transilluminator. Image masks of the halo plates were generated by threshold and quantified using ImageJ.

2.4.5 Batch secretion and activity measurements

Candidate cultures were inoculated at $2x10^7$ conidial spores for a 100 mL flask of 1% glucose minimal medium cultures. Cultures were then incubated at 200rpm at 25ºC for 30h, which then 5 mL of 20% cellulose (Avicel PH-101, Sigma) suspension in water was introduced to the cultures to produce a 1% cellulose induction. This total amount of 2% carbon results in full digestion by the end of 120h, using WT strains, thus, we used this induction procedure as a reference for screening future strains. Activity was measured by the addition of 5 µL of culture supernatant to a 1% cellulose suspension in 100 mM Sodium acetate solution (pH 5.0). Suspensions were incubated for 16h at 50ºC with nutation. Post nutation, cellulose was allowed to settle, and the supernatant was measure for sugar released from cellulose using 2 mM DCPIP and CDH-1 protein isolated from *P. pastoris* (Fang, 2013) for measuring full redox potential.

2.4.6 Genomic pooling and analysis

Bulk pools were inoculated at 10^6 spores/mL into 24 well plates with 3mL of 2% sucrose + 1x Vogel's medium. Cells were grown for 24h from which each strain was washed with ddH_2O, and placed in 2mL screw top centrifuge tubes with 200mg of glass beads and flash frozen. Tubes were cracked slightly and lyophilized for 24h or until each tube was completely dry. Tubes were closed tightly and bead beat for 3x 30 secs, and 400µL lysis buffer (50mM NaOH, 1mM EDTA, 1% TritonX-100) was added. Lysate cultures were bead beat for 1min and incubated at 65ºC for

30min to 1hr with occasional vortexing. Lysis reaction was neutralized with 80uL of 1M Tris HCl (pH 7.0) and 400µl Phenol: Chloroform: Isoamylalcohol 25:24:1 was added. Cultures were mixed by vortexing and centrifuged at 5m at 12,000g. 300µL of upper phase was transferred into a clean tube, and 2µL RNAase A (Life biotech) was added and incubated at 37ºC for 30min. 5uL was run onto a 1% agarose gel for quantification. DNA was pooled to equal quantities estimated by the genomic DNA band. Pooled samples were cleaned with the Qiagen Blood and Tissue Kit and submitted for sequencing. Sequences received were mapped to the reference genome (Galagan, Calvo et al. 2003) using Bowtie (Langmead and Salzberg 2012). SNPs and deletions were called by Samtools (Li, Handsaker et al. 2009) and annotated by Snpeff (Cingolani, Platts et al. 2012)

2.4.7 Cloning and plasmids

Ectopic cloning into the *his-3* locus was performed into pTS91, a pMF272 plasmid with a swapped sGFP with a superfolding GFP. All cloning to pTS91 was performed between the BamHI and the PacI of the plasmid for expression under the p*ccg-1* promoter. Constructs cloned into the pCSR, a plasmid that was ectopically integrated into the genome the *csr-1* locus were ligated into the NotI and PacI sites of the multiple cloning site of the vector.

2.4.8 Microscopy

Germlings for microscopy were grown on Vogel's medium supplemented with 0.2%, 0.04% and 0.008% cellobiose, at 25° for 8 h or 16 h. Germlings were observed using a 63x or 100× 1.4 NA oil immersion objective on a Leica SD6000 microscope attached to a Yokogawa CSU-X1 spinning disc head with a 488nm laser.

CHAPTER 3: ROLE OF FLIPPASES IN ENZYME SECRETION

3.1 Introduction

Lipids constitute the basis of all biological membranes, and their role as a barrier to diffusion is as important as their intracellular function of signaling and vesicle transport (van Meer, Voelker et al. 2008). Within the cell, lipid membranes form an interconnected network that help to transport proteins, small molecules, and lipids between organelles and the plasma membrane (Graham 2004, van Meer, Voelker et al. 2008, Hankins, Baldridge et al. 2015). While the ER is the major site of lipid synthesis, the lipid membranes of organelles are specific in their constitution and orientation of lipid head groups. Thus, the lipids comprising individual leaflets of the membrane bilayers of vesicles and organelles are largely organized from highly heterogeneous constituents; each lipid component providing a structural or signaling role to the lipid network (Graham 2004, van Meer, Voelker et al. 2008). In fact, phospholipids, the largest component of lipid membranes, are highly asymmetric in their distribution across the membrane bilayer and are maintained on the correct leaflet by the families of lipid transporters. Lipids that are maintained on the cytoplasmic leaflet are transported by the superfamily of P4 ATPases, or more colloquially known as flippases. The best-studied flippases are the members of the P4 ATPases in *Saccharomyces cerevisiae,* Dnf1p, Dnf2p, Dnf3p, Drs2p, and Neo1p. As a result of the function of these five flippases, phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphoinositol (PI) are maintained on the cytoplasmic or inner leaflet, whereas phosphatidylcholine and sphingosine are maintained on the outer or luminal leaflet. Phosphatidylserine (PS) is the only aminophospholipid component of the membrane that contains an anionic head group, and in total, cells contain <10% PS. PS is exquisitely maintained on the cytoplasmic face of the membrane, as dysfunction of the PS asymmetry is an early marker of cells undergoing apoptosis (Hankins, Baldridge et al. 2015).

In fungi, PS regulation on the cytoplasmic leaflet is maintained in cells by the flippase, Drs2p. The *S. cerevisiae* Drs2p is the best-characterized PS-specific P4 ATPase and is found in a complex with Cdc50p to form a heterodimer (Chen, Wang et al. 2006). Drs2p functions at the trans-Golgi network to maintain PS polarity on the trans-Golgi network and the plasma membrane. PS localization can be determined through the maintenance of NBD-PS, a fluorescent analogue of PS (Hua, Fatheddin et al. 2002) and the LactC2-GFP, the C2 domain of human Lactadherin protein fused to GFP, which has been used as a marker for the in-vivo localization of PS (Yeung, Gilbert et al. 2008). Both methods of PS localization (NBD-PS and LactC2-GFP) in WT *S. cerevisiae* cells results in the fluorescent labeling of plasma membrane (Sartorel, Barrey et al. 2015). LactC2-GFP relocalizes to the cytoplasm when the *DRS2* is deleted from cells, or if PS synthesis is altered through deletion of *cho1*Δ, the mutant of the phosphatidylserine synthase. These data indicate that the role of Drs2p is specific for PS maintenance, however, the loss of Drs2p is not necessary for viability (Ripmaster, Vaughn et al. 1993).

In fact, the flippases of *S. cerevisiae* are largely redundant for viability (Hua, Fatheddin et al. 2002, Roelants, Baltz et al. 2010). Of the five phospholipid flippases in *S. cerevisiae*, *neo1*Δ is

the only flippase that is inviable as a single mutant, while the other four are functionally redundant (Hua and Graham 2003). Only a single flippase (either Dnf1p, Dnf2p, or Drs2p) is necessary for the maintenance of viability (Hua, Fatheddin et al. 2002, Roelants, Baltz et al. 2010, Sartorel, Barrey et al. 2015). These observations implicate the ability of a single flippase to maintain regulation of enough lipid polarity for viability despite the previous investigations that each flippase has a preference for a particular substrate as determined by rates of aminophospholipid flipping with fluorescently labeled phospholipids. Dnf1p has been shown to have a high specificity for PE (Stevens, Malone et al. 2008), Drs2p is specific toward PS (Natarajan, Wang et al. 2004, Baldridge and Graham 2012), Dnf3p is more poorly investigated, but has some specificity toward phosphatidylcholine (Alder-Baerens, Lisman et al. 2006), Neo1p has been recently shown to prefer PE over PS (Takar, Wu et al. 2016). While the mechanism of phospholipid discrimination is not well understood, forays into substrate swapping have focused on mutations on the transmembrane segments 1, 2 and 3 of both Drs2p and Dnf1p (Baldridge and Graham 2012, Baldridge and Graham 2013) and the role of the heterodimer partner subunits in substrate discrimination has not been adequately explored (Andersen, Vestergaard et al. 2016).

Work in *S. cerevisiae* has shown that Cdc50p, Lem3p and Crf1p are paralogs that complement specific subsets of flippases for proper function. Cdc50p dimerizes with Drs2p (Saito, Fujimura-Kamada et al. 2004, Lenoir, Williamson et al. 2009), Lem3p is in a heterodimer with Dnf1p or Dnf2p (Hachiro, Yamamoto et al. 2013) and Crf1p dimerizes with Dnf3p (Tanaka, Fujimura-Kamada et al. 2011, Yamamoto, Fujimura-Kamada et al. 2017). All of the heterodimer partners contains two transmembrane domains and are hypothesized to help form a hydrophobic gate with the 10 transmembrane domains of their respective P4-ATPases to aid in lipid binding (Lenoir, Williamson et al. 2009, Andersen, Vestergaard et al. 2016).

DRS2 was originally identified due a cold sensitive dysfunction in the mutant of ribosomal assembly (Ripmaster, Vaughn et al. 1993) While the original mechanism for cold sensitive ribosomal assembly has not been properly elucidated, this phenotype has largely been used to find the genetic interactors with *DRS2* and have helped to shape the understanding of what regulates flippase activity and specificity of pathways that use flippases to function.

The role of Drs2p in *S. cerevisiae* is varied and has been shown to promote late secretory pathway function by helping to manipulate the curvature of the trans-Golgi membrane. As the major phospholipid flippase for PS, the only negatively charged aminophospholipid, the resulting patches of PS lipid rafts formed by translocation promotes the increased curvature of the cytoplasmic leaflet of the membrane through charged group repulsion (Xu, Baldridge et al. 2013). This manipulation of curvature promotes the proper localization of Gcs1p ADP-ribosylation factor GTP activating protein (ARF-GAP), through its positively charged residues in coordination with the ALPS motif, a membrane deformation dependent binding domain (Xu, Baldridge et al. 2013), which allows for the proper localization of Snc1p, a v-SNARE necessary for vesicle trafficking and regulated exocytosis (Xu, Baldridge et al. 2013). *DRS2* has also been shown to be a part of the AP1 pathway, as deletion of *gga1*Δ and *gga2*Δ (Golgi localized, γ-ear-containing, Arf-binding proteins that facilitate trans-Golgi to late endosome transport in *S. cerevisiae*) has a synthetic lethal phenotype when mutated along with *drs2*Δ*,* and Drs2p has direct interaction with Apl4p, the large gamma subunit of the Ap1 complex (Liu, Surendhran et al. 2008).

Endocytosis is also impaired in a *drs2*Δ mutant, as Rcy1p, an F-box protein that is required for the recycling of Snc1p, directly binds to Drs2p (Hanamatsu, Fujimura-Kamada et al. 2014). Knock out and critical point mutations of the interaction sites between Rcy1p and Drs2p results in mis-localization of Snc1p-GFP to trans-Golgi compartments from the plasma membrane. This may indicate that the *drs2*Δ mutant promotes a gross defect in vesicle trafficking and has dysfunctional secretion. Other direct interactors of Drs2p include Gea2p and Arl1p, where Gea2p, a guanine nucleotide exchange factor for ARFs, which directly binds to the C-terminal domain of Drs2p and facilitates the binding of Arl1p, an ARF-like protein, that is necessary for the subsequent recruitment of the golgin Imh1p to the Golgi (Tsai, Hsu et al. 2013)

Flippase function in filamentous fungi is less well characterized mechanistically. Previous work looking the genetic causes of cell polarity defects have isolated an allele of *drs-2* and the putative upstream kinase regulator *stk-50* (*YPK1* ortholog) and the potential downstream genetic interactor, *apl-4* (Seiler and Plamann 2003). The localization of flippases has been performed in *Aspergillus nidulans*. DnfA, the orthologue of Dnf1p, localizes to the Spitzenkörper ring, a vesicle organizing center that marks the polarity of hyphal growth and the endocytic collar (Schultzhaus, Yan et al. 2015). Endocytosis of the DnfA is dependent on an NPFxD motif. The Drs2p homolog (DnfB) localizes to the core of the Spitzenkörper and is not associated with the endocytic collar (Schultzhaus, Yan et al. 2015).

These data together along with this work, we elucidate the role of flippases in cellulolytic enzyme secretion and the possible mechanisms by which flippase activity is able to enhance the accumulation of cellulolytic proteins. Previously, we have found that *drs-2* plays a role in inhibiting cellulase secretion and accumulation into the supernatant, and in this chapter, we characterize the role it has in transcriptional regulation, vesicle sorting, and the potential genetic interactors.

3.2 Results

3.2.1 Protein accumulation on sucrose is additive with the mis-expression of CLR-2

To investigate whether the cellulase accumulation phenotype of Δ*drs-2* was independent of the induction of the cellulolytic regulon, we made double mutants using previously established hypersecreting strains of *N. crassa*. Both known hypersecreters show increases in transcription of the cellulolytic regulon, and we hypothesized that due to the localization of Drs2p to the trans-Golgi compartments of *S. cerevisiae,* the phenotype of protein accumulation may be additive with the known transcriptional hypersecreters. To test this hypothesis, we mated Δ*drs-2* with a strain containing the mis-expression of CLR-2, *pccg1-clr-2.* This mis-expression strain is able to constitutively transcribe CLR-2, the transcription factor that directly binds the promoter region of cellulases, and upregulates the transcription of cellulase enzymes. This mis-expression of cellulases results in the secretion of cellulases under normally non-inducing conditions (Coradetti, Xiong et al. 2013). We isolated cross progeny with Δ*drs-2*, WT, *pccg-1-clr-2*, and Δ*drs-2*; *pccg1-clr-2* genotypes and assayed the strains on 1% w/v glucose medium until starvation at 30h with an additional 1% w/v sucrose induction. We then measured the accumulation of cellulases over the span of 120h.

As the model of cellulase induction would predict, the WT strains would not be induced for cellulase activity under sucrose conditions. Similar to WT, the Δ*drs-2* knock did not increase the amount of cellulase enzyme into the supernatant to a significant extent. These data indicate that the Δ*drs-2* is not sufficient to increase secretion through aberrant induction of cellulase enzyme transcription under non-inducing conditions (Fig 3.1a)

In contrast to WT and Δ*drs-2* secretion levels, the p*ccg1-clr-2* mis-expression strain is highly upregulated for cellulase transcription (Coradetti, Xiong et al. 2013) and is able to secrete cellulases at the level of 175 mg/L under normally non-inductive conditions. When the Δ*drs-2* mutation is stacked with the mis-expression strain, the result is a significant >2-fold increase in cellulase secretion under non-inductive conditions (p<.001) (Fig 3.1a) as compared to the p*ccg-1-clr-2* mis-expression strain. This result indicates that the increase in secretion observed in the Δ*drs-2* mutant under cellulolytic conditions is not working through primary transcriptional induction of the cellulases.

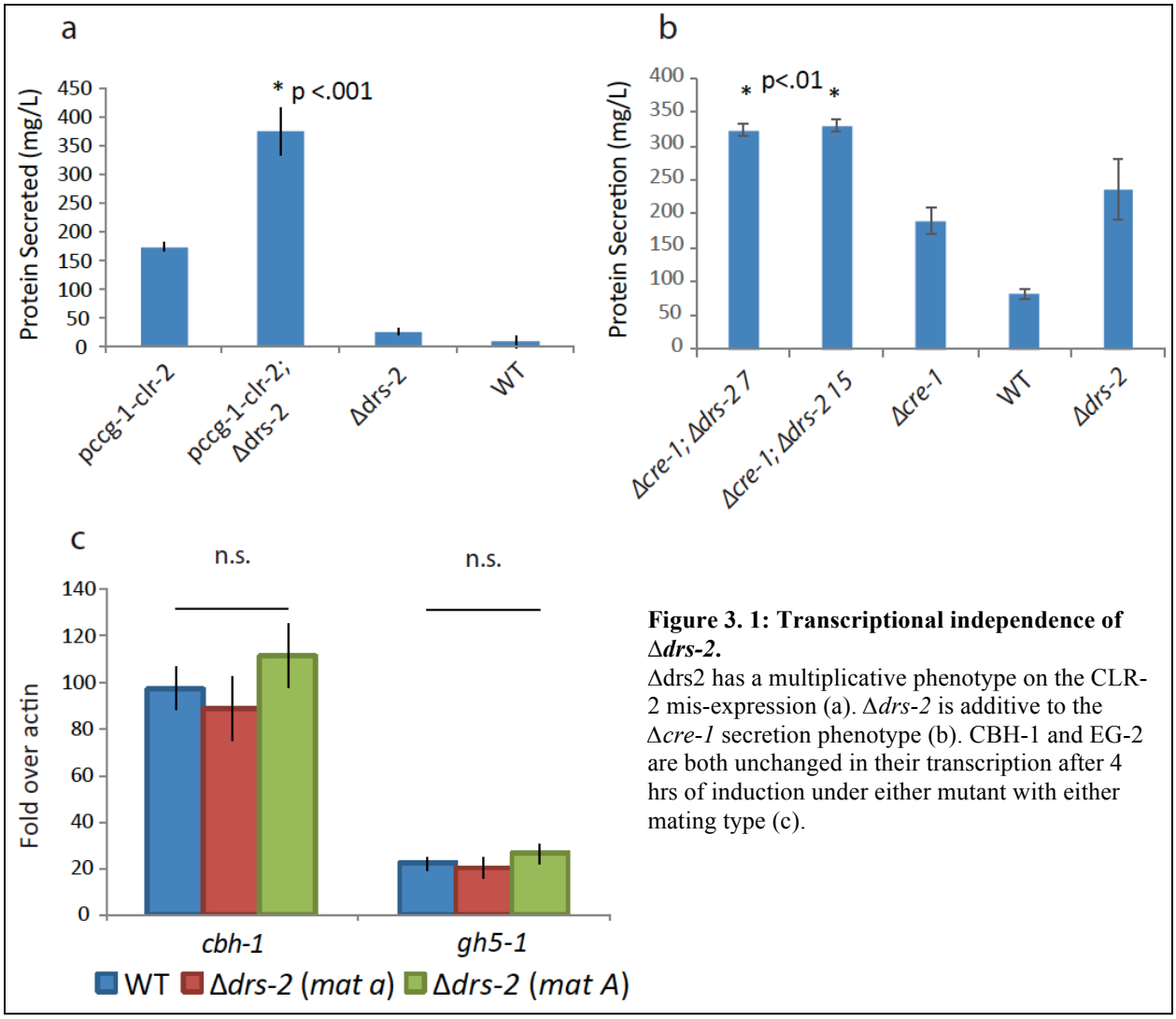

Figure 3. 1: Transcriptional independence of Δ*drs-2*.
Δdrs2 has a multiplicative phenotype on the CLR-2 mis-expression (a). Δ*drs-2* is additive to the Δ*cre-1* secretion phenotype (b). CBH-1 and EG-2 are both unchanged in their transcription after 4 hrs of induction under either mutant with either mating type (c).

3.2.2 Δ*drs-2* is independent of Δ*cre-1* secretion phenotype

Another manner by which Δ*drs-2* may be regulating transcription is through the de-repression of carbon catabolite repression, CCR. The major regulator of cellulose CCR in fungi is the transcription factor, CRE-1. *cre-1* was found to be deleted in the major industrial *T. reesei* strains (Le Crom, Schackwitz et al. 2009), and in *N. crassa*, a strain carrying the deletion of Δcre-1 enhances the amount of cellulase accumulation in the supernatant (Sun and Glass 2011). If the role of DRS-2 is to de-repress transcription of cellulases through the *cre-1* pathway, the double deletion strain, Δ*drs-2;* Δ*cre-1* should not accumulate more secreted protein than either of the single mutants, Δ*drs-2* or Δ*cre-1*.

We mated a strain bearing a deletion in Δ*cre-1* with a strain with the knock out of Δ*drs-2* and isolated cross progeny with WT, Δ*drs-2*, Δ*cre-1*, and Δ*drs-2;* Δ*cre-1* genotypes. The progeny of the cross were tested on cellulose media as the inducer. We corroborated the results of previous studies (Sun and Glass 2011, Xiong, Sun et al. 2014) using a 1% w/v cellulose induction after 30h of 1% w/v glucose growth. The Δ*cre-1* single mutants showed a >2 fold increase in secreted protein levels over WT under cellulosic growth conditions ($p<.05$) (Fig 3.1b). The Δ*drs-2* single mutant showed a 2.5 fold increase in secreted protein levels over WT strains, ($p<.01$), while the double mutant, Δ*drs-2*; Δ*cre-1* showed a significant additive secretion level phenotype that was 3.5 fold over WT and the single mutants ($p<.01$). Since the Δ*drs-2* mutation compounds the secretion phenotype of the Δ*cre-1* mutation, these data may indicate that DRS-2 does not act on the cellulolytic machinery from the standpoint of induction, or modulation of carbon catabolite repression.

3.2.3 Δ*drs-2* does not have a transcriptional effect on *cbh-1* and *gh5-1*

To further test this hypothesis, and as a direct test of transcriptional alterations in the Δ*drs-2* mutant, we assessed the transcription of the major cellobiohydrolase, *cbh-1*, and the endoglucanase that we used for screening, *gh5-1* in the Δ*drs-2* mutant relative to the WT strain. We modeled our experiments on transcription based on previously established conditions for *N. crassa* induction on cellulose (Coradetti, Craig et al. 2012). We isolated mRNA from cellulose induced cultures and found through qRT-PCR of *cbh-1* and *gh5-1* that a quantifiable change of expression levels of either gene in the Δ*drs-2* mutant was not observed relative to WT (Fig 3.1c).

From these data, we can conclude that the role of the Δ*drs-2* mutant on enzyme secretion and accumulation is not due to increased transcription of *cbh-1* and *gh5-1*, two of the major enzymes of the cellulolytic response (Phillips, Iavarone et al. 2011). Δ*drs-2* may play a role that is independent of transcription. This is especially surprising to us because insofar, the major modulators of secretion in the literature have exclusively been explained through transcriptional changes. These transcriptional data led us to hypothesize that Δ*drs-2* mutant may play a role downstream of transcription.

3.2.4 Altered secretion of Δ*drs-2* on sucrose indicates enzyme-specific hypersecretion

If *drs-2* were functioning independently downstream of transcription, then the Δ*drs-2* mutant would have a secretion phenotype regardless of the enzyme transcribed. The p*ccg1-clr-2;* Δ*drs-2*

compounded secretion phenotype already indicated to us that the Δ*drs-2* secretion phenotype was not cellulose specific. Thus, we tested xylan and sucrose substrates to examine whether the effect of Δ*drs-2* secretion was specific to a subset of enzymes. Different carbon substrates are metabolized at different rates (Tian, Beeson et al. 2009) and require a unique subset of CAZymes to degrade, import and metabolize each substrate (Tian, Beeson et al. 2009, Sun, Tian et al. 2012, Benz, Chau et al. 2014). In order to measure the enzymes secreted, we performed a time course of protein secreted on cellulose and the hemicellulase activity of Δdrs-2 and WT cultures on xylan, and invertase activity on sucrose comparing the Δ*drs-2* mutant to the WT strain over the course of 120h.

We observed that after 1% w/v cellulose induction, protein secretion rate was significantly higher at the 24 to 72h time points in the Δ*drs-2* mutant, which resulted in a >2.5 fold final accumulation of protein (fig 3.2a). Cellulase activity determined by cellulose degradation was proportionally increased to the protein secreted. We saw that after the first 72h of induction, we see a significant increase in the protein production corroborating the initial characterization of the Δ*drs-2* mutant.

When WT and Δ*drs-2* mutant cultures were induced with 1% w/v beechwood xylan, a model substrate for hemicellulose, we observed that xylanase activity was significantly increased in the Δ*drs-2* mutant across all time points measured. The increased xylanase activity indicated that the Δ*drs-2* mutant secreted a significantly higher amount of hemicellulases into the supernatant (fig 3.2b). From these data, we concluded that plant cell wall induced enzyme secretion of both

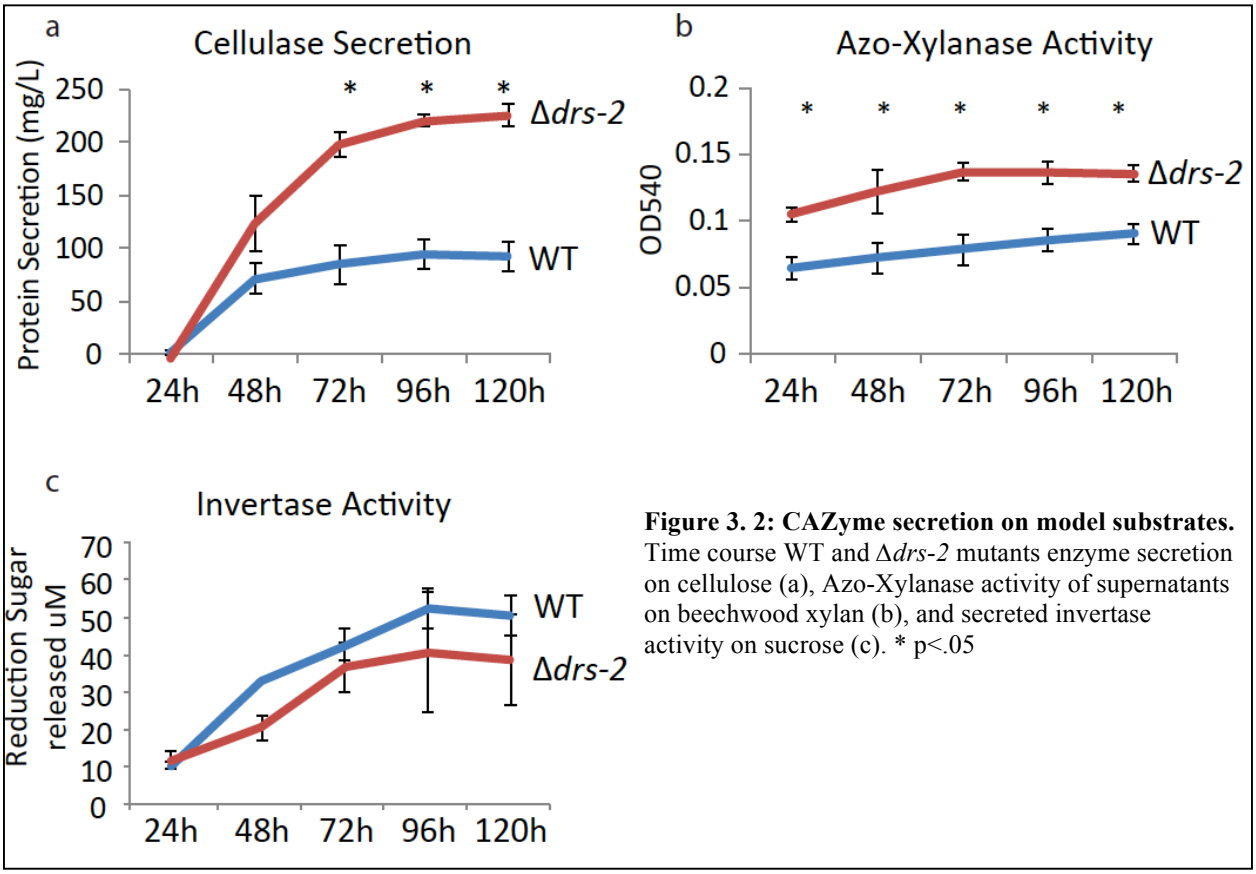

Figure 3. 2: CAZyme secretion on model substrates. Time course WT and Δ*drs-2* mutants enzyme secretion on cellulose (a), Azo-Xylanase activity of supernatants on beechwood xylan (b), and secreted invertase activity on sucrose (c). * p<.05

cellulases and hemicellulases significantly increased as a result of the mutation Δ*drs-2*. When WT and Δ*drs-2* mutant cultures were induced with 1% w/v sucrose. Surprisingly, invertase secreted in the supernatant, as assayed by sucrose hydrolysis activity, had a lower, but statistically not significant change between Δ*drs-2* and WT cultures over the time course.

These time course data indicated to us that the Δ*drs-2* mutant is hyper secreting cellulases and over accumulating hemicellulases, while not altering invertase secretion. This can be explained by a few possibilities: the first reason may be that the Δ*drs-2* mutant phenotype may only hypersecrete cellulases and hemicellulases. Another reason for the altered trafficking of invertase may be due to a trivial aspect of invertase secretion or activity. We decided to follow up with the first line of inquiry and probe whether the Δ*drs-2* phenotype was specific toward specific enzymes.

3.2.5 Lignocellulases are over abundant and cell wall proteins are under represented on the secretome

We determined the altered trafficking of proteins in the Δ*drs-2* mutant relative to the WT by performing ultra-high performance liquid chromatography coupled tandem mass spectroscopy (UPLC-MS/MS) on protein supernatant of cellulose-induced cultures. UPLC-MS/MS gave a semi-quantitative measure of protein abundances in the cellulose secretome. These protein abundances were normalized to total protein detected and compared between Δ*drs-2* and WT. Of the two strains tested, WT and Δ*drs-2*, we observed significant differences in the amount of protein that was detected between the two culture supernatants (Table 3.1).

Of the major carbohydrate active enzymes, CAZymes, CBH-1 is the major representative protein. However, despite the increased secretion of total protein in the Δ*drs-2* cultures, CBH-1 is not overly represented in the total amount of protein. From these data, we deduced that the relative amount of CBH-1 accumulates to similar levels between the Δ*drs-2* and WT supernatants. Other CAZymes that are secreted at comparative levels between the two genotypes include the endoglucanase, GH5-1, used at the onset for sorting hypersecretion mutants, cellobiose dehydrogenase CDH-1, the accessory soluble protein that is the electron donor to various polysaccharide monooxygenases (PMO/AA9) for oxidative hydrolysis of cellulose and other polymers. Two of the major PMO/AA9 genes were also observed to be relatively similar in their abundance in Δ*drs-2* and WT supernatants.

We were surprised by the relative over-abundant and under-represented proteins of the Δ*drs-2* supernatant compared to WT. CBH-2, the major non-reducing cellobiohydrolase enzyme is the second most abundant protein is the *N. crassa* cellulose proteome, and makes up 13% of the secretome of WT supernatant cultures (Phillips, Iavarone et al. 2011). From our data, CBH-2 of the Δ*drs-2* culture supernatant was 10-fold over represented relative to WT. This increase is accompanied by many hemicellulose active enzymes, including acetylxylan esterases, CE5-2 and CE5-3, and endo-1,4-β-xylanase, GH11-2.

Table 3. 1: **Ranked protein abundances on normalized supernatant of WT and Δ*drs-2* mutants.**

Proteins of similar representation between Δ*drs*-2 and WT supernatants

#NCU	Annotation	High Genotype	Fold Change	Anova(p)
NCU07340	**CBH1, GH7-1**	**WT**	**1.015191352**	**0.989619506**
NCU00762	**EG2, GH5-1**	**Δ*drs-2***	**1.423991801**	**0.145254998**
NCU00206	cellobiose dehydrogenase, CDH-1	Δ*drs-2*	1.042020082	0.780824373
NCU02240	AA9-1	Δ*drs-2*	3.501483033	0.034053978
NCU01050	AA9-4	Δ*drs-2*	2.188881824	0.037771324

Proteins over-represented in Δ*drs-2* supernatant relative to WT supernatant

#NCU	Annotation	High Genotype	Fold Change	Anova(p)
NCU00811	hypothetical protein	Δ*drs-2*	3768.045094	0.000623615
NCU09691	hypothetical protein	Δ*drs-2*	62.49325759	0.000808296
NCU05751	cellulose-binding protein, CE3-2	Δ*drs-2*	5.679695804	0.000953827
NCU09680	**CBH2, GH6-2**	**Δ*drs-2***	**9.030067172**	**0.001142286**
NCU05159	acetylxylan esterase CE5-2	Δ*drs-2*	63.28261795	0.001471928
NCU02916	AA9-3	Δ*drs-2*	26.41008792	0.001971112
NCU01076	hypothetical protein	Δ*drs-2*	17.00820415	0.004028914
NCU09664	acetylxylan esterase CE5-3	Δ*drs-2*	10.35037642	0.006255771
NCU07225	endo-1,4-beta-xylanase 2 GH11-2	Δ*drs-2*	15.77282442	0.00944744

Proteins under-represented in Δ*drs-2* supernatant relative to WT supernatant

#NCU	Annotation	High Genotype	Fold Change	Anova(p)
NCU09285	*zinc-containing alcohol dehydrogenase, mig-6*	*WT*	*98228.56035*	*1.65E-06*
NCU04883	**chitinase 1, GH18-4**	**WT**	**243.3749575**	**2.13E-05**
NCU10641	*hypothetical protein*	*WT*	*237.4844887*	*2.35E-05*
NCU01063	hypothetical protein	WT	9.458979849	5.73E-05
NCU07324	conidiation-specific protein 13, con-13	WT	28738.85683	9.06E-05
NCU04202	nucleoside diphosphate kinase ndk-1	WT	25.77489739	0.000112024
NCU03100	*6-phosphogluconate dehydrogenase*	*WT*	*581.7299411*	*0.000136716*
NCU04471	hypothetical protein	WT	232.0546169	0.000138181
NCU05534	hypothetical protein	WT	7125.953604	0.000221144
NCU09734	hypothetical protein	WT	13055.61955	0.000416075
NCU02136	*transaldolase*	*WT*	*15.52971742*	*0.000613953*
NCU08412	*endo-beta-1,4-mannanase*	*WT*	*10.09657476*	*0.001225169*
NCU04431	endo-1,3(4)-beta-glucanase	WT	49.8565729	0.001736799
NCU06512	*methionine synthase*	*WT*	*805.7881484*	*0.002015294*
NCU07914	*phosphoglycerate kinase*	*WT*	*25.28172102*	*0.002417749*
NCU00673	serine protease p2	WT	6.993102388	0.005349923

Protein supernatants of WT and Δ*drs-2* cultures were ran on SYNAPT-G2si for semi-quantitative comparative protein measurement. Protein detected was normalized to total protein abundance. **Bolded** entries are proteins studied in this work. *Italicized* entries are supernatant proteins lacking predicted signal peptides by SignalP and Phobius

The under-represented proteins of Δ*drs-2* supernatants included many proteins that did not have a predicted signal peptide. These proteins are some of the most highly expressed genes of the genome under all previously reported RNA sequencing conditions (Coradetti, Craig et al. 2012), including alcohol dehydrogenase (NCU09285), 6-phosphogluconate dehydrogenase (NCU03100), transaldolase (NCU02136), methionine synthase (NCU06512), phosphoglycerate kinase, (NCU07914). These under represented proteins also have very well characterized roles in primary metabolism, and are normally cytosolic, so the release of these proteins into the supernatant indicated to us that cell lysis may be occurring in WT cells along with protein secretion.

Of the proteins that were predicted to contain signal peptides *and* be underrepresented in the Δ*drs-2* supernatant were two proteins that have putative functions on cell wall modification: chitinase (NCU04883, GH18-4), and the endo-1,3(4)- β-glucanase, (NCU04431, GH16-3). We found it interesting that Δ*drs-2* functioned to enhance plant cell wall degrading enzymes while also repressing the secretion of fungal cell wall modifying enzymes. To test this result further, we wanted to confirm the altered secretion phenotypes between the enzymes CBH-2 and GH18-4.

3.2.6 Western blot analysis of secretome confirms mass spectroscopy data

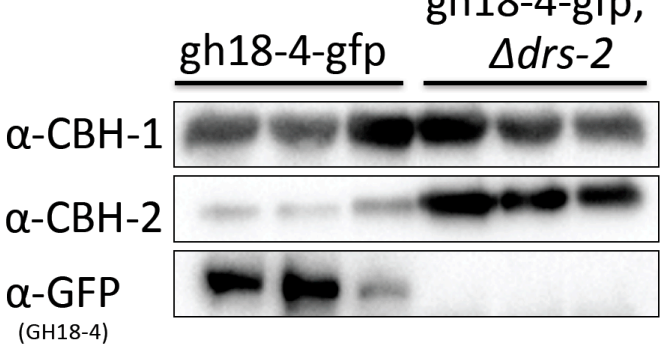

Figure 3. 3: Western of GH18-4-GFP and Δ*drs-2*; GH18-4-GFP

In order to confirm the disparate secretion rates that were observed from the mass spectroscopy results, we constructed a strain containing a natively tagged GH18-4 with superfolder green fluorescent protein, sfGFP. We crossed the gh18-4::*gh18-4-GFP* strain with the Δ*drs-2* mutant to generate progeny with genotypes *gh18-4*::*gh18-4-GFP* and *gh18-4::gh18-4*; Δ*drs-2*. We cultured strains of these two genotypes in triplicate, and performed a western blot on the normalized supernatants after 72h of cellulose induction, probing for with α-CBH-1, α-CBH-2 and α-GFP antibodies for the detection of secreted CBH-1, CBH-2, and GH18-4, respectively.

From the α-CBH-1 western, secreted CBH-1 levels were nearly equal between culture supernatants from the Δ*drs-2* and WT strains. This similar level of relative CBH-1 abundance is reflective of the UPLC-MS/MS data (Fig 3.3). CBH-2, the major cellulase that was over represented between the culture supernatants of the Δ*drs-2* mutant relative to the WT strains also was qualitatively over represented of the Δ*drs-2* mutant under normalized supernatants, confirming the MS measurements of relative CBH-2 abundance. The α-GFP antibody detected

the presence of GH18-4-GFP in the supernatant of the WT cultures, but GH18-4-GFP was not detected in the Δ*drs-2* culture supernatant. The detection of the similar abundance of CBH-1, CBH-2 over-representation and GH18-4-GFP under-representation of Δ*drs-2* supernatants relative to WT supernatant corroborated the UPLC-MS/MS data. The role of DRS-2 in selective enzyme secretion was intriguing, and we investigated the selective accumulation of CBH-2 and GH18-4 at the level of transcription of these genes.

3.2.7 The Δ*drs-2* mutant has a transcriptional phenotype along with a secretion phenotype

One of the hypotheses that would explain the change in relative protein secretion rates between CBH-2 and GH18-4 in the Δ*drs-2* mutant and WT would be a change in relative transcription of these genes. While previous data implicated no difference in the transcription of *cbh-1* and *gh5-1* transcription in the Δ*drs-2* mutant relative to the WT strain (fig3.1c), expression levels of *cbh-2* and *gh18-4* were not previously assayed by the earlier qRT-PCR experiments.

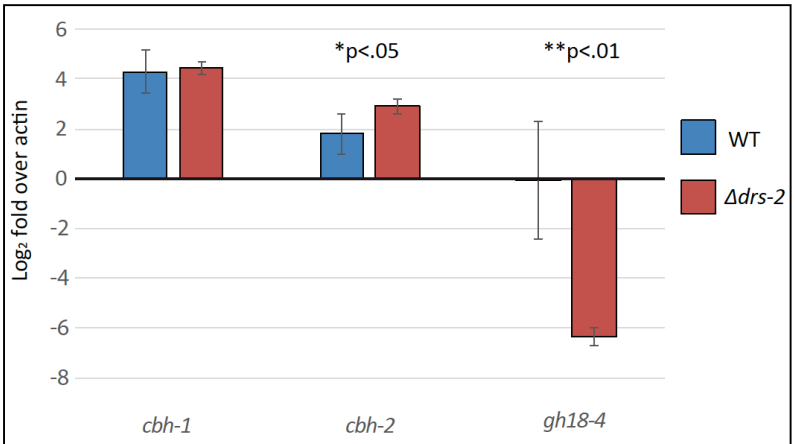

Figure 3. 4: Transcription of cellulases and GH18-4 on induced WT and Δ*drs-2* cultures.

Because the level of protein accumulation was occurring in 1% w/v cellulose induced 1% w/v glucose cultures, we performed a time course of *cbh-1*, transcription over the span of 48h to determine a time point of maximum induction of induction in culture. We observed the greatest amount of *cbh-1* induction at the 24h time point. Thus, we measured the transcription of *cbh-1*, *cbh-2*, and *gh18-4* at 24h (fig 3.4).

While *cbh-1* transcription was relatively similar between WT and Δ*drs-2* cultures, *cbh-2* transcription was significantly upregulated in Δ*drs-2* cultures ($p<.05$). *gh18-4* transcription in Δdrs-2 cultures, was significantly down regulated relative to WT ($p<.01$). The trends of relative transcription reflected the trend of secreted proteins, and the specificity of over secreted *cbh-2* and under secreted protein *gh18-4* is at least partially explained by the transcription of these genes in the Δ*drs-2* mutant.

3.2.8 The Δ*drs-2* mutation results in mis-trafficking of the GH18-4-GFP to the vacuole

Despite the decrease in transcription of *gh18-4,* we were surprised at the complete absence of secreted GH18-4-GFP from the western blot and UPLC-MS/MS data. Thus, we hypothesized that the lack of GH18-4-GFP secretion of Δ*drs-2* cultures was also due to intracellular mis-localization of the enzyme. We tested this hypothesis by observing the GFP fluorescence of the natively tagged *gh18-4*::*gh18-4-GFP* strain in comparison to the *gh18-4*::*gh18-4-GFP*; Δ*drs-2* strain.

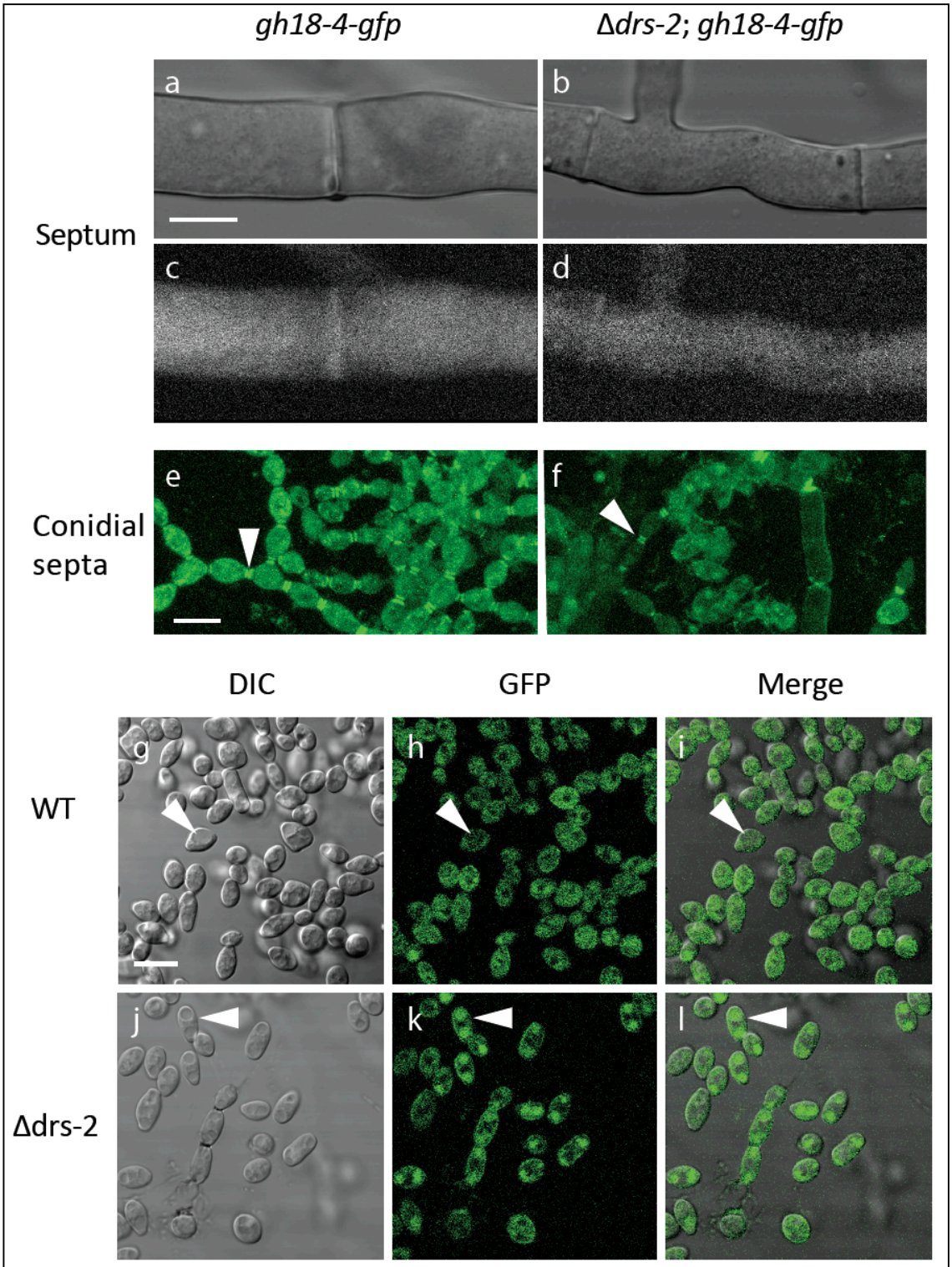

Figure 3. 5: Localization and mistrafficking of GH18-4-GFP.
DIC (a,b) and GFP fluorescence (c,d) of GH18-4-GFP(a,c) and Δ*drs-2*;GH18-4-GFP(b,d). Conidial septal staining of GFP in GH18-4-GFP(e) and Δdrs-2;GH18-4-GFP(f) arrows indicate septa. GH18-4-GFP staining in conidia of GH18-4-GFP(g,h,i) and Δdrs-2; GH18-4-GFP (j,k,l), arrows indicate conidial vacuoles. Scale bars represents 10 µm.

In WT cells, we observed septal localization of GH18-4-GFP (fig 3.5c). GH18-4-GFP fluorescence was also observed in the Δ*drs-2* background (fig 3.5d). GH18-4-GFP was also observed at conidial septa of both WT and Δ*drs-2* (fig3.5e, f, respectively). While both localizations were comparable, we observed that once the conidia separated from each other, there was a noticeable difference in GFP accumulation between the two strains within the conidial cells.

Once the conidia separated, GH18-4-GFP was no longer localized to the site of conidial separation. In WT cells, GH18-4-GFP diffused and remained diffuse in the cell after conidial separation. However, once the conidia containing the Δ*drs-2* mutant separated, instead of remaining diffuse in the cell, the GH18-4-GFP accumulated to the conidial vacuole (fig 3.5k). This observation indicated to us that GH18-4-GFP is being mis-trafficked in the conidium. This

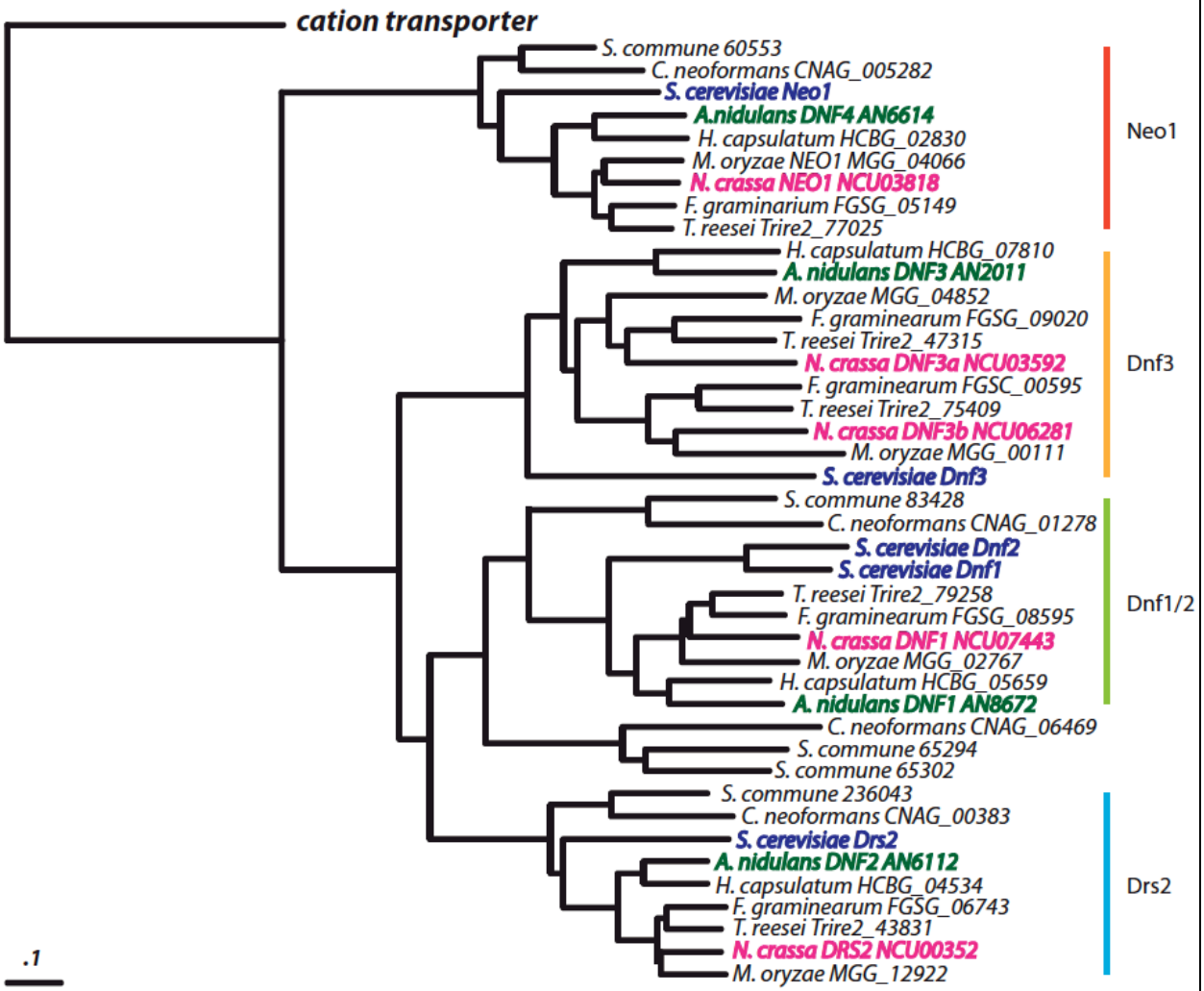

Figure 3. 6: Gene tree of P4 ATPase superfamily.
N. crassa DRS-2 was used for identifying P4 type ATPases in the genomes of *Aspergillus nidulans* FGSC A4, *Cryptococcus neoformans* var. grubii H99, *Fusarium graminarium* PH-1, *Histoplasma capsulatum* G186AR, *Magnaporthe oryzae* 70-15, *Neurospora crassa* OR74A, *Saccharomyces cerevisiae* S288c, *Schizophyllum commune* H4-8, *Trichoderma reesei* QM6a.

mis-localization may be indicative of a broader mechanism of mis-trafficking in hyphae and an explanation of why the GH18-4-GFP in the Δ*drs-2* background is undetectable on the Western blot and in MS data. From these secretome analyses, we wondered whether the secretion phenotypes observed in Δ*drs-2* cultures were specific to the Δ*drs-2* members of the P4-type ATPases.

3.2.9 The P4-type ATPase superfamily in Ascomycota and Basidiomycota

The phenotypes attributed to Δ*drs-2* may be largely redundant. Any single flippase of Dnf1p, Dnf2p, and Drs2p is sufficient for viability in *S. cerevisiae* (Hua, Fatheddin et al. 2002, Roelants, Baltz et al. 2010). Or, the phenotypes attributed to *drs2*Δ may be specific to the flippase alone. Cold sensitivity and protein mis-trafficking has played a large role in isolating Drs2p interactors, most of which are Drs2p specific (Chen, Wang et al. 2006, Hankins, Sere et al. 2015, van Leeuwen, Pons et al. 2016). Many of the specific functions are attributed to the phosphatidylserine specific function of *drs2*Δ. From previous DRS-2 ortholog localization in *A. nidulans*, and the dissimilar trafficking to the DNF-1 ortholog, we hypothesized that the secretion dysfunction of the Δ*drs-2* knock out may be DRS-2 specific (Seiler and Plamann 2003, Schultzhaus, Yan et al. 2015, Schultzhaus and Shaw 2016, Schultzhaus, Zheng et al. 2017).

To begin testing this hypothesis, first wanted to identify all members of the P4-ATPases we performed BLASTp searches to the NCBI database using the *N. crassa* DRS-2 protein sequence. We focused our results to organisms where data on Δdrs-2 has been previously reported (*S. cerevisiae, Aspergillus nidulans, N. crassa, Cryptococcus neoformans*), studied ascomycete fungi with economic value (*Magnaporthe oryzae, Histoplasma capsulatum, T. reesei, Fusarium graminarium*), and a putative basidiomycete outgroup (*Schizophyllum commune*). We placed a similarity threshold of sequences for significance of E value < 10^{-10} and generated a maximum likelihood phylogenetic gene tree from the remaining sequences. We observed from the superfamily of flippases, with a single common ancestor of the gene family evolved from a cation transporter, which we used as the outgroup for rooting our gene tree. We used the *S. cerevisiae* gene members to define the four flippase gene clades, *NEO1*-clade, *DRS2*-clade, *DNF1*-clade, and the *DNF3*-clade (Fig 3.6).

The basal-most clade of the Dikarya gene tree is the *NEO1*-clade. Neo1p plays an essential role for *S. cerevisiae* (Takar, Wu et al. 2016, van Leeuwen, Pons et al. 2016). The other notable clade for single orthologs is the *DRS2*-clade. The gene tree of the *DRS2*-clade is reflective of the evolutionary phylogeny of fungi and does not yet contain evidence of gene duplication and loss. The *DNF1*-clade has a gene duplication of *S. cerevisiae*, and the *DNF3*-clade contains a gene duplication in the Sordariomycetes and a loss of a clear orthologue in basidiomycete organisms. The basidiomycetes, *C. neoformans* and *S. commune*, however, have a duplication of the *DNF1*-clade. This sister clade to the *DNF1*-clade may be compensatory and may have neofunctionalized to perform new functions, and gaining the heterodimer partner, *cdc-50* (Huang, Liao et al. 2016).

While the superfamily of P4-ATPases are well conserved among eukaryotes, radiations of certain genes have promoted the idea of redundancy in maintaining the lipid asymmetry. As a

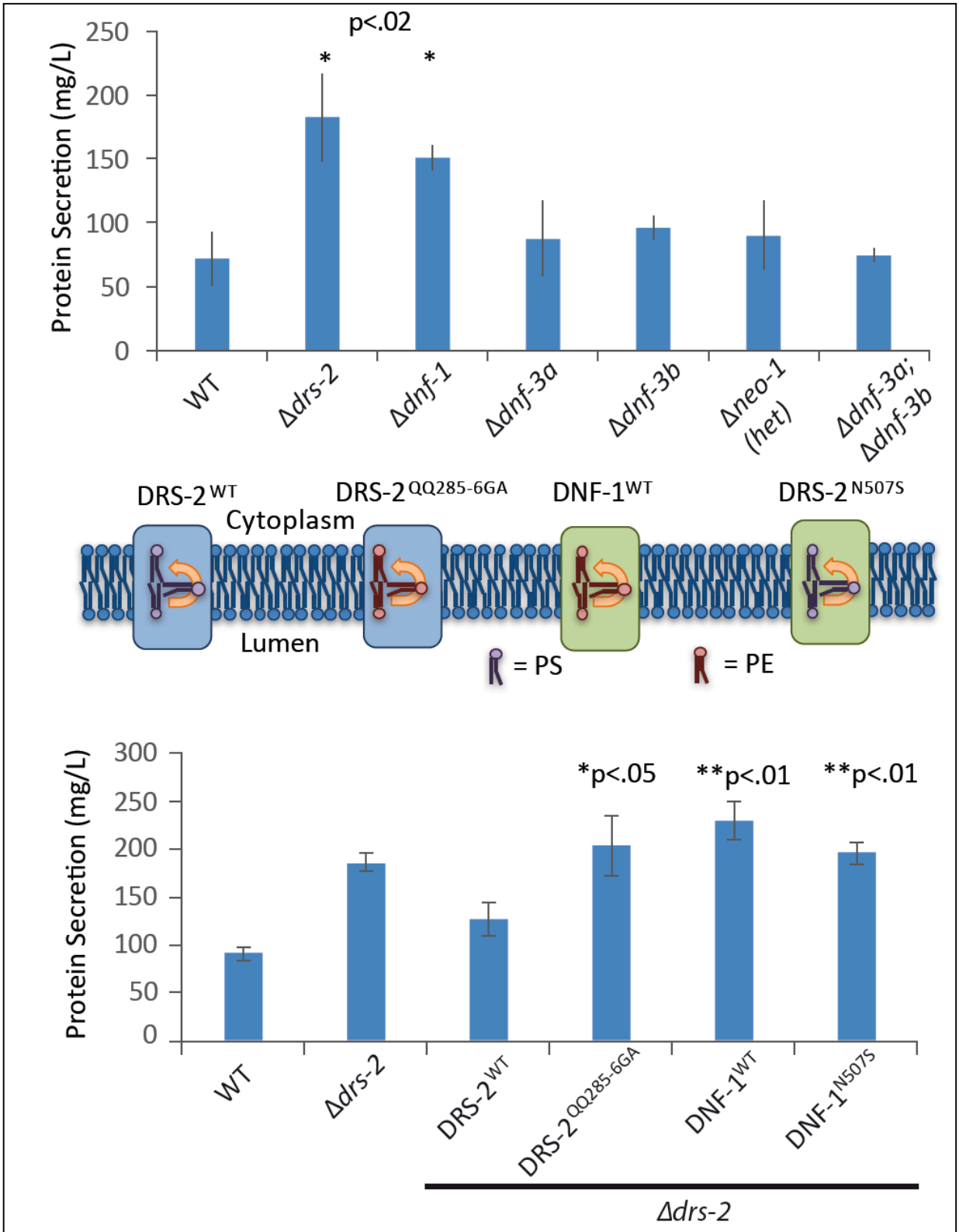

Figure 3. 7: Survey of flippases and mutant complementations.
Single deletion mutant secretion phenotypes and Δdnf-3a;Δdnf-3b double knockout (a). Cartoon of substrate swapping of DNF-1 and DRS-2 (Baldridge and Graham 2012, Baldridge and Graham 2013), (b). Secretion phenotypes substrate swapping of DNF-1 and DRS-2 (c). Asterisks represent significance over Δdrs-2; DRS-2^{WT}

result of the evolution of gene duplications leading to paralogs of flippase activity, each flippase is able to maintain asymmetry of a preferred lipid. While specificity of certain lipids has been a major area of study for flippases, the *DRS2*, *DNF1*, *DNF3* and *NEO1* clades are well maintained among all Ascomycete species listed. However species like *S. cerevisiae* contains a duplication of the *DNF1* gene, *DNF2*. *N. crassa* contains a duplication of the *DNF3* gene, ΔNCU03592 (*dnf-3a*) and ΔNCU06281 (*dnf-3b*).

3.2.10 The Δ*drs-2* and Δ*dnf-1* mutants have cellulase secretion phenotypes

From the flippase superfamily tree, we deduced that 5 flippase genes are encoded in the *N. crassa* genome, which we named for their orthologs in *S. cerevisiae*, *neo-1* (NCU03818), *dnf-1* (NCU07443), *dnf-3a* (NCU03592), *dnf-3b* (NCU06281) and Δ*drs-2* (NCU00352). Single mutants were obtained from the knock out library and the Δ*dnf-3b* knock out was generated using a nourseothricin resistance (NatR) replacement of the *dnf-3b* coding sequence. Δ*neo-1* was a heterokaryon in the deletion library, and grown on hygromycin containing media for nuclear bias, and the *DNF3* homologues were tested both as single mutants and as a double mutant for deletion of the *DNF3*-clade.

Screening through all the flippase mutants, we observed secretory phenotypes in the Δ*drs-2* and Δ*dnf-1* mutants. The Δ*dnf-1* mutant accumulated protein levels 2-fold over WT levels ($p<.02$) (fig 3.7a). We did not observe secretion phenotypes in Δ*dnf-3a*, Δ*dnf-3b*, Δ*dnf-3a*; Δ*dnf-3b* mutants or the Δ*neo-1* heterokaryon strains.

3.2.11 Non-complementation of Δ*drs-2* with the switched-lipid substrate flippases

It was interesting to us that secretion phenotypes of both Δdrs-2 and Δdnf-1 resulted in significant increases over WT. Previous studies in S. cerevisiae have shown that Drs2p is specific to PS flipping, while Dnf1p is preferential to PE flipping (Baldridge and Graham 2012). However, point mutations in the transmembrane domains resulted in proteins that switch the substrate specificity of the flippase. By altering two sequential glutamine residues to glycine and alanine, $Drs2^{QQ237\text{-}8GA}$, the specificity of Drs2p to PS is altered to PE. Conversely, by mutating an asparagine residue to a serine, $Dnf1p^{N550S}$, the substrate specificity was changed from PE to PS (Baldridge and Graham 2013, Hankins, Sere et al. 2015)(fig 3.7b).

The orthologous mutations in N. crassa DRS-2 and DNF-1 were constructed and integrated ectopically into the genome in a Δ*drs-2* background strain. The complemented strain with $drs\text{-}2^{WT}$ allele was able to complement the Δ*drs-2* secretion phenotype, so ectopic integration of the *drs-2* allele was functional and able to rescue the Δ*drs-2* phenotype. Introduction of the $drs\text{-}2^{QQ285\text{-}6GA}$ allele, however, did not rescue the secretion phenotype. This indicated to us that the Δ*drs-2* secretion dysfunction is dependent on the PS recognition and flipping.

When we introduced a $dnf\text{-}1^{WT}$ allele into the Δ*drs-2* background, we also did not see a functional rescue of Δdrs-2 secretion phenotype, which was to be expected. However, in contrast to expectations, the introduction of the PS-flipping $dnf\text{-}1^{N507S}$ construct into the Δ*drs-2* mutant failed to rescue the secretion dysfunction (fig 3.7c). The inability for the $dnf\text{-}1^{N507S}$ allele to

complement the secretion defect could potentially be explained by the disparate localization of the two flippases in the cell (Schultzhaus, Yan et al. 2015).

3.2.12 DRS-2-mCherry Spitzenkörper localization

We hypothesized that the N. crassa DRS-2 may show similar Spitzenkörper core localization as previously shown in *A. nidulans* (Schultzhaus, Yan et al. 2015). Schultzhaus and colleagues indicated that the *dnf-1* ortholog (DnfA) and the *drs-2* ortholog (DnfB) localized to the Spitzenkörper in *A. nidulans*, but the DNF-1 ortholog was localized to the macro vesicles of the outer Spitzenkörper ring, while the DRS-2 ortholog was localized to the microvesicles of the Spitzenkörper core. This exclusion of the vesicles may be an explanation why DNF-1^{N507S} was not able to functionally rescue the secretion phenotype of Δ*drs-2* mutant.

We constructed ectopically integrated *his-3::pccg-1-drs-2-mCherry* strain, and visualized growing tips using swept field confocal microscopy. DRS-2-mCherry fluorescence was exclusively localized at the Spitzenkörper of actively growing hyphal tips (fig 3.8d). This corroborated the *A. nidulans* observation of the DRS-2 ortholog localizing at the Spitzenkörper core. Spitzenkörper stratification has been investigated in *N. crassa* previously (Verdin, Bartnicki-Garcia et al. 2009) and the micro-vesicles of the Spitzenkörper core have been shown to selectively shuttle chitin synthase components (Riquelme, Bartnicki-Garcia et al. 2007, Verdin, Bartnicki-Garcia et al. 2009, Sanchez-Leon, Verdin et al. 2011).

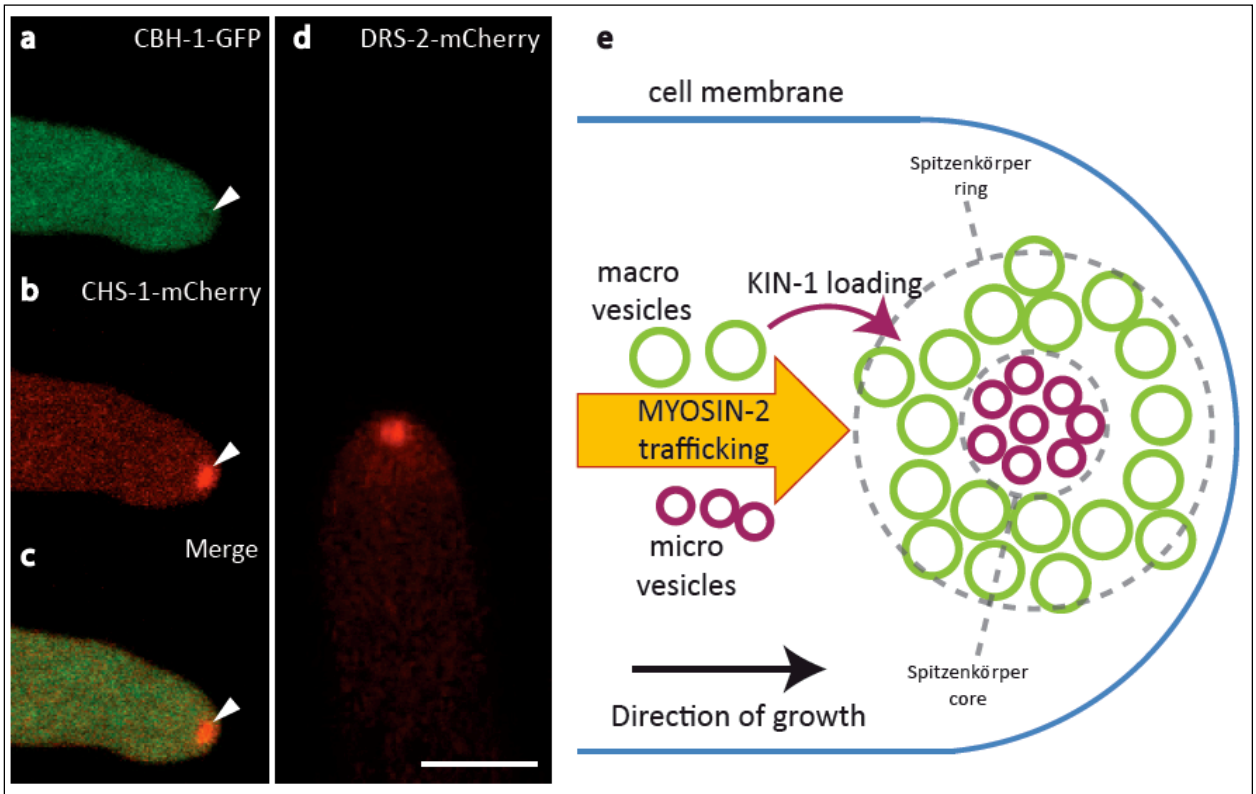

Figure 3. 8: Localization of CBH-1, CHS-1, and DRS-2.
Localization of CBH-1-GFP (a), CHS-1-mCherry(b) and the merged image (c), where arrows indicate spitzenkorper core. DRS-2-mCherry localization (d). Cartoon of the stratified Spitzenkörper (e) is based on (Verdin, Bartnicki-Garcia et al. 2009, Penalva, Zhang et al. 2017)

3.2.13 CBH-1-GFP and CHS-1-mCherry are trafficked in distinct pathways through the Spitzenkörper

Studies of previous Spitzenkörper visualization have shown that the chitin synthase, CHS-1-mCherry, CHS-3-GFP, and CHS-6-GFP can be visualized at the Spitzenkörper core, which is composed primarily of microvesicles. In contrast, the glucan synthase regulator GS-1-GFP localizes to the Spitzenkörper ring, which is composed primarily of macrovesicles. These data indicate distinct vesicle trafficking routes for proteins in Spitzenkörper core microvesicles versus macrovesicles that constitute the Spitzenkörper ring (fig 3.8e).

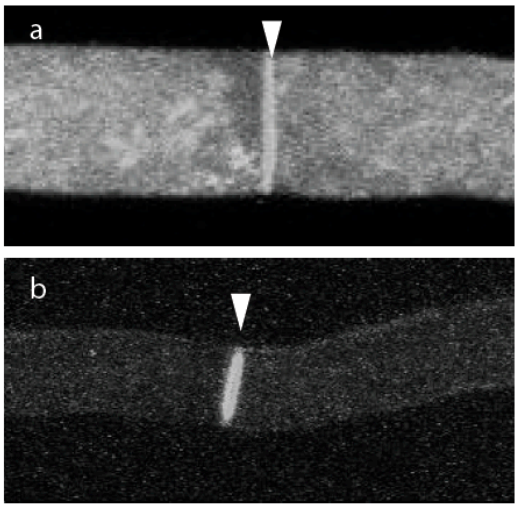

Figure 3. 9: Septal secretion of CAZymes. Septal accumulation of CBH1-GFP (a) and septal accumulation of CBH2-GFP (b).

To visualize the trafficking of cellulase components through the Spitzenkörper, we constructed a strain co-labeled with CHS-1-mCherry and native CBH-1-sfGFP. When grown under 6% w/v carboxy-methyl cellulose, we visualized the CBH-1-sfGFP in the hyphal tip and saw that CBH-1-sfGFP was excluded from localization with CHS-1-mCherry (3.8 a,b,c). These observations indicated that while CHS-1-mCherry labels the Spitzenkörper core, CBH-1-sfGFP is shuttling through the Spitzenkörper ring for secretion from the growing hyphal tip.

Previous studies have shown that the sole site of secretion does not happen at the hyphal tip (Hayakawa, Ishikawa et al. 2011). From our work, it is possible that septal secretion may be a significant site of protein secretion and a route that cellulolytic enzymes are released. GFP labeling of CBH-1, CBH-2 and GH18-4 showed that all three enzymes are secreted at the septum (3.9a,b, 3.5c). With DRS-2 localized to the hyphal tip and enzyme cargo localized at the septum, DRS-2 may play a role in both sites for secretion.

3.2.16 A Δ*cdc-50* (NCU01165) strain does not phenocopy the Δ*drs-2* secretion phenotype

Flippases are components of a heterodimer complex, and the role of the heterodimer partner of the flippases has not been well described in its mechanistic contributions to lipid headgroup or backbone discrimination (Graham 2004). However, deletion of the partner subunit results in a phenocopy of the flippase mutants in many cases (Saito, Fujimura-Kamada et al. 2004, Chen, Wang et al. 2006, Lenoir, Williamson et al. 2009) indicating a contribution of the heterodimer partner to flippase function. Thus, identifying the heterodimer partner of the DRS-2 flippase would be essential to understanding function of the flippase. We looked at putative *N. crassa* orthologs of the Cdc50p, Lem3p, and Crf1p proteins as they are paralogs of one other and heterodimer partners for Drs2p, Dnf1p/2p, and Dnf3p, respectively. From a BLASTp search of

the *N. crassa* proteome, we found only a single homolog of Cdc50p/Lem3p/Crf1p, *NCU01165* (termed *cdc-50*) with 42%, 36%, and 38% protein sequence identity to Cdc50p, Lem3p and Crf1p, respectively. When we extended the search to other fungi, we observed only one homolog of Cdc50p/Lem3p/Crf1p within all the predicted proteomes of the fungi surveyed. *cdc-50* has a similar architecture to Lem3p/Cdc50p/Crf1p as it contains two transmembrane domains.

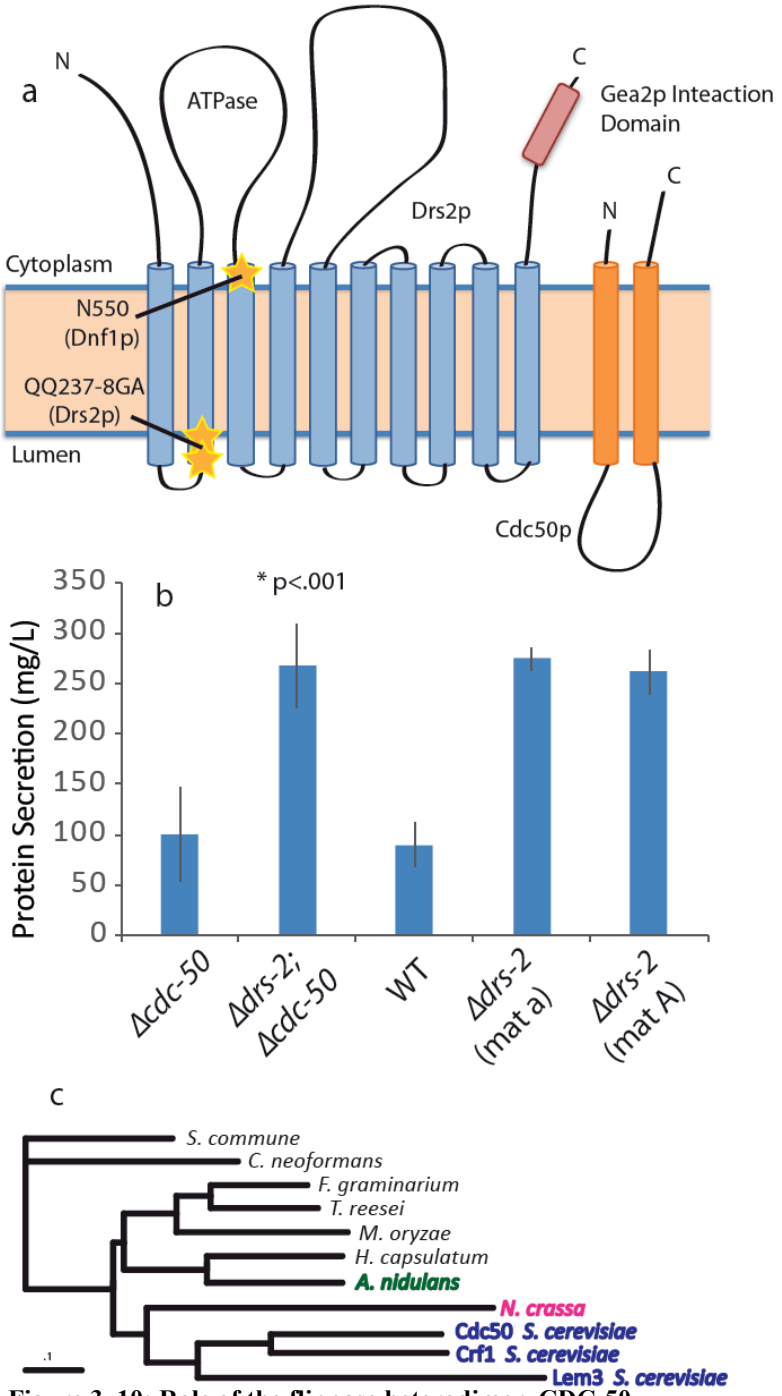

Figure 3. 10: Role of the flippase heterodimer, CDC-50. Cartoon of the S. cerevisiae Cdc-50p/Drs2p heterodimer complex (a). Secretion of the Δ*drs-2*; Δ*cdc-50* knockouts (b). A gene tree of the homologs of *S. cerevisiae* Cdc50p implicates a single gene homolog in the filamentous fungi (c).

We constructed a deletion strain of *cdc-50* in *N. crassa* by the nourseothricin resistance cassette replacement of the *cdc-50* coding sequence, and mated this knock out strain to the Δ*drs-2* knock out to isolate Δ*drs-2; Δcdc-50* double mutant progeny. The Δ*cdc-50* single mutant did not show a significant phenotype of protein accumulation over WT. The Δ*drs-2; Δcdc-50* double mutant phenocopied the single mutant Δ*drs-2*. From this, we concluded that CDC-50 may not be the functional heterodimer partner with DRS-*2* in *N. crassa*.

3.2.17 Mutant of upstream flippase kinase regulator, *nrc-2*, is synthetically lethal in liquid cultures with Δ*drs-2*

We began to probe other pathways to confirm conservation of flippase regulation. Flippase function is regulated by a series of kinases upstream that have been previously elucidated in S. cerevisiae (Nakano, Yamamoto et al. 2008, Roelants, Baltz et al. 2010). When we searched the *N. crassa* proteome for orthologues

of flippase activators, Fpk1p and Kin82p from *S. cerevisiae* using BLASTp, we identified a single homologue, *nrc-2,* with 61% and 67% respective amino acid identities to the paralogs Fpk1p and Kin82p. Fpk1p/Kin82p in *S. cerevisiae* has been shown to activate membrane flippases Dnf1p and Dnf2p, and has a synthetic lethal phenotype with the *cdc50*Δ*,* a strain that lacks the heterodimer partner for Drs2p (Nakano, Yamamoto et al. 2008). We hypothesized that if NRC-2 is conserved in its activating function of the flippase DNF-1 in *N. crassa*, we would observe a cellulase hyper-secreting phenotype for the Δ*nrc-2* mutant. Furthermore, we predicted that a Δ*nrc-2;* Δ*drs-2* double mutant would show synthetic lethality.

To test these hypotheses, we isolated cross progeny of the single mutants, and genotyped strains that carried both the single mutations, Δ*drs-2* and Δ*nrc-2* and the double mutations Δ*nrc-2;* Δ*drs-2*. Superficially, this indicated to us that our second hypothesis about the synthetic lethality of the double mutant was incorrect. When we tested for the cellulase protein secretion, we observed a 2-fold level of increased protein accumulation in the Δ*nrc-2* mutant relative to WT. (fig 3.11d, 3.7a). This increase in protein accumulation, from conservation to *S. cerevisiae*, could potentially function through activation of either DRS-2 or DNF-1 to cause the secretion phenotype.

When we placed the double mutant, Δ*drs-2*; Δ*nrc-2* in liquid culture for the secretion experiment, we did not see any growth of the mycelia. We subsequently isolated more progeny of the double mutant, and observed the same synthetic phenotype in liquid culture that persisted in all Δ*drs-2*; Δ*nrc-2* progeny. This data indicated that the synthetic lethality of Δnrc-2 and Δdrs-2 may be conserved between *S. cerevisiae* and *N. crassa*, and may further implicate a conserved role of Δ*nrc-2* in the same genetic pathway as Δ*dnf-1*.

3.2.18 Putative downstream regulators by DRS-2 do not contribute to Δ*drs-2* secretion phenotype.

The Ap1 complex is the heterotetrameric clathrin coat adaptor protein complex that facilitates the trafficking between the trans-Golgi network and early endosomes (Gomez-Navarro and Miller 2016). Apl4p is the γ- adaptin large subunit of the AP-1 complex (Kirchhausen 2000). Studies in *S. cerevisiae* have implicated that the Drs2p is an activator of Apl4p function because of the synthetic lethality of the mutant *drs2*Δ with the mutants *gga1*Δ*; gga2*Δ and direct binding of the Drs2p with Apl4p (Liu, Surendhran et al. 2008). While the knock out the Apl4 homolog, APL-4 was not available in the *N. crassa* deletion collection, previous work in *N. crassa* generated an *apl-4* temperature-sensitive allele that was missing 3 amino acids at the N terminus resulting in a colonial growth phenotype at restrictive growth temperatures (37ºC) (Seiler and Plamann 2003). Previous work in the lab showed that GH5-1-GFP accumulated in endosomes in *apl-4*^{ts} mutant grown at restrictive temperature. While the consequence of this accumulation is still unclear, it could be a re-routing of GH5-1 into endosomes for vacuolar processing, or a result of GH5-1 secretory processing stopping in the *apl-4*^{ts} mutant, which prevents further downstream trafficking of GH5-1 (Scheurg, unpublished results). When the *apl-4*^{ts} mutant is grown at permissive temperature and induced for cellulase production, the amount of protein secreted is increased, but not significant. We then tested the amount of protein accumulation of the single *apl-4*^{ts} under 37ºC restrictive temperature on cellulose induction in liquid culture, and the secreted protein of the *apl-4*^{ts} mutant was not observed to be increased compared to WT. It is

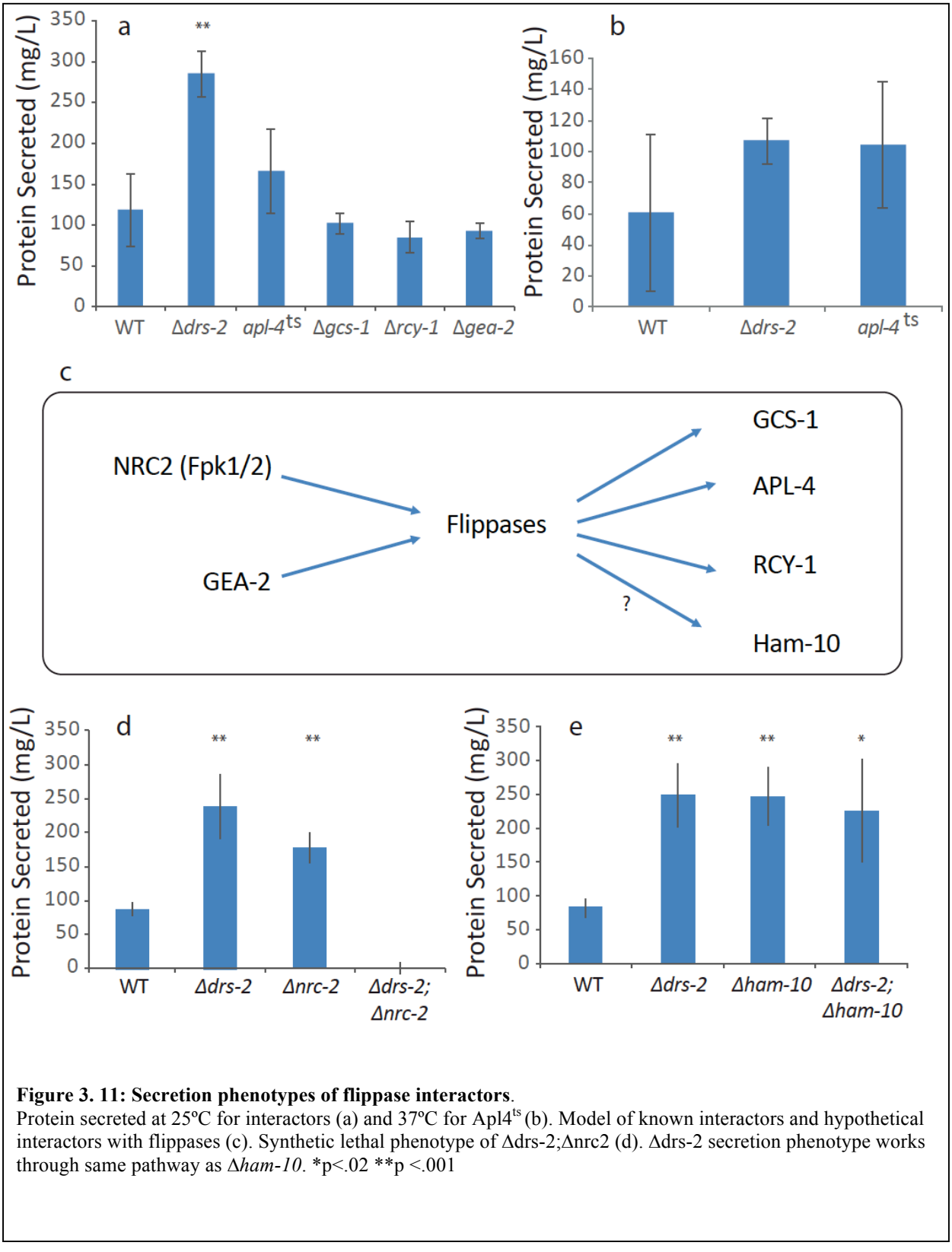

Figure 3. 11: Secretion phenotypes of flippase interactors.
Protein secreted at 25ºC for interactors (a) and 37ºC for Apl4^{ts} (b). Model of known interactors and hypothetical interactors with flippases (c). Synthetic lethal phenotype of Δdrs-2;Δnrc2 (d). Δdrs-2 secretion phenotype works through same pathway as Δ*ham-10*. *p<.02 **p <.001

also notable that cellulase hyper-secretion phenotype of the Δ*drs-2* mutant is abolished at a higher temperature (fig 3.11a,b).

Previous work in *S. cerevisiae* showed that Drs2p enhances the curvature of the trans-Golgi membrane to recruit binding to the Gcs1p ALPS motif by flipping the repellant anion headgroups of phosphatidylserine to the cytoplasmic leaflet. The localization of Gcs1p is crucial for proper recruitment of the v-SNARE, Snc1p (Xu, Baldridge et al. 2013). When knock outs of Δ*gcs-1* single mutants in *N. crassa* were grown under cellulosic condition, we hypothesized that the genetic interaction with DRS-2 might have a role in positively regulating GCS-1 and the Δgcs-1 mutant would phenocopy the Δdrs-2 single mutant phenotype. However, we did not observe an increased protein accumulation phenotype for Δ*gcs-1* over WT. These data indicated that the positive regulation of GCS-1 by DRS-2 may not be causing the secretion defect of the Δ*drs-2* mutant (fig 3.11a).

Other genetic interactors of Drs2p in S. cerevisiae are Rcy1p, an F-box protein that is required for the proper recycling and localization of the v-SNARE Snc1p and has been shown to directly interact with Drs2p (Hanamatsu, Fujimura-Kamada et al. 2014). However, when we grew the Δ*rcy-1* mutant under cellulosic induction conditions, the culture did not accumulate protein over the levels observed in WT cultures (fig 3.11a).

Gea2p, an ARF GTPase exchange factor (ARF-GEF) that directly interacts with Drs2p in *S. cerevisiae*, helps to coordinate the binding of Arl1p to regulate membrane dynamics at the trans-Golgi network (Tanaka, Fujimura-Kamada et al. 2011, Tsai, Hsu et al. 2013). Gea2p may play a similar role in activating or being activated by Drs2p. We hypothesized that a Δgea-2 knock out in *N. crassa* may display a Δdrs-2 phenotype. However, we were unable to detect an increase in the amount of protein secreted over wild type secretion levels in Δgea-2 mutant cultures (fig 3.11a). These results suggest that the Δ*drs-2* secretion phenotype is not subject to the myriad of Drs2p interactions in *S. cerevisiae* for the secretion of cellulases.

3.2.19 Screening of potential lipid binding domain proteins identifies genetic interaction of *drs-2* with *ham-10*

Other downstream genetic interactors of DRS-2 in N. crassa may not be direct interactors, but may actually be proteins that are able to bind to the phosphatidylserine asymmetry through known PS binding domains (Stace and Ktistakis 2006). We set out to identify potential genetic interactors with canonical lipid binding motifs as potential downstream interactors with DRS-2. We searched the *N. crassa* proteome with BLASTp for sequence similarity to human proteins with documented lipid binding domains and found a single annexin like protein, (NCU04421/*anx-14*), and the C2 domains of known lipid binding proteins, phospholipase C (NCU01266/*plc-2*, NCU06245/*plc-1*, NCU02175/*inl-15* and NCU11415/*inl-7*), protein kinase C (NCU06544/*pkc-1*), synaptotagmin (NCU02833/*ham-10*, NCU03947/*ubi-6*, NCU01004/*chol-15*, NCU01510/*mup-3*, NCU08064/*mup-2*) and rabphilin-3 (NCU03263/*syt-1*). *N. crassa* did not have significant hits to discoidin-like C2 domains nor to coagulation factor Gla-like domains which have been previously shown interact with PS (Stace and Ktistakis 2006).

Utilizing the KO collection, we screened the single mutants of these annexin and C2-domain containing proteins for potential cellulase secretion and found that in the strain bearing a knock out of *ham-10*, a C2 –containing protein with a predicted PFAM domain with key member Munc13, showed a >2-fold increase in protein accumulation over WT ($p<.001$) (fig 3.11e). *ham-10* has been identified previously as hyphal anastomosis mutant, and found to be incapable of cell-to-cell fusion in both hyphae and germlings (Fu, Iyer et al. 2011).

To investigate whether HAM-10 and DRS-2 interact in the same pathway, we mated the Δham-10 knock out with the Δdrs-2 mutant to isolate cross progeny. If the genes interact in parallel pathways, we would expect a synthetic phenotype like that of the Δ*nrc-2;* Δ*drs-2* mutant and if the genes interact in the same pathway, we would not expect a change either in growth or an additive protein accumulation phenotype. When we cultured the Δham-10; Δdrs-2 double mutant on cellulose medium, we saw significant protein accumulation over WT levels ($p<.02$)(fig. 3.11e) and a similar protein accumulation rate as either the Δ*drs-2* or Δ*ham-10* single mutants. This indicated to us that DRS-2 may be functioning through HAM-10 to influence the secretion rate of cellulases.

3.3 Discussion

The aminophospholipid flippase Drs2p in *S. cerevisiae* has a multifaceted role in maintaining membrane asymmetry of the anionic phospholipid, phosphatidylserine at the trans-Golgi network and endocytosis at the plasma membrane (Ripmaster, Vaughn et al. 1993, Gall, Geething et al. 2002, Natarajan, Wang et al. 2004, Alder-Baerens, Lisman et al. 2006, Chen, Wang et al. 2006, Liu, Surendhran et al. 2008, Hankins, Sere et al. 2015). In this work, we identify DRS-2 as being a modulator of cellulase secretion rate, transcriptional regulation, and in the same genetic pathway as the C2-lipid domain binding protein, HAM-10.

From our work, we have shown that the Δdrs-2 mutant has an increased secretion rate between 24 and 72h post induction, and is capable of accumulating levels of cellulases in the supernatant up to 2.5-fold higher than WT culture supernatant (3.2a). Additionally, the increase of secretion compounds on the phenotype of strains carrying modification of the already known transcriptional upregulators, *pccg-1-clr-2* and Δ*cre-1.* We found that Δ*drs-2* is capable of regulating transcription of the major cellulase gene *cbh-2* and the chitinase, *gh18-4.* It remains unclear how Δ*drs-2* regulates the transcription of these genes, as it does not localize to the nucleus, and would seem to indirectly alter the transcription of these genes through feedback mechanisms. Further studies on transcriptomics of Δ*drs-2* will help to implicate the specific machinery that is necessary for the changes in gene transcription and the specificity for altering *cbh-2* and *gh18-4* transcription but not altering *cbh-1* and *gh5-1*.

DRS-2 is not the only flippase that when knocked out, contributes to a cellulase hyper-producing phenotype. The primary PE flippase, DNF-1, when mutated, was observed to have increased cellulase secretion. Previous mutagenic work looking at a substrate swapped allele of Drs2p and Dnfp1 in *S. cerevisiae* indicated that the substrate swapped $Dnf1^{N550S}$ is be able to rescue the *drs2*Δ phenotypes, but the orthologous mutations in *N. crassa* did not rescue the secretion phenotype. This inability to rescue the secretion dysfunction may be explained by the alternate localization of the DNF-1 ortholog, DnfA in *A nidulans* relative to the DRS-2 ortholog, DnfB.

While both flippases in *A. nidulans* localizes to the Spitzenkörper, they localize to different vesicle populations. The DNF-1 ortholog populates macrovesicles of the Spitzenkörper ring, while the DRS-2 ortholog localizes to vesicles in the Spitzenkörper core.

From the *A. nidulans* flippase localization, DRS-2-mCherry localization, and microscopy of CBH-1-GFP and CHS-1-mCherry co-localization, we observed that DRS-2-mCherry may be decorating the Spitzenkörper core microvesicles containing the chitin synthase machinery. The exclusion of CBH-1-GFP from the CHS-1-mCherry would indicate that the cargo of plant cell wall degrading enzymes and fungal cell wall modifying enzymes may be me sorted for differential trafficking to the growing hyphal tip. If chitin synthase machinery were to co-localize with DRS-2-mCherry, it could imply that DRS-2 has a role in trafficking cell wall modifying enzymes to the plasma membrane.

Furthermore, DRS-2 may play be playing a biophysical role in maintaining the curvature of the Spitzenkörper microvesicles by the flipping of PS by Drs2p. In S. cerevisiae, binding of the Gcs1p ALPS motif to the trans-Golgi network requires Drs2p function to facilitate the curvature of the membrane from flipping PS to the cytosolic leaflet. Microvesicles are described as 30-40nm in diameter vesicles that are major sites of chitin synthase activity. The small diameter of microvesicles compared to the macrovesicles, sized at 70-100nm (Bartnicki-Garcia 2006) may require a higher amount of membrane distortion. And the anionic headgroups of PS asymmetry on the microvesicle cytoplasmic membrane leaflet may help to facilitate the curvature of these vesicles.

Surprisingly, though, the genetic interactors of Drs2p in yeast like Gcs1p, among other genes identified in the Drs2p pathway of *S. cerevisiae*, do not to contribute as single mutants to the secretion phenotype (fig 3.10a). The mutant of the flippase activating kinase Fpk1p ortholog, NRC-2, has a synthetic phenotype with the Δdrs-2 mutant corroborates previous data on Fpk1p regulation of Dnf1p and Dnf2p. By having a synthetic phenotype, the pathways of function are not fully redundant.

Conservation of the *S. cerevisiae* Drs2p pathway may occur at the regulation of Fpk1/2p homolog, NRC-2 on activating DNF-1. The synthetic phenotype of the Δ*nrc-2;* Δ*drs-2* double mutant may represent the conserved interaction of *fpk1/2*Δ synthetic lethality with S. cerevisiae *cdc50*Δ. However, we were able to find a mutant of HAM-10, a protein that contains a lipid binding C2 domain. The Δ*ham-10* mutant phenocopied the Δ*drs-2* as a single mutant, but also was not synthetically lethal with the Δ*drs-2* mutant, nor additive in secreted cellulases as a Δ*drs-2;* Δ*ham-10* double mutant. This indicated to us that a novel function of *drs-2* may be genetically interacting with the lipid binding *ham-10* for the protein secretion phenotype.

ham-10 has been previously identified as a potential vesicle trafficking mutant (Fu, Iyer et al. 2011) due to the presence of a C2 domain. Insofar the function of the C2 domain and the genetic interactions have not been well elucidated. *Ham-10* has an essential role in germling and hyphal fusion and contains a PFAM domain that has a prominent member of Munc13, a mammalian uncoordinated mutant, that in neuronal cells, have been shown to facilitate priming of the SNARE complex at the fusion membrane (Ma, Li et al. 2011, Yang, Wang et al. 2015), and may represent a novel interaction of the flippase, DRS-2.

While the Δ*ham-10;* Δ*drs-2* double mutant is neither synthetically lethal nor synergistic for protein accumulation, this does not preclude other functions of the *ham-10* pathway. In this manner, *ham-10* may be functioning in the same pathway as *dnf-1* for cellulase secretion, as the C2 domain of Ham-10 is still unknown in its lipid specificity. The key next experiment would be to test whether the Δ*drs-2*; Δ*dnf-1 and* Δ*dnf-1;* Δ*ham-10* double mutants has a similar, non-synergistic, non-synthetically lethal phenotype as the Δ*drs-2*; Δ*ham-10* double mutant. If both double mutant strains have non-synergistic protein accumulation, and non-synthetically lethal phenotypes, this would in turn, imply the role of HAM-10 as a key component of both *drs-2* and *dnf-1* protein accumulation pathways.

3.4 Methods

3.4.1 Media and strain maintenance

N. crassa strains were cultured in Vogel's medium (Vogel 1956) agar supplemented with 2% sucrose for 7 days at 25ºC light to obtain conidia for experimentation. Crosses were performed on Westergaard's media (Westergaard 1947). WT strain (FGSC2489), and the gene deletion strains (Colot, 2006) were obtained from the Fungal Genetic Stock Center (FGSC, University of Missouri, Kansas City, Missouri, USA)(McCluskey 2003).

3.4.2 Batch secretion

Candidate cultures were inoculated at 2E7 conidial spores for a 100mL flask of 1% glucose minimal medium cultures. Cultures were then incubated at 200rpm at 25ºC for 30h, which then 5mL of 20% avicel (PH-101, Sigma) suspension in water was introduced to the cultures to produce a 1% cellulose induction. 20% solutions of xylan (Beechwood, Sigma) and Sucrose (Fisher Scientific) were used for xylan and sucrose inductions, respectively. This total amount of 2% carbon results in full digestion by the end of 120hrs, using WT strains, thus, we used this induction procedure as a reference for screening future strains.

3.4.3 Protein and activity measurements

Protein secretion was measured by Bradford protein assay reagent(Bio-rad) using BSA as a protein standard. Sucrose hydrolysis was measured by mixing 1:1 culture supernatant: 400mM sucrose in 50mM Sodium Acetate pH 5.0 by volume, incubating for 30 minutes at 37ºC and stopped by chilling at 4ºC. Reducing sugar concentration was measured by 1:20 dilution of incubation: DNS assay reagent, and incubation at 100ºC for 5 min, OD was read at 540nm with a 50% glucose, 50% fructose standard.

Hemicellulase activity was assayed using Azo-xylan (Birchwood) reagent (Megazyme). 1:1 culture supernatant: 1% w/v Azo-xylan was incubated at 50ºC for 10 minutes, 2.5 volumes 95% ethanol was added to terminate the reaction, assays were vortexed, centrifuged at 1000g for 10 min, and supernatant measured at OD590.

3.4.4 Mass Spectroscopy

72h induced culture supernatant was filtered through a Buchner funnel with glass fiber Whatman filter (GE). Proteins were precipitated with addition of 45% Ammonium sulfate (Fisher Scientific) and allowed to equilibrate at 4ºC for 30 min. Protein suspensions were centrifuged at 14000rpm in J14 rotor Beckman floor centrifuge for 30min to pellet total protein. Protein pellet was resuspended in 20mM Sodium Phosphate buffer pH 7.0, and measured via Bradford using BSA standard. Protein solution was denatured with 6.27 urea and 9.8mM DTT final concentration and incubated at 55º for 20 min. Alkylation was performed with 19mM iodoacetamide final concentration and incubated at 25ºC for 30 min in dark and quenched with the addition of 27.5mM DTT at 25ºC for 20 min. Trypsin digest was performed at 25ºC for 16 h with 500ng trypsin, 50mM Tris pH 7.0, 1mM $CaCl_2$. UPLC-MS/MS was performed as previously reported in Wu(Wu, Dana et al. 2017). Proteins were curated using a threshold of significance $p < .001$ and a >5 fold change in abundance.

3.4.5 Phylogenetics

Neurospora DRS-2 protein sequences was searched against the NCBI database for the genomes of Aspergillus nidulans FGSC A4, Cryptococcus neoformans var. grubii H99, Fusarium graminarium PH-1, Histoplasma capsulatum G186AR, Magnaporthe oryzae 70-15, Neurospora crassa OR74A, Saccharomyces cerevisiae S288c, Schizophyllum commune H4-8, Trichoderma reesei QM6a using BLASTp. Multiple alignment was performed using Constraint-based Multiple Alignment Tool (COBALT), and tree generated using fast minimum evolution (Desper and Gascuel 2004)

3.4.6 Microscopy

Observations of growing cells was performed on an Olympus FluoView FV1000 Confocal Microscope (Olympus, Japan), equipped with Ar/2 ion and He/Ne1 lasers to detect GFP (λex = 488, λem = 505–530 nm) and mCherry (λex = 543, λem = 600–630 nm). We used a Plan Apochromat 60× oil objective, and images were false colored and analyzed using ImageJ.

3.4.7 qRT-PCR

RNA from 3mL of 24h post induction cultures were extracted using 50mg of glass beads. 1 mL of Trizol reagent was added to sample and nutated at 25ºC for 5 min. 200µL chloroform was added, vortexed quickly and centrifuged at 4ºC for 15 min at 12,000g. Upper phase was transferred to a new tube and a ethanol precipitation was performed. RNA was subsequently digested with DNAse I (Life Technologies), and the result was confirmed for full digestion by running on a 1% agarose gel. qRT-PCR was performed with the EXPRESS One-Step SYBR Green ER™ Kit, with premixed ROX (Life Technologies). The standard cycling protocol in a 20µl reaction was used with 2ng total RNA as the input. Amplification was performed on a CFX Connect™ Real-Time PCR Detection System (Biorad). Transcript abundance was normalized to actin (NCU04713).

3.4.8 Western Blot

24μL of 72hr normalized culture supernatant was loaded with 4x SDS loading buffer and ran on a 10% 29:1 bis/tris acrylamide gel. Protein gels were subjected to western blot and probed with α-CBH-1 antibody(1:1000 dilution), α-CBH-2 (1:1000 dilution) antibody and α-GFP (1:1000 dilution, Roche). α-CBH-1 and α-CBH-2 were a kind gift of Trevor Starr (Energy Biosciences Institute). Each experiment was repeated 3 times in triplicate with identical results.

CHAPTER 4: ASSAYING THE TRANSLOCATION OF CELLULASE ENZYMES IN VIVO

4.1 Introduction

Despite the broad understanding of protein secretion, the initial step of secretion, protein translocation, still has not been comprehensively described (Pechmann, Chartron et al. 2014, Chartron, Hunt et al. 2016). In order for a protein to be secreted, it must transverse the ER membrane by one of two methods: one that depends on a signal recognition particle (SRP-dependent)(Walter and Blobel 1981), which uses translational forces to translocate the protein across the membrane, and the other route of translocation is independent of the SRP, and has been theorized to translocate a nascent peptide across the membrane post-translationally (SRP-independent)(Deshaies and Schekman 1987, Deshaies, Koch et al. 1988). The translocation pore is a similar complex of proteins between the two translocation pathways, but Sec62p, an ER membrane bound protein is potentially a unique subunit to the translocation pore in the SRP-independent pathway, and has been shown to aid in the translocation of both soluble proteins into the ER lumen and incorporation of transmembrane proteins into the lipid membrane (Deshaies and Schekman 1987).

Studies in S. *cerevisiae* have predicted that nearly 45% of the secretome may be translocated through an SRP-independent pathway (Ast, Cohen et al. 2013). However, our understanding of protein delivery to the ER is not comprehensive, as many SRP-independent translocation events in *S. cerevisiae* are still under investigation (Ast and Schuldiner 2013). Previous studies have shown that Get3p, a cytosolic protein shuttle, in coordination with the rest of the GET pathway is responsible for the recruitment and delivery of GPI anchored proteins and tail-anchored proteins to the ER membrane without the reliance on SRP machinery (Schuldiner, Collins et al. 2005, Schuldiner, Metz et al. 2008, Ast and Schuldiner 2013), however the GET pathway has not been implicated in soluble secreted proteins. Recent studies in ribosomal profiling in *S. cerevisiae* have shown that mRNAs of secreted proteins are actively enriched toward the ER membrane surface, although the mechanism is still unclear (Chartron, Hunt et al. 2016). This enrichment to the membrane of mRNAs encoding secreted proteins can be signal peptide independent, and is proposed to depend on the SRP being recruited by cis–acting sequences in the mRNA molecule that act as a targeting motif (Chartron, Hunt et al. 2016).

From RNA-seq data performed on WT strains of *N. crassa* challenged with sucrose, no sugar, and cellulose, fragments mapped per kilobases measured (FPKM), is increased at least 2 fold for all components of the SRP-independent translocation pore (Coradetti, Craig et al. 2012, Benz, Chau et al. 2014, Xiong, Coradetti et al. 2014). Strikingly, subunits of the SRP and SRP receptor, a protein complex that recruits SRP to the ER membrane, are not induced by the same fold change, and are instead maintained at a constant level of expression between the three media conditions. These results suggest that the translocation of upregulated proteins under cellulosic conditions occurs through the SRP-independent pathway.

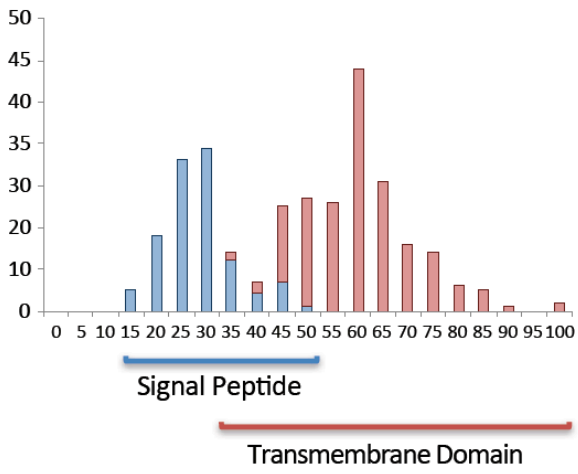

Figure 4. 1: Bifurcation of signal hydrophobicity in proteins that traverse the membrane. Bifurcation is explained by a transmembrane domain population and a more hydrophilic signal peptide distribution.

In the current model of SRP-independent translocation, cytosolic chaperones help shuttle the SRP-independent, fully translated, unfolded protein to the ER translocon for membrane translocation. With the increase in SRP independent translocation during cellulose induction, we presume that the cytosolic chaperones would increase in their transcription level in order to compensate for the protein that needs to be maintained in an unfolded state in the cytosol. However, we observed that the major subunits of the heat shock proteins (HSP70) and activating proteins (HSP40) that were increased in transcription all contained the presence of a SP. This indicated that upon cellulose induction, cells respond by increasing the ER luminal HSP70 and HSP40 proteins through the UPR (Benz, Chau et al. 2014, Xiong, Coradetti et al. 2014, Fan, Ma et al. 2015, Qin, Wu et al. 2017), but a similar HSP70 and HSP40 upregulation was not found in the cytoplasmic chaperones (Coradetti, Craig et al. 2012). If the cytoplasmic chaperones were necessary for post translational translocation of the cellulase proteins into the ER, this model for SRP-independent translocation of soluble proteins was not well corroborated by the RNA-seq data. This informed our hypotheses that (1) cellulases undergo SRP-independent translocation, and (2) the current model of SRP-translocation is not sufficient to explain the route by which SRP-independent substrates travel to the ER. We began by investigating the first hypothesis, and we will provide routes by which to investigate a potentially novel pathway for the delivery of SRP-independent substrates to the ER.

4.2 Results

4.2.1 Predictions of Signal hydrophobicity reveals bimodality is due to inherent hydrophobicity of Signal Peptides and Transmembrane domains

As the nascent protein is translated and the first signal (signal peptide or transmembrane fragment) appears outside of the ribosome exit tunnel, the hydrophobicity of the signal recruits SRP to bind the signal and traffic the ribosome nascent protein complex to the ER (RNP). Previous work has attempted to predict that the subset of signal sequences that utilize SRP-independent delivery to the ER through hydrophobicity of the signal sequence. When plotted in a histogram, all signal sequences of the *S. cerevisiae* genome have hydrophobicities that lay in a bimodal distribution (Ast, Cohen et al. 2013). The hypothesis was then that the hydrophilic distribution of the bimodal plot would be putative SRP-independent substrates while SRP-dependent substrates would constitute the hydrophobic portion of the distribution. From the predicted signals of *S. cerevisiae*, we wanted to plot the predicted hydrophobicities of *N. crassa* as a prediction of potential SRP-independent substrates. When we utilized the algorithm to

predict the hydrophobicity of the *N. crassa* secretome, our data also showed a bimodal distribution of hydrophobicities similar to that of *S. cerevisiae*. However, when we highlighted the signal peptide containing population of proteins to the transmembrane containing proteins, a clear explanation of the bifurcation appears (fig 4.1). The bimodality of the hydrophobicity plot is indicative of the role of the signals, where signal peptides are inherently predicted to be more hydrophilic than transmembrane domains.

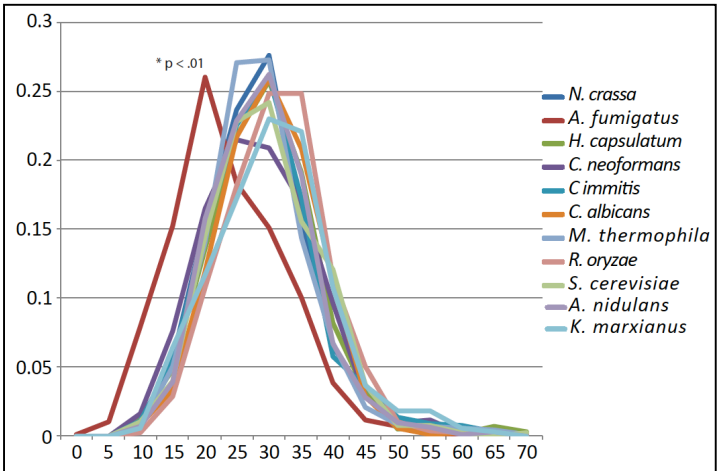

Figure 4. 2: Hydrophobicity distribution of signal peptides from thermophilic and pathogenic organisms. Differences in hydrophobicity are insignificant except for Aspergillus fumigatus (red) which has a significant hydrophobic shift (p<.01)

4.2.2 Signal peptide hydrophobicities of signal peptides of model and pathogenic fungi

Proteins increase their hydrophobicities as temperatures are increased (Chen, Huang et al. 2003), and given the particular environmental niche, local environmental niches select for the most reproductive fungal strains (Ellison, Hall et al. 2011). As such, if the environmental temperature is selecting for an optimal hydrophobic signal for a protein, and if we extrapolated this algorithm to various fungi from varying environmental niches, we would expect to see a shift to a more hydrophilic signal peptides as the optimal environmental temperatures for each fungus increases. We surveyed human opportunistic pathogenic fungi (*Aspergillus fumigatus, Histoplasma capsulatum, Crytococcus neoformans, Coccoides immitis, Candida albicans*, *Rhizopus oryzae*), a thermophilic fungus (*Myceliophthora thermophila,*), mesophilic fungi (*Neurospora crassa and Aspergillus nidulans*), a thermophilic yeast (*Kluyveromyces marxianus*) and the model yeast (*Saccharomyces cerevisiae*).

When we plotted the signal peptide hydrophobicities of each of these fungi, surprisingly, the majority of all fungi are very similar in their signal peptide hydrophobicity distribution (fig 4.2). Most surprising was the similar distributions of the thermophilic model fungus, *M. thermophi*la to that of *N. crassa*. Despite the growth temperature difference between the optimal growth of these two model organisms (30ºC for *N. crassa* and 45ºC for *M. thermophila*) the hydrophobicities of both of the signal peptide distributions were nearly indistinguishable. Despite the trend for heat to increase protein hydrophobicity and alter protein interactions, the hydrophobicity of signal peptides (SP) are not correlated with the environmental niche temperatures that these fungi may inhabit.

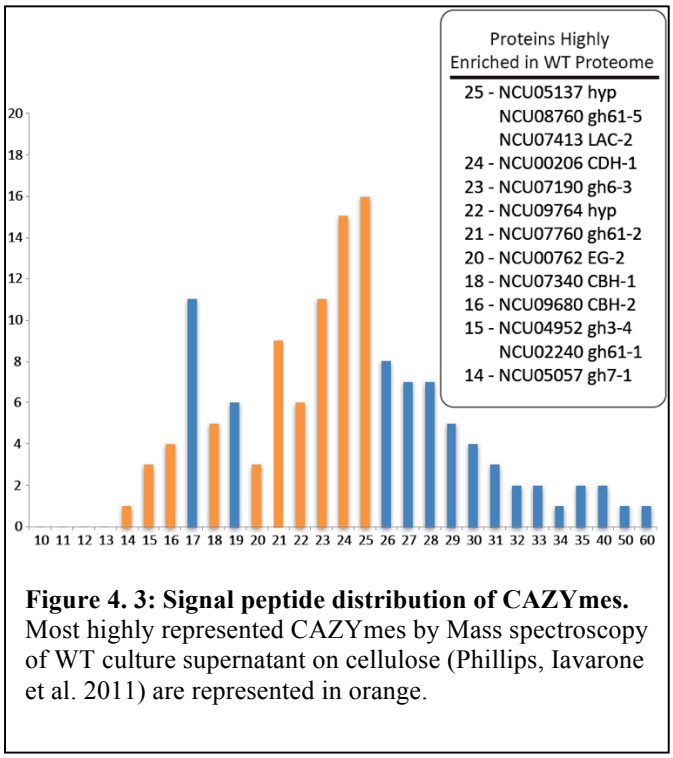

Figure 4. 3: Signal peptide distribution of CAZYmes. Most highly represented CAZYmes by Mass spectroscopy of WT culture supernatant on cellulose (Phillips, Iavarone et al. 2011) are represented in orange.

Notably, the hydrophobicity distribution of *Aspergillus fumigatus* was shifted significantly to the hydrophilic size of the spectrum ($p<.01$). *Aspergillus fumigatus* is one of the most prolific human fungal pathogens and is a ubiquitous airborne fungus (Pringle, Baker et al. 2005) that has even adapted to the environment on the International Space Station (Knox, Blachowicz et al. 2016). *A. fumigatus* is a compost fungus, and thus, able to tolerate thermophilic conditions in compost piles of 60ºC (Bhabhra and Askew 2005) and foster mesophilic growth at 25ºC (Millner, Marsh et al. 1977). The distribution change in hydrophobicities, may reveal a species-specific adaptation to a SRP-independent mechanism for the secretion of *A. fumigatus* peptides.

4.2.3 Highly Secreted CAZYmes contain hydrophilic signal peptides

To determine if the shifted distribution of hydrophobicity was one that was physiologically relevant to SRP-independent delivery to the ER of *A. fumigatus,* our first task was to define the SRP-independent and dependent-translocating proteins. From our work, *N. crassa* signal containing proteins represent 30% of the predicted proteins in the genome as predicted by Phobius (http://phobius.sbc.su.se/) (Kall, Krogh et al. 2007). For the rest of this work, we focused on signal peptide hydrophobicities because the shift in the hydrophobic spectrum was found in signal peptides of *A. fumigatus*.

Due to the massive 1000-fold upregulation of genes encoding cellulases when *N. crassa* is induced on cellulose (Coradetti, Craig et al. 2012), we hypothesized that cellulase signal peptides would be efficient in traversing the translocon by employing the SRP. However, the pattern of transcriptional and proteome response to cellulose induction led us to hypothesize that SRP was not recruited for translocation of cellulase proteins(Benz, Chau et al. 2014, Xiong, Coradetti et al. 2014). Thus, we plotted the cellulases on the hydrophobicity plot of SP containing proteins, and observed the hydrophobic distribution of proteins that were highly enriched in the secretome (Phillips, Iavarone et al. 2011) were skewed toward the hydrophilic distribution of the hydrophobicity spectrum. These data would corroborate the hypothesis that the most highly expressed carbohydrate active enzymes (CAZymes) contained the most hydrophilic SPs in the genome, and may utilize SRP-independent methods of traversing the ER membrane.

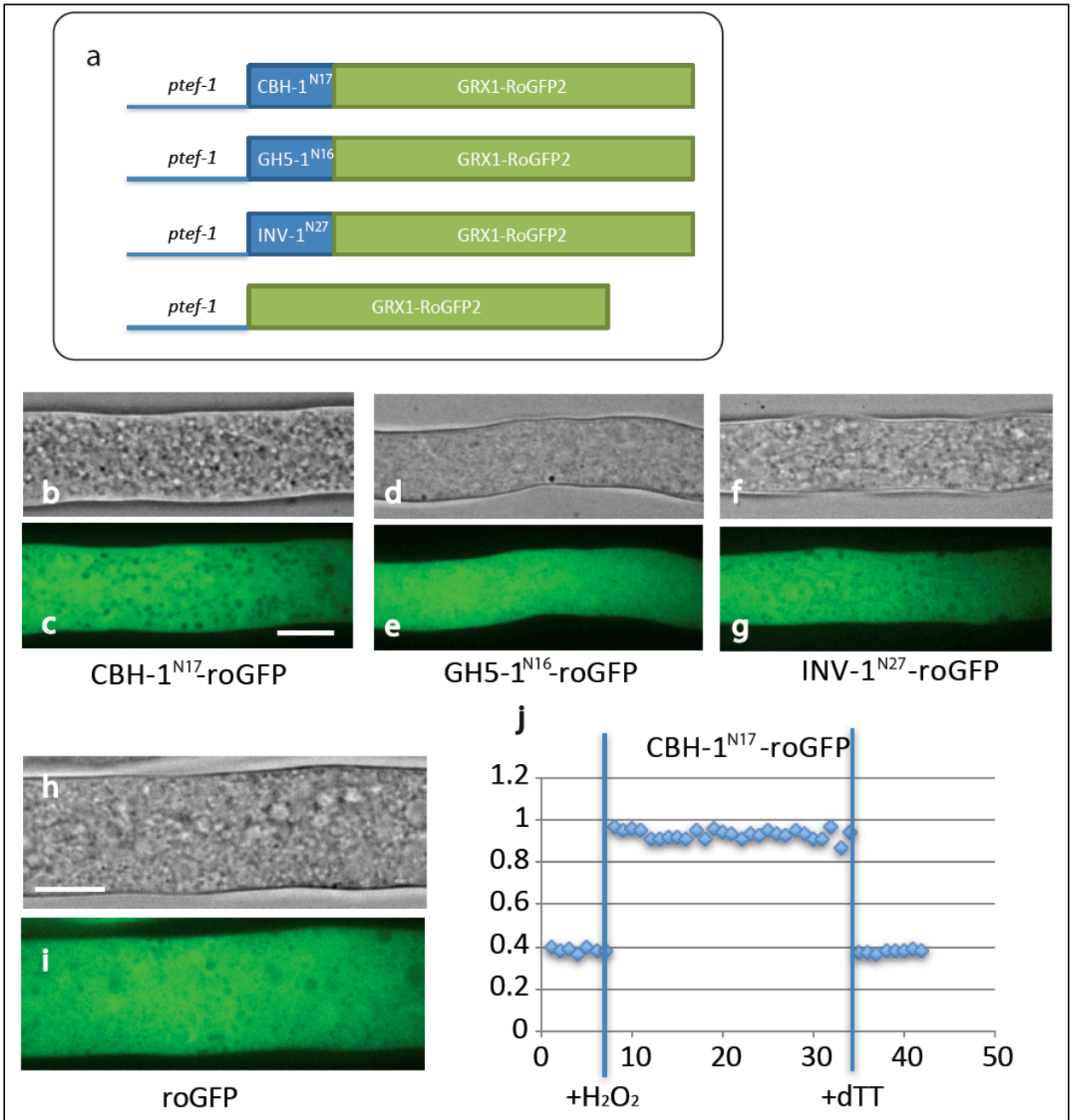

Figure 4. 4: : Signal peptide of CAZymes do not translocate roGFP.
Schematics of CAZy signal peptide cloning (a). DIC (b,d,f,h) and GFP (c,e,g,i) of CBH-1^{N17}-roGFP (b,c), GH5-1^{N16}-roGFP (d,e), INV-1^{N27}-roGFP (f,g), and cytosolic roGFP (h,i). Oxidative (H_2O_2) and reductive(dTT) treatment of the CBH-1^{N17}-roGFP indicates a fully cytosolic roGFP (j).

4.2.5 Establishing a system to track translocation in vivo

To begin testing the SRP-independence of CAZymes, we had to establish a system by which to track a substrate molecule through translocation *in vivo.* The most prominent way of establishing a system of tracking depended on utilizing the environmental differences between the cytoplasm and ER lumen (Birk, Ramming et al. 2013). Previous work utilized redox sensitive GFP (roGFP) as a marker for ER redox potential (Heller, Meyer et al. 2012, Birk, Ramming et al. 2013) and the roGFP2 construct has been used previously as a marker for mapping transmembrane protein topology (Brach, Soyk et al. 2009, Meyer and Brach 2009). Thus, we adapted the roGFP2 construct by initially tagging various SPs of CAZYmes to measure the ER translocation oxidative potential of these proteins.

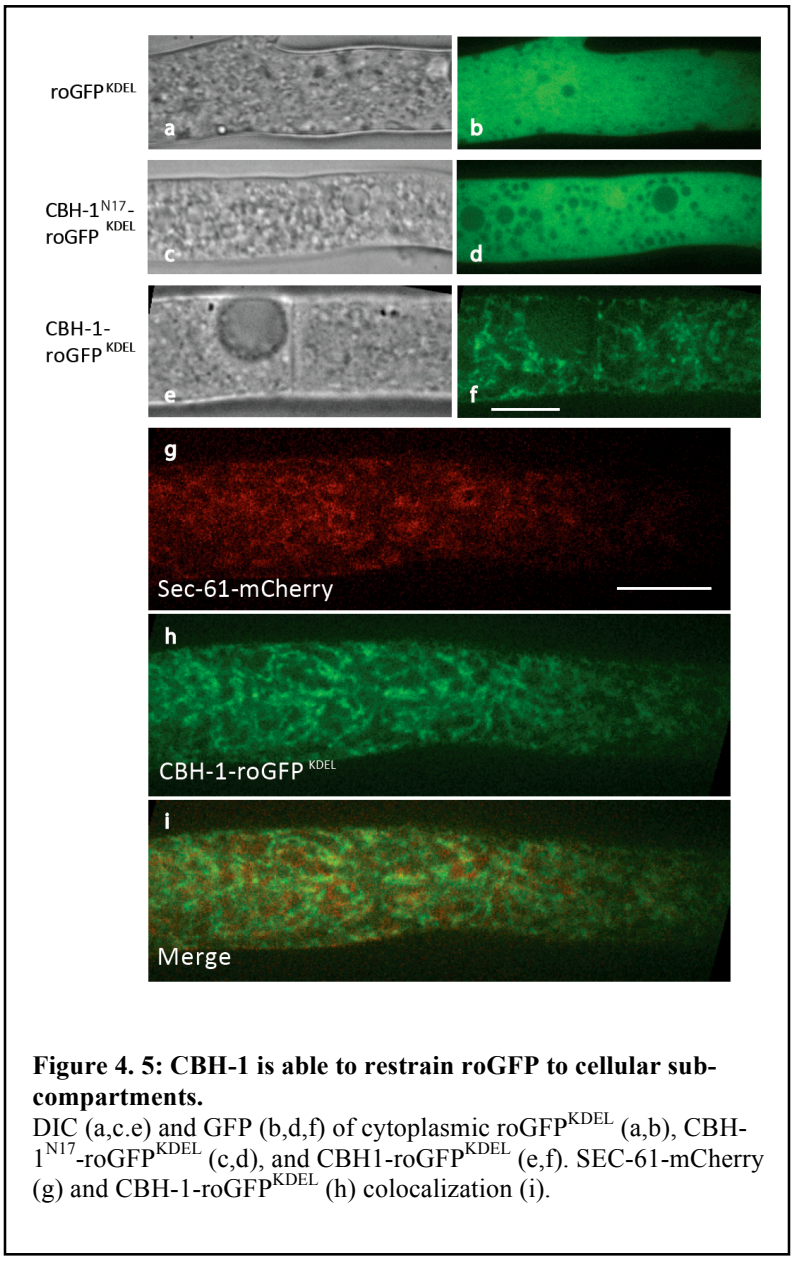

Figure 4. 5: CBH-1 is able to restrain roGFP to cellular sub-compartments.
DIC (a,c.e) and GFP (b,d,f) of cytoplasmic roGFP^{KDEL} (a,b), CBH-1^{N17}-roGFP^{KDEL} (c,d), and CBH1-roGFP^{KDEL} (e,f). SEC-61-mCherry (g) and CBH-1-roGFP^{KDEL} (h) colocalization (i).

When we expressed the free roGFP in *N. crassa* cells, we visualized the untagged protein localization, which, as expected, remained in the cytoplasm (fig 4.4i). We then made a series of chimeric proteins that expressed the roGFP with the SP of CBH-1, GH5-1and INV-1. However, when we expressed these constructs under the same conditions as the free roGFP, we observed that these chimera proteins localized to the cytoplasm (Fig 4.4 c, e, g). These results were confounding to us as these are well-established secretory proteins and crystal structures (Divne, Stahlberg et al. 1994, Tan, Kracher et al. 2015{Divne, 1994 #1300) corroborated the cleavage site of these SPs for CDH-1 and CBH-1. We developed two explanations for this result, either (1) the SP of these proteins was not sufficient for the translocation of the roGFP2 constructs, which is why we see the accumulation in the cytoplasm, or (2) the maturation of proteins in the ER may be rapid enough that the oxidized population of roGFP2 could not be visualized.

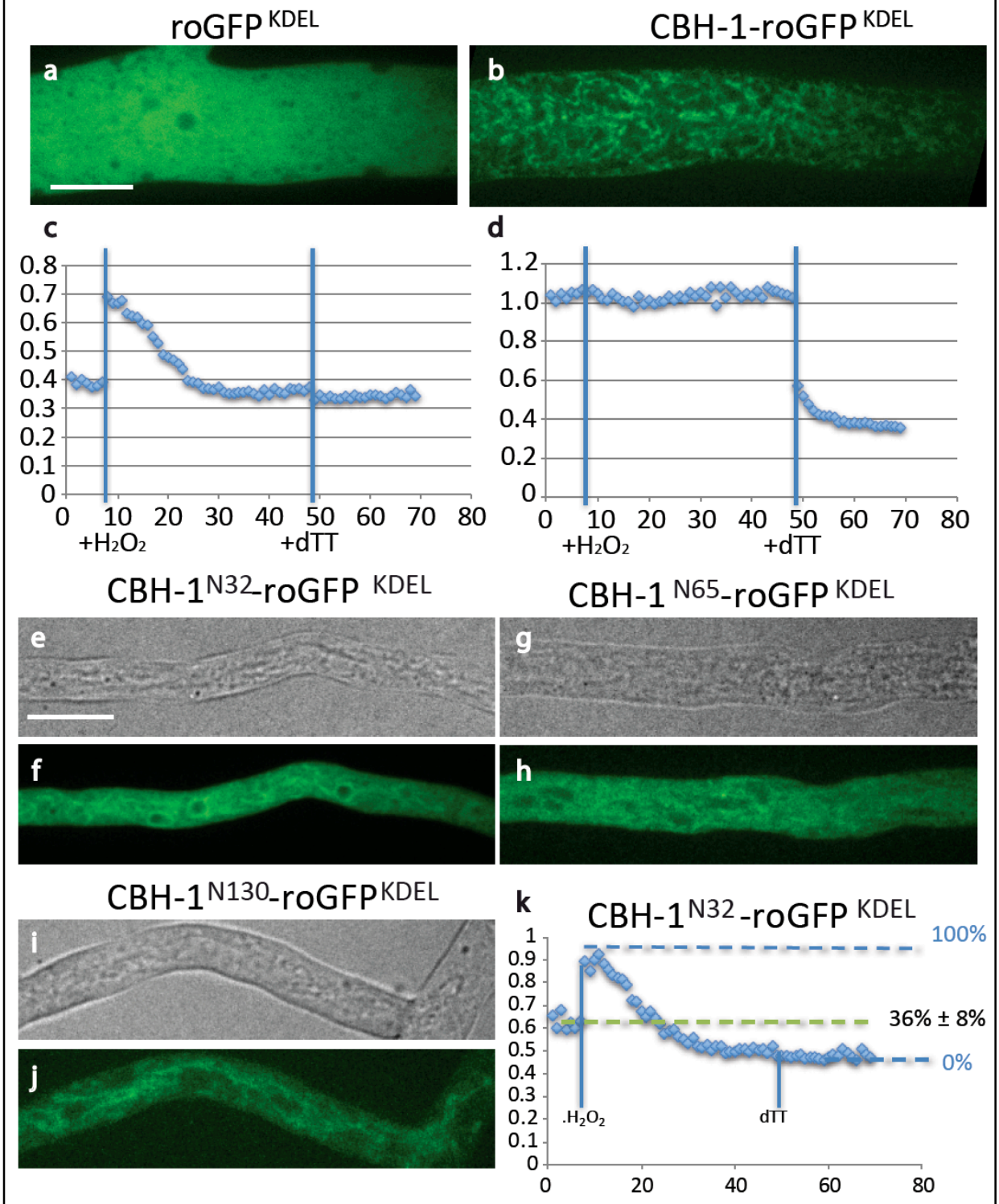

Figure 4. 6: CBH1 length dependent translocation.
roGFP localization of cytoplasmic roGFP (a) and CBH-1-roGFP (b), oxidative and reductive treatments of roGFP (c) and CBH-1-roGFP (d). DIC (e,g,i) and GFP (f,h,j) of CBH-1^{N32} (e,f), CBH-1^{N65} (g,h), CBH-1^{N130} (i,j). Oxidative and reductive treatment of CBH-1^{N32}-$roGFP^{KDEL}$ (k). Scale bar represents 10µm.

4.2.6 Restraining the roGFP chimeras to the ER

In order to address the possibility that roGFP secretion was preventing our ability to measure the accumualiton of the roGFP in the ER, we restrained the constructs that we generated with the roGFP2 by using a C-terminal KDEL tag, which would prevent further downstream trafficking of the chimeras. Restraining the roGFP2 in the ER would then allow us to visualize the translocation of these enzymes.

When we tagged the roGFP with a KDEL sequence and expressed the construct under minimal media conditions, we saw that free roGFP2^{KDEL} was restricted to the cytoplasmic space, implicating the inability of the construct to translocate into the ER, as expected. Furthermore, the KDEL sequence did not have a noticeable defect in localization or mis-trafficking within the cytoplasm. After adding the CBH1 signal peptide (CBH-1^{N17}), consisting of the first 17 amino acids before the glutamine that is cyclized as the protein is secreted (Dana, Dotson-Fagerstrom et al. 2014, Wu, Dana et al. 2017), the chimeric protein was not translocated and still remained soluble in the cytoplasmic space.

As a positive control, we used the full-length CBH-1 protein tagged with the roGFP^{KDEL} construct to visualize whether the whole CBH-1 protein was able to be secreted. The protein translocated out of the cytoplasm (fig 4.5b), and the resulting quantification of the roGFP fluorescence implicated a 100% translocation rate (4.6 b, d). The visualization of the CBH-1-roGFP^{KDEL} shows filamentous structures within the cell that was not reminiscent of ER, so we used a strain containing a SEC-61-mCherry construct in a dual labeled heterokaryotic strain. Co-localization of the CBH-1-roGFP^{KDEL} and SEC-61-mCherry indicated that while the CBH-1 construct was able to be translocated across to a higher oxidative potential environment, it does not co-localize with the SEC-61-mCherry (fig 4.5 g, h, i). Thus, the construct may be mis-trafficking to other organelles, perhaps tubular vacuoles.

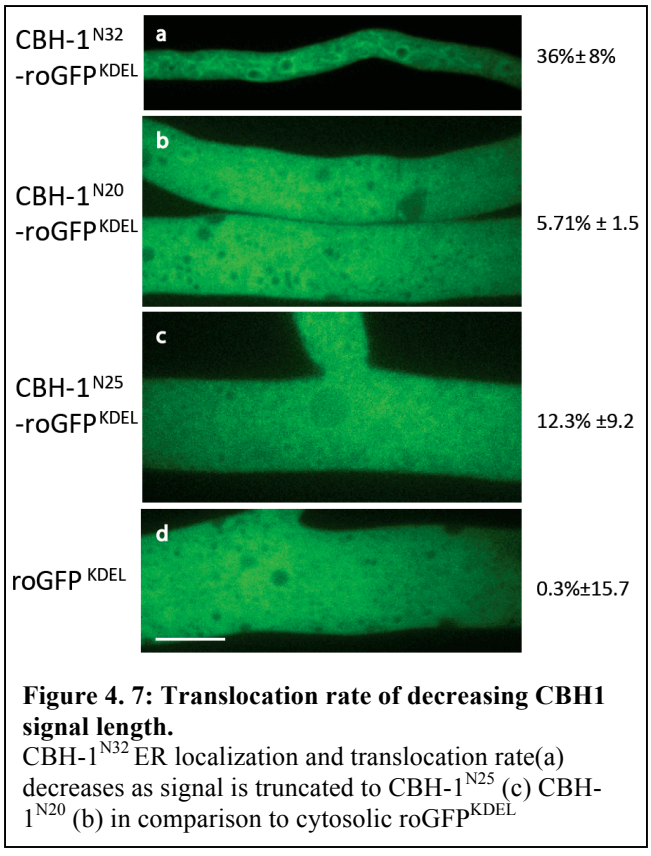

Figure 4. 7: Translocation rate of decreasing CBH1 signal length.
CBH-1^{N32} ER localization and translocation rate(a) decreases as signal is truncated to CBH-1^{N25} (c) CBH-1^{N20} (b) in comparison to cytosolic roGFP^{KDEL}

This data brings up several points of interest. First, the CBH-1^{N17} is not sufficient for efficient translocation of proteins to the ER. Secondly ER-processing signals are able to clear the ER of excess protein when full length CBH-1 is translocated despite the restraint of the KDEL sequence. And third, CBH-1 full-length protein is able to translocate across the ER membrane.

4.2.7 Additional residues to the CBH1 signal peptide aids in translocation of the roGFP

Since the CBH-1^{N17} was not a sufficient sequence for translocation while full length CBH-1 was, we hypothesized that as we appended gradually longer sequences of CBH-1 to the SP, we would be able to see a gradient of translocation as the protein signal becomes sufficient. Starting from 1/4th of the CBH-1 protein (CBH-1^{N130}), we observed strong translocation (fig 4.6j). We generated a series of truncations for the N-terminal 1/8th (CBH-1^{N65}) and 1/16th (N32) of the CBH-1^{N32} protein (fig 4.6h,f). At the CBH-1^{N32} construct, the translocation rate was 36% ± 8% of the protein into the ER.

We subsequently made shorter deletions of the amount of the N32 portions of the protein assess the potential for the minimal, sufficient sequence for the CBH-1 that would signal for translocation. Adding 3 amino acids to CBH-1 signal peptide, CBH-1^{17}, we quantified a near total loss in CBH-1^{N20} translocation. We then tried a 25-amino acid portion of the protein (CBH-1^{N25}), the addition of 8 amino acids to the signal peptide enough to increase translocation to a detectable amount to 12.3% ± 9.2(fig 4.7). These data together indicated that translocation of the CBH-1 peptide is a function of the signal peptide plus additional sequence. As we gradually appended more sequence to the signal peptide we observed a correlated increase in translocation.

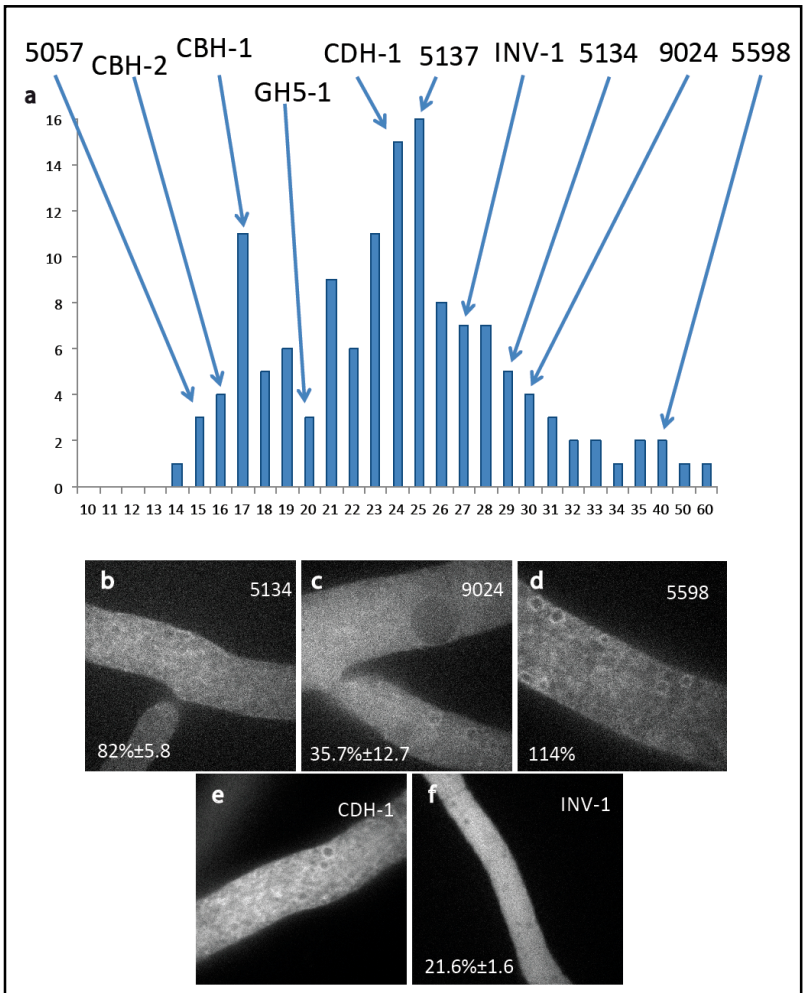

Figure 4. 8: Translocation of hydrophobic signals.
Proteins located on the hydrophobic half of the signal peptide spectrum were assayed (a). NCU05134^{N32} (b) NCU09024^{N32} (c), NCU05598 (d), NCU00206 (e) and NCU04265 (f) were assayed and visualized for translocation

4.2.8 N32 of hydrophobic proteins is sufficient to encode for translocation

Using the hydrophobic distribution to examine hydrophobicities of SP in proteins that are secreted, we hypothesized that the more hydrophobic SP of proteins would have sufficient signal in the first 32 amino acids to be able to translocate across the ER membrane.

By establishing the truncation of CBH-1 that is necessary for translocation, we surveyed enzymes that would have an alternative translocation rate than the CBH-1^{N32}. We surveyed proteins that were highly hydrophobic, did not contain a transmembrane domain, the signal peptide had to be well predicted by the major programs, SignalP and Phobius, and finally, the signal peptide had to be cleaved by the first 32 amino acids. We surveyed the first 32 amino acids of 5 proteins that lay on the hydrophobic half of the predictive scale, NCU09024, NCU05134, CDH-1 (NCU00206), ASD-1 (NCU05598) and INV-1 (NCU4265). After constructing the signals as a fusion protein with the roGFP, we transformed these strains into a *his-3* strain, FGSC6103 and screened for GFP fluorescence.

Transformed strains were visualized through confocal microscopy (Fig 4.8) and assayed via oxidative/reductive assays for the basal rate of translocation. From the visualized constructs and the assay results, INV-1^{N32} showed a decreased level of translocation with a basal translocation rate of 21.6%±1.6 SD (fig 4.8f). The NCU09024^{N32} had a similar rate of translocation to CBH-1^{N32} despite the near doubling of hydrophobicity (NCU09024 = 30 vs CBH-1 = 17) (fig 4.8c). This result is indicative that hydrophobicity may not be the sole factor that predicts whether proteins are able to translocate through SRP-dependent means. A strain containing a CDH-1^{N32}-roGFP^{KDEL} resulted in a morphological mutant that did not grow or conidiate like the other strains tested, so it was excluded from continued analysis. However, the visualizing the roGFP through microscopy indicated that the roGFP strongly associated with the ER which would indicate translocation of the CDH-1^{N32}-roGFP substrate.

We did, however, find two proteins where the first 32 amino acids encoded enough information for high to near full translocation. NCU05134, that has a relatively mild SP hydrophobicity score of 29, resulted in a near full translocation rate (fig 4.8b). NCU05598, ASD-1^{N32} was also fully translocated (114%±27%) (Fig 4.8d). These data indicated to us that the ASD-1 signal may engage the SRP most efficiently at a hydrophobicity of 42. This hydrophobicity is well into the range of transmembrane domain proteins thus, we predicted that the the ASD-1^{N32} signal would engage the SRP for recruitment into the ER.

4.2.10 ASD-1 and CBH-1 use alternative translocation strategies

If ASD-1 utilized SRP to translocate into the ER, then we would predict that CBH-1 and ASD-1^{N32} would use to distinct mechanisms to traverse the ER membrane due to the disparity in translocation efficiencies between the two chimeras. To investigate the potential dependence of translocation on the SRP, we modeled our studies of determining the SRP-dependent mechanisms of *N. crassa* similar to previous work in *S. cerevisiae* where knock downs of SRP-independent translocation pathway and SRP-dependent pathways allowed the authors to infer the potential pathway of translocation (Ast, Cohen et al. 2013).

To investigate this, we identified the orthologs of the SRP (SRP-14/NCU02405, SRP-19/NCU03485 SRP-54/NCU09696, SRP-68/NCU10927, SRP-72/NCU01455), the components of the heterotetrameric SRP-independent translocation complex (SEC-62/NCU06333, SEC-63/NCU00169, SEC66/NCU02681, SEC-72/NCU07746) and the SEC61 translocon itself (SEC-61a/NCU08897, SEC-61b/NCU08379, SEC-61g/NCU04127). Of the genes we could gather from the knock out collection as viable homokaryotic knock outs, we obtained one mutant of the

SRP, Δ*srp-14*, one component of the SRP-independent heterotetramer complex, Δ*sec-66*, and one translocon subunit, Δ*sec-61b*. We hypothesized that the Δsec-61b mutant would reduce translocation efficiency of all substrates tested, that Δsrp-14 would prevent translocation of substrates that depended on the SRP to be delivered to the ER but continue to allow SRP-independent substrates to the ER, and that Δsec-66 would prevent translocation of SRP-independent substrates while allowing translocation of SRP-dependent substrates.

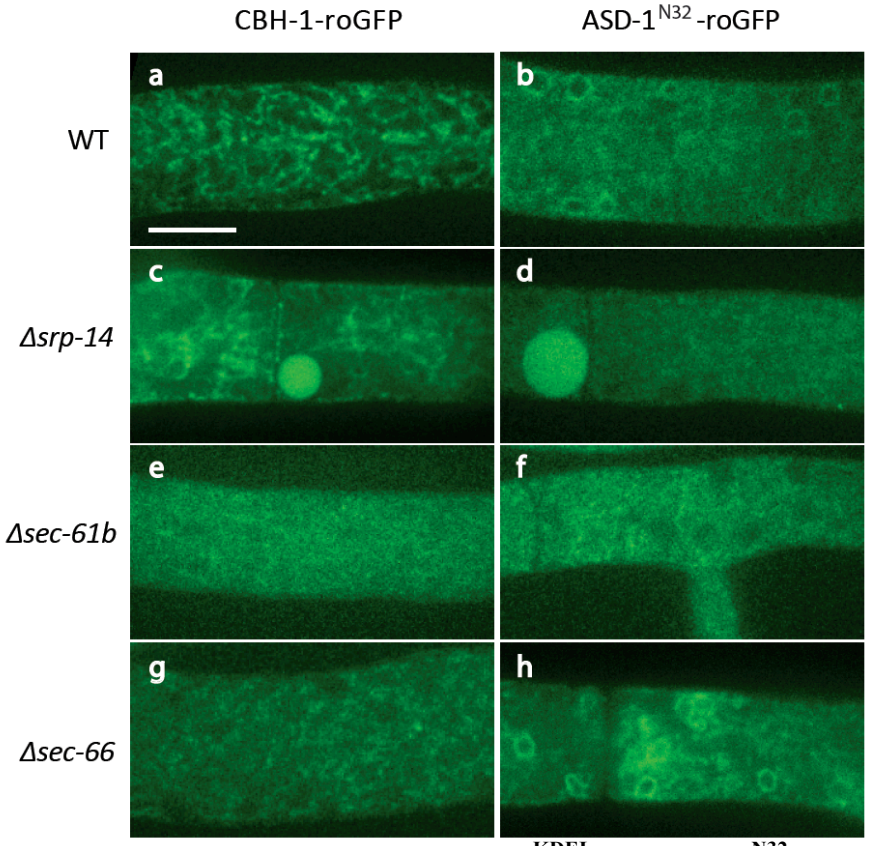

Figure 4. 9: Routes for CBH-1-roGFP^{KDEL} and ASD-1^{N32}-roGFP^{KDEL} translocation.
CBH-1-roGFP^{KDEL} (a,c,e,g) and ASD-1^{N32}–roGFP^{KDEL} (b,d,f,h) under WT (a,b), Δ*srp-14* (c,d), Δ*sec-61b* (e,f), and Δ*sec-66* (g,h). Scale bar represents 10 µm.

In order to test these hypotheses, we mated the mutants with the ASD-1^{N32}–roGFP^{KDEL} construct and the full length CBH-1-roGFP^{KDEL} construct, and isolated cross progeny contained all six combinations. Under the Δ*sec-61b* knockout background, as expected, both substrates of the ASD-1^{N32}–roGFP^{KDEL} and CBH-1-roGFP^{KDEL} failed to translocate in an efficient manner compared to the WT strains bearing these constructs (fig 4.9a, b, e, f). This indicated that both substrates were inefficiently being translocated. Under the Δsrp-14 background, we could visualize localization to the tubular vacuoles for CBH-1-roGFP^{KDEL} (fig 4.9c) while the localization to the ER is abolished for ASD-1^{N32}-roGFP^{KDEL} (fig 4.9d) compared to WT (fig 4.9a, b). This suggested that ASD-1^{N32}-roGFP^{KDEL} is dependent on the SRP for proper translocation. Finally, when we visualized the constructs in the Δ*sec-66* background, the ASD1^{N32} translocation to the ER was maintained like the WT background (fig 4.9h, fig 4.9 b), while the full length CBH1 translocation to the ER and accumulation to the tubular vacuoles was prevented by the deletion of Δ*sec-66* (fig 4.9g). These data together indicated to us that the translocation between using the ASD1^{N32} and CBH1 utilize different mechanisms for efficient translocation. ASD1^{N32} is dependent on the SRP complex and the CBH1 is dependent on the SRP-independent translocon heterotetramer.

4.3 Discussion

From the algorithm laid out by Ast et al (Ast, Cohen et al. 2013), we analyzed the hydrophobicities of the predicted *N. crassa* proteome. When the hydrophobicities were sorted in a histogram, like the *S. cerevisiae* data, the *N. crassa* proteome contained a bi-modal distribution. The bifurcation of the distribution, however, was most likely not due to SRP-independent and dependent substrates as was first hypothesized. Rather, the bifurcation was better explained by the hydrophilic signal of proteins that traversed the ER through a signal peptide while the hydrophobic distribution was largely representing the proteins that traversed the ER with a transmembrane domain. From these data, we set out to better define the set of SRP-independent substrates. From our application of the algorithm, we saw a distribution of signal peptides that varied across the spectrum with hydrophobicities . We observed that proteins that were highly secreted on cellulose medium were highly represented on the hydrophilic end of the hydrophobicity spectrum (fig 4.2). These data indicated that highly secreted CAZYmes on cellulosic media may traverse the ER membrane through SRP-independent means.

When we performed the analysis of proteome hydrophobicities on a wide variety of fungi, we observed that the hydrophobicity spectrum of the secretion signals span a similar hydrophobic space, regardless of environmental niches. The single organism that we looked at that was significantly shifted toward the hydrophilic series of the distribution came from the cosmopolitan human pathogenic fungus, *A. fumigatus*. We hypothesized that this would indicate an evolution of secreted enzymes to prefer the SRP-independent route of translocation. In order address this hypothesis, we developed a trackable fluorophore to measure the translocation state of protein signals *in vivo*.

By utilizing the changes in fluorescent states of the redox active GFP (roGFP) between oxidized and reduced states, we utilized the difference in environmental states between the cytoplasm and ER as a tractable readout for protein translocation. We engineered this marker protein with an ER retention KDEL signal that maintains the protein in an oxidized state when it translocates. This tag also prevents the maturation of the roGFP into downstream vesicles for secretion and gives a strong cumulative signal of GFP fluorescence in the oxidizing environment within the ER or other membrane bound compartments. By utilizing exogenous oxidating (H_2O_2) and reducing (dTT) agents, we established a fully oxidized and reduced state for in vivo normalization of the translated roGFP.

We appended a multitude of signal peptides with a variety of hydrophobicities to track innate translocation abilities of the signal peptides alone. We were surprised that the highest represented protein in the secretome, CBH-1, encodes a signal peptide, what when appended to the roGFP is a poorly translocated substrate. These data indicate that CBH-1 required additional sequence on top of the signal peptide to provide translocation into the ER to be secreted. Through screening of hydrophobic signal peptides, we determined that ASD-1 (NCU05598) was highly hydrophobic by the predictive algorithm at a hydrophobic score of 40. When the roGFP2 was fused to the ASD-1^{N32}, the peptide sequence was sufficient for translocation into the ER. We evaluated the translocation of CBH-1-roGFP^{KDEL} and ASD-1^{N32}-roGFP^{KDEL} in viable knock out mutants of a subunit of the SRP *(Δsrp-14*) which should be specific to the SRP-dependent pathways, a subunit of the SRP-independent translocon accessory complex *(Δsec-66*) and a

subunit of the translocon that may slow translocation of both pathways *(Δsec-61b*). We saw that CBH-1 translocates in the Δsrp-14 condition, which would indicate preferential trafficking through the SRP-independent pathway, while ASD-1^{N32} translocated under the Δsec-66 background while not translocating as efficiently under the Δsrp-14 background indicating the use of the SRP-dependent pathway for delivery to the ER.

From these data, we have concluded that the CBH-1 cellulase, the highest secreted protein in the supernatant of cellulolytic cultures is not dependent on the SRP for delivery to the ER. However, this brings up the possibility that full length CBH-1 still depends on an accessory protein for efficient delivery to the ER and translocation across the ER membrane. While we did not address either the accessory protein that may help bring CBH-1 to translocate at the ER, nor did we extrapolate the roGFP2 system to *Aspergillus fumigatus* proteins, we were successful in elucidating the inherent abilities of the signal peptides to translocate across the ER membrane. As a result, we established constructs that can be used as model substrates for future studies of translocation pathways in *N. crassa.*

4.4 Methods

4.4.1 Media and strain maintenance

N. crassa strains were cultured in Vogel's medium (Vogel 1956) agar supplemented with 2% sucrose for 7 days at 25ºC light to obtain conidia for experimentation. Crosses were performed on Westergaard's media (Westergaard 1947). WT strain (FGSC2489), and the gene deletion strains (Colot, 2006) were obtained from the Fungal Genetic Stock Center (FGSC, University of Missouri, Kansas City, Missouri, USA)(McCluskey 2003).

4.4.2 Cloning and plasmids

Ectopic cloning into the *his-3* locus was performed into a pMF272 plasmid with a swapped pTEF1 promoter and roGFP2. All cloning into pMF272 was performed between the BamH1 and the EcoR1 of the plasmid and transformed into *his-3* strain FGSC6103.

4.4.3 Microscopy

Mycelia for microscopy was grown on VM agar supplemented with 2% sucrose at 25°C overnight. Mycelia were observed using a 100× 1.4 NA oil immersion objective on a Leica SD6000 microscope attached to a Yokogawa CSU-X1 spinning disc head with a 488 nm and/or 561 nm laser. Dual-color imaging was accomplished using automated acquisition of the two wavelengths in series with a time differential for emission filter changeover.

4.4.4 RoGFP fluorescence measurement

Conidia were inoculated at 10^6 cells per mL into 2% sucrose + Vogel's salts. Cultures were incubated for 16 hours at 25ºC and shaking, and 200 µL of cultures were washed with fresh medium and placed in triplicate in wells of a black bottom 96-well plate (Corning). Initial

measurements were taken every 30s for 3 min for roGFP2$_{oxidized}$ (λex = 405, λem = 525 nm) roGFP2$_{reduced}$ (λex = 488, λem = 525 nm). 20 µL of 100mM H_2O_2 was added to each well and shaken for 10s. Measurements were taken every 30s for 20 min. 2.5µL of 1M dTT was added to each well and measured every 30s for an additional 10 min. Ratios of roGFP2$_{oxidized}$/ roGFP2$_{reduced}$ were plotted and normalized to maximum H_2O_2 treatment (100% oxidized) and dTT treatment (0% oxidized).

4.4.5 Hydrophobicity measurements

Hydrophobicity plot was generated similar to (Ast, Cohen et al. 2013). Protein sequences were obtained from the Joint Genome Institute (jgi.org) and The Broad Institute (broadinstitute.org). Prediction files of signal peptide and transmembrane helices were generated using Phobius (http://phobius.sbc.su.se/) Average hydropathy values were determined using scanning windows of the first 100 amino acids of protein sequence between 7-15 using Kyte and Doolittle hydropathy values (Kyte and Doolittle 1982). Maximum window value was multiplied by predicted helix length predictions to generate hydrophobicity value.

CHAPTER 5: CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Deconvolution of alleles and future mutagenesis screening

From mutagenesis and screening, we identified two hypersecreting strains that are increased in their protein production by 8-fold over the wild type strain. The mutagenesis and screening has been successful in finding strains that consistently hyper produce proteins, but more work needs to be performed to dissect out the alleles that may be altering protein production. Beyond the work of *drs-2* and other flippases, we also have described two alternative alleles that were inferred by phenotype from the knock out collection (strains carrying Δ*stk-12* and Δ*ad-15*). In future work, it would be possible to use the bulk segregation analysis protocol outlined in this work to identify the causal allele. We also have two genomic intervals that could use further work to reconstitute SNPs in causal alleles that may be causing a secretion defect. Results from either, the study on the alternative alleles or the two known causal intervals, may help elucidate conserved mechanisms for fungal secretion.

In parallel, our investigation of alleles that may compound with the Δdrs-2 phenotype has not been adequately investigated. Due to the iterative nature of the cellulase screening, we could transform in a hygromycin resistance cassette to knock out the *drs-2* coding sequence in the parental screening background to generate a Δ3βG; Δ*drs-2* strain for quickly screening compounding alleles and generate further candidate alleles for hypersecretion. Due to the power of FAC sorting, a low amount of mutagen can be used for the production of fewer SNPs in the genome. By using a smaller amount of mutagen, the causal allele can be identified much more rapidly, as deconvoluting the genomic sequence to the causal allele is the major challenge of screening.

Due to the FAC sorting of the initial library of secretion mutants, we identified mutants that were sorted due to their ability to retain GH5-1-GFP fluorescence intracellularly. Strains that have been prevented from secreting the protein may be rerouting proteins to the vacuole, or accumulating proteins in intracellular compartments. Work performed by our lab has identified 12 hypo-secreting phenotypes among the sorted library strains. This results in a 1% hypo-secretion phenotype among all the strains that were sorted.

5.2 Conservation of DRS-2 and CDC-50 heterodimer

One of the motivating aspects of this study was to use the genetic tools available to *N. crassa* to dissect the hypersecreting strains so that the information could be extrapolated broadly to other filamentous fungi. The use of *N. crassa* as a genetic model system for determining novel alleles for cellulase hypersecretion has been largely successful, as we have now added four new genes associated with a hypersecreter phenotype that have not been described previously (*drs-2, dnf-1, nrc-2, ham-10*). The mutant alleles of these four genes can be utilized to engineer other filamentous fungal hyper-producing hosts. Conservation of the *drs-2* pathway will be crucial to understanding if similar mechanisms of hypersecretion work in diverse filamentous fungi. Given

that *Trichoderma reesei* is in the same phylogenetic family as *N. crassa,* and the five orthologs of flippases are conserved between the two fungi, we hypothesize that the role of DRS-2 in secretion would also be conserved.

One of the caveats to our work on flippases has been a lack of study in the mechanistic flipping of PS lipids by *N. crassa* DRS-2. While in *S. cerevisiae*, modular growth, FACS analysis tools and well established protocols have been beneficial for the discovery of substrate specific lipid flipping, in *N. crassa*, hyphal growth and heterogeneous cultures have prevented us from investigating the conservation of DRS-2 function. Future work could focus on identifying the specific flippase substrate of DRS-2 by using heterologous expression of *N. crassa* DRS-2 in *S. cerevisiae*. If the *N. crassa* DRS-2 is able to complement the cold sensitivity of *drs2*Δ mutation, then the function of this flippase may be conserved. If DRS-2 is capable of rescuing the cold sensitivity of *S. cerevisiae*, established methods for substrate flipping would help to determine the preferences of the *N. crassa* DRS-2.

Sorting of DRS-2-mCherry to the Spitzenkörper core of the hyphal tip has been intriguing because of the stratification that the Spitzenkörper has to keep distinct populations of vesicles at the hyphal tip. The origin of sorting cargo proteins destined to either the Spitzenkörper core or the Spitzenkörper ring is still unclear and would be beneficial to decode what provides the signal for sorting. Furthermore, data from this work among others have indicated that septal secretion may have a major effect on the accumulation of proteins in the supernatant. Since many enzymes are secreted at the septum, it would be advantageous to characterized septal secretion, and whether fungi contain septal specific vesicles.

Other aspects of the DRS-2 mechanisms that remain unclear is why Δ*cdc-50* may not have a role in secretion as the predicted heterodimer subunit. The heterodimer partners of fungal lipid flippases have been shown to be important in evolutionarily different fungi (Chen, Wang et al. 2006, Andersen, Vestergaard et al. 2016). *Cryptococcus neoformans*, a pathogenic basidiomycete yeast has been shown to depend on the *cdc-50* subunit for proper lipid asymmetry maintenance, anti-fungal drug sensitivity and iron uptake (Hu, Bandyopadhyay et al. 2011, Huang, Liao et al. 2016, Hu, Caza et al. 2017). One explanation for the importance of *cdc-50* in *C. neoformans* and the disposable function of *cdc-50* in Neurospora may be due to the role of the *DNF1* clade duplication of the basidiomycete flippases. The role of this new DNF-1 duplication has not been well elucidated, but evidence suggests that the *C. neoformans* Δ*cdc-50* mutant allele has phenotypes most similar to the Δ*dnf-1* mutant (Hu, Caza et al. 2017). Thus it may be evidence that when *DNF-1* duplicated in the basidiomycetes, either of the *DNF-1* duplicates may have neofunctionalized by evolving heterodimer function with *cdc-50.*

One possible reason for the altered function of *cdc-50* in N. crassa is due to the altered transmembrane domains of the N. crassa flippases. The S. cerevisiae flippases all have 10 transmembrane domains. However, transmembrane domains in the N. crassa flippases vary between 7 (DRS-2, DNF-3a, DNF-3b), 9 (DNF-1), and 5 (NEO-1). While the transmembrane domains of flippases vary in importance (Baldridge and Graham 2012, Baldridge and Graham 2013, Baldridge, Xu et al. 2013), the total number of transmembrane domains may be indicative of how many are necessary for proper function of the Drs2p-Cdc50p complex. The explanation of for low transmembrane prediction could also be trivial, where the algorithm for predicting

transmembrane domains is not optimized for filamentous fungi proteins, and as a result, the algorithm will be mis-predicting the number of transmembrane domains. However, with the hypothesis that transmembrane domains of the dimer should total 12, it would be possible to screen through the knock outs of all 58 five transmembrane domain proteins that are able to complement the 12 transmembrane domains of the flippase heterodimer with DRS-2 for 2-fold increased protein accumulation, and subsequent western blot for CBH-1 and CBH-2 accumulation in the supernatant for CBH-2 relative hyper-production compared to WT. However, the most straight-forward way to determine what might be the heterodimer partner for DRS-2 is to perform mass spectroscopy on the interacting proteins with DRS-2 after an immunoprecipitation. In this manner, the DRS-2-mCherry is ready for immunoprecipitation, which could lead to the identification of a potential filamentous fungal-specific heterodimer. The evolution of the flippases may implicate that the heterodimer partner of DRS-2, once discovered, may be evolutionarily conserved in the sordariomycetes, a large class of agricultural pathogens.

5.3 Heterologous protein ER translocation

One of the most important steps of secretion is the primary introduction of the protein into the ER for proper processing and folding. By analyzing the signal peptides that could traverse the ER membrane without additional information besides the signal peptide, we have found that signal peptides do not encode the same amount of translocation potential. This is important as the field of heterologous expression of proteins accounts for a majority of the secreted proteins and the tremendous interest in using filamentous fungi as a boutique protein production system. By having a large library of signal peptides, we can, theoretically, tune the rate at which proteins are able to enter the ER for proper maturation. By defining the proteins that undergo translocation, with just the signal peptide, our understanding of protein translocation will make a robust system for engineering heterologous secreted protein production in filamentous fungi.

Overall, this work has highlighted several aspects of hypersecretion and heterologous protein production that have not been well investigated. By populating the genetic toolbox that the field of secretion can use to produce protein hyper-producing strains of filamentous fungi, we can influence our future to more rapidly develop renewable cellulosic biofuels and more efficiently produce enzymes for a more environmentally friendly bio-based processing industry. By distributing the knowledge we have gained from this study, we can apply our understading of hypersecretion to more efficiently produced the enzymes that are necessary for food, fuel, and textiles, en route to a more sustainable bio-based economy.

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