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Targeting the TORC1 network improves survival in models of congenital disorders of glycosylation

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“Wenn man auf einem Elefanten sitzt,
sollte man ihn auch reiten.“

Michael Poglitsch

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Abstract

Defects in the human glycosylation machinery are responsible for a group of rare but devastating diseases called congenital disorders of glycosylation (CDG). CDG are often connected with severe health problems in central and peripheral nervous system and frequently end in early death. While over 100 different subtypes of CDG have been discovered so far, treatment is only available for three of the CDG defects, which makes investigations in that area even more important.

Following up preliminary data by others, this work shows that the destructive effects in a glycosylation-defective *Saccharomyces cerevisiae* strain, which holds a mutation in the *wbp1* gene, can be rescued by rapamycin treatment. Rapamycin treatment improves lifespan in this defective strain by TORC1 downstream signaling. Moreover, we could show that the TORC1 target Sch9, the yeast homolog to the human serine-6-kinase, is involved in the rescue. Interestingly, even though autophagy activation is one of the major hallmarks of rapamycin treatment, defective glycosylation does not seem to inhibit autophagic flux and rapamycin continues to rescue cells with disrupted autophagic signaling. To identify downstream targets of Sch9 that are involved in the recovery we analyzed different deletion strains in yeast, but no downstream effectors were identified so far. Instead, rapamycin, at least in part, requires the autophagy-related protein and TORC1 target Atg1 for its positive effects. Importantly, the herein presented data also validates the application of rapamycin for higher eukaryotes, since administration to glycosylation-defective *Drosophila melanogaster* Alg6 RNAi strains resulted in increased survival during development, which is of great interest as most CDG subtypes lead to developmental defects in humans. This study suggests rapamycin as a candidate for further investigation against CDG and a possible treatment option. Since rapamycin is already used in medicine to treat patients, for example after organ transplantations, there already exists valuable data on drug tolerance, side effects and other parameters relevant for therapeutic use.

Zusammenfassung

Defekte in der Glykosylierungsmaschinerie von Proteinen sind verantwortlich für eine seltene Gruppe an Krankheiten, welche teilweise durch sehr schwere Symptomatik begleitet werden. Diese Defekte werden kollektiv als „Congenital Disorder of Glycosylation“ bezeichnet, oder kurz CDG. CDG geht einher mit gesundheitlichen Problemen, welche meist das zentrale und periphere Nervensystem betreffen und häufig zu einem frühen Tod führen. Bis heute sind über 100 verschiedene Subtypen der Krankheit bekannt, jedoch ist nur für drei der bekannten Defekte eine Behandlung verfügbar. Diese Tatsache definiert die Forschung auf diesem Gebiet als essenziell.

Aufbauend auf Daten vorhergehender Studien, zeigt die hier präsentierte Studie die destruktiven Folgen und Effekte des N-Glyksoylierungsdefektes in *Saccharomyces cerevisiae*. Um so einen Defekt zu simulieren wurde eine Mutation im *wbp1* Gen eingebracht. Träger dieser Mutation zeigen wiederum durch Behandlung mit Rapamycin verbessertes Überleben, welches nahezu an das Normlevel heranreicht. Rapamycin wirkt über TORC1. Außerdem konnten wir nachweisen, dass auch Sch9, ein Target von TORC1 und ein Hefehomolog zur humanen Serin-6-Kinase, an der positiven Wirkung beteiligt ist. Obwohl die Aktivierung von Autophagie eines der Hauptmerkmale von Rapamycinsupplementation ist, scheint der autophagische Flux im glykosylierungsdefekten Hefestamm nicht gestört zu sein. Um weiter Targets im Signalweg abwärts von Sch9 zu finden, welche am positiven Effekt von Rapamycin mitwirken, wurden infrage kommende Proteine in Hefe deletiert und die Auswirkungen auf die Effektivität der Rapamycinwirkungen getestet. Monumentaler jedoch, die hier gezeigten Daten validieren die Supplementation von Rapamycin in höheren Eukaryoten. Die Behandlung des glykosylierungsdefekten *Drosophila melanogaster* Alg6 RNAi Stamms führte zu erhöhten Überlebensraten während der Entwicklung. Dies könnte sich als Fund höchsten Interesses innerhalb der folgenden Arbeit hervortun, da die meisten CDG Subtypen zu Beeinträchtigungen in der menschlichen Embryonalentwicklung und pränatalem Tod führen. Schlussfolgernd schlagen wir Rapamycin als mögliche Substanz zur Behandlung von Glykosylierungsdefekten vor. Dies wird gestützt durch die bereits etablierte medizinische Anwendung von Rapamycin, wie etwa nach Organtransplantationen. Zudem wurden schon Daten zu Nebenwirkungen und anderen Parameter, welche für den Einsatz von Rapamycin als Medikation relevant wären, publiziert.

1 Introduction

1.1 The glycosylation pathway: an overview

Protein glycosylation plays a central role in post translational modification and is ubiquitously found in all domains of life. Protein-linked glycans play a crucial role biologically and physiologically. They are not only responsible for mediating the final localization of proteins in the cell (e.g. secretory proteins or membrane proteins, like receptors), but also support proper folding of the proteins or ensure their stability (Colley, Varki, and Kinoshita 2015).

Today, we know that approximately 700 proteins are required for the full diversity of mammalian glycans, which are assembled from only 10 monosaccharides such as fucose (Fuc), galactose (Gal), glucose (Glc), *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), glucuronic acid (GlcA), iduronic acid (IdoA), mannose (Man), sialic acid (SA) and xylose (Xyl). Around 200 enzymes, so called glycosyltransferases are involved in the machinery. (Nairn et al. 2008) They use lipid-linked or nucleotide sugars as donor substrate and anchor those to a growing glycan chain. Glycans are attached to polypeptide structures in different ways, including amide linkages to asparagin acid (Asn) side chains (*N*-glycosylation), through glycosidic linkages (*O*-glycosylation) to side chains of serine/threonine (Ser/Thr), hydroxylysine (collagen) or tyrosine (Tyr) (glycogenin), or through C-C linkages to the C2 position of tryptophan (Trp) (*C*-mannosylation). Most of the proteins and polypeptides got assembled, folded, subjected to the quality control and translocated through the ER-Golgi-Pathway (Johansen, Marshall, and Neuberger 1961) (Moremen, Tiemeyer, and Nairn 2012).

In this work, I will focus on N-glycosylation, which is the most common form of protein glycosylation and most CDGs are caused by defects in N-glycosylation-associated pathways. Nearly half of the human proteins are glycoproteins and most are modified with N-glycan anchors (Apweiler, Hermjakob, and Sharon 1999). The N-glycan biosynthesis starts with a highly conserved pathway at the ER membrane: the synthesis of the dolichol precursor. This dolichol phosphate is located on the cytoplasmic site of the ER membrane. There, it receives different monosaccharides from different enzymes, which belong to the ALG (asparagine-linked glycosylation) protein family, to form a lipid-linked oligosaccharide (LLO) precursor (Patricie Burda and Aebi 1999) (Kornfeld and Kornfeld 1985). The LLO is extended to a certain point

and then flipped through the membrane of the ER to the luminal side, which is mediated by the transmembrane protein Rft1, a flippase (Helenius and Aebi 2002). Further elongations of the dolichol phosphate precursor, including further branching, are catalyzed by other ALG proteins, until it received 14 sugars (Figure 1, lower right) and is now called N-glycan precursor (P. Burda et al. 1999). Of note, mannose moieties are synthesized on the outside of the ER and are subsequently attached to the dolichol phosphate precursor. A pathway consisting of glusocephophate-6-isomerase (GPI), mannosephosphate-6-isomerase (MPI) and phosphomannomutase 2 (PMM2) yields mannose-1-phosphate from glucose-6-phosphate. (McConville and Menon 2000) (Helenius et al. 2002)

During translation at the ER, the growing polypeptide chain enters the ER lumen via the Sec61 complex. An oligosacherlytransferase (OST) complex catalyzes the attachment of the N-glycan precursor to the polypeptide chain at the amino group of the asparagine residue at Asn-X-Ser/Thr motifs (I. Nilsson and Von Heijnes 1993). One part of the yeast OST-complex, which consists of eight different subunits, is Wbp1 (Figure 1, upper left) (Imperiali and Hendrickson 1995).

This newly synthesized and modified glycoprotein gets now trimmed and folded through chaperones. Only correctly folded proteins are packaged for the transport to the Golgi. If this is not the case, they can get degraded through the ERAD (ER-associated degradation) (I. Nilsson and Von Heijnes 1993)

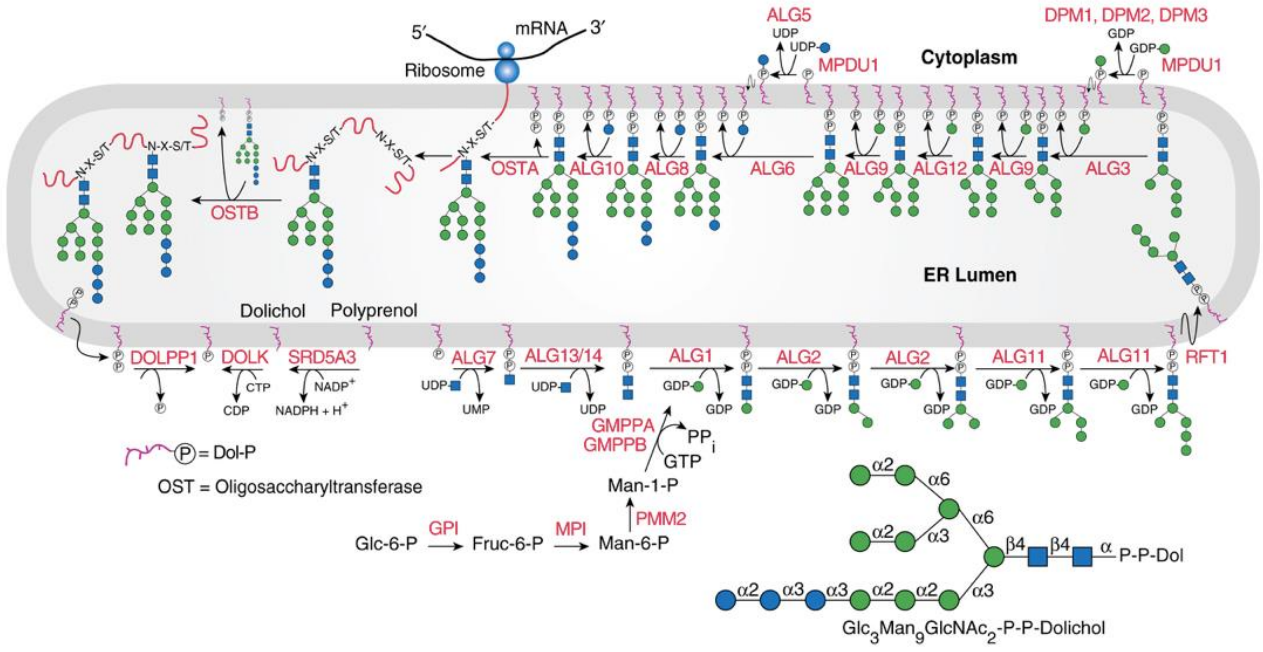


Figure 1: Scheme of the N-glycosylation steps of the luminal and cytoplasm side of the ER. It starts with the dolichol phosphate (red squiggle) which receives different monosaccharides such as

glucose (blue circle), mannose (green circle) and N-acetylglucosamine (blue box). These sugars are attached through different ALG enzymes. During maturation the precursor is flipped from the cytoplasmic side to the luminal side throughout the flippase RFT1 (Helenius et al. 2002). After further monosaccharide elongations to the LLO precursor, the OST links the N-glycan precursor to an Asn residue of the newly synthesized polypeptide (Figure and figure legend (Stanley, Taniguchi, and Aebi 2015a)).

After leaving the ER, the precursor proteins are passaging the Golgi apparatus. At this stage, both trimming, and extension of the glycan chain may occur. The maturation steps of the glycoproteins take place in a *cis* to *trans* Golgi trafficking manner, so the cisternal organization of the Golgi is most likely the main factor of the sequential process. In general, enzymes acting early in the pathway localize to the *cis* and medial Golgi cisternae, for instance N-acetylglucosaminyltransferase or mannosidase II, whereas those acting later on in the pathway (e.g. galactosyltransferase and sialyltransferase) are localized in the *trans* Golgi cisternae and the *trans*-Golgi-network (Rabouille et al. 1995) (T. Nilsson et al. 1993).

Glycosylated proteins traverse the different Golgi compartments through vesicles, and the correct trafficking of these vesicles is another critical step in the correct localization and folding of N-glycosylated proteins. There are several different models of how this trafficking takes place and how the spatial organization of glycosylation enzymes in the Golgi mediates specific modifications of the glycan chain (Glick and Luini 2011). Besides “classical” trafficking-associated proteins, such as COP-I, an additional factor of glycan maturation has been identified, namely the conserved oligomeric Golgi complex (COG). It consists of eight subunits and importantly, deficiencies in these genes also lead to CDG. It is believed that COG functions as a cytoplasmic tethering complex that links incoming vesicles to their target compartments before vesicle fusion (Balch et al. 1984)(Pokrovskaya et al. 2011)(Smith and Lupashin 2008). The process starts with the interaction of COG and the SNARE (SNAP (soluble NSF attachment protein) receptor) protein syntaxin 5. Additionally, it has been suggested that COG does not only interact and recruit SNARE proteins, but also stabilizes the final SNARE fusion machinery. Through knockout strains it has been elucidated that COG interacts with GEAR, a collective term for the Golgi mannosidase II, four glycosyltransferases, the Golgi SNARE protein syntaxin 5 and several golgins (Shestakova et al. 2007) (Laufman et al. 2009). The first phase of vesicle tethering begins with a longer phase where golgins locate and later attach to the membrane. Afterwards, either the COG-complex or another complex is recruited to the tethered vesicle and induces SNARE

complex-mediated vesicle fusion to the next compartment (Figure 2, A) (Sohda et al. 2007) (Oka et al. 2004). Through immunoprecipitation, an interaction between COG-complex and subunits of the vesicular coat complex I (COP-I) complex has been demonstrated. In addition, in COG knock out strains, COP-I subunits mis-localize, which further corroborates a role of COG in correct vesicle transport (Figure 2, B) (Oka et al. 2004). Its major role could be the sorting of the glycosyltransferases. COG may also give the opportunity for another quality check before the commitment of the vesicles (Reynders et al. 2011) (Moremen, Tiemeyer, and Nairn 2012).

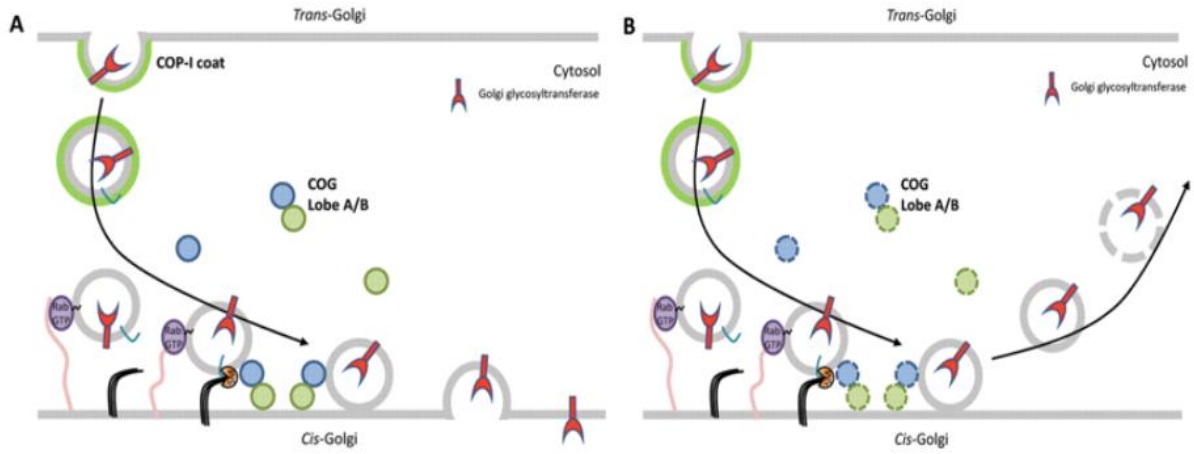


Figure 2: The hypothetical model of the COG complex in vesicle tethering. Key proteins that are involved in the process are COP-I, small GTPases, like Rab here indicated in purple (the activated form), vesicle SNAREs (vSnares here in green) and target SNAREs (tSNAREs here in black) and the SM protein in orange Furthermore, the COG complex lobes A and B, which are indicated by blue and green circles. A) indicates physical conditions and the vesicles can fuse normally with the Golgi. If there is an COG deficiency, such as in cases of CDG, the vesicles are unable to fuse and do not deliver to the right Golgi compartment, B. (Figure and figure legend by Reynders et al. 2011.).

Among the N-glycans, there are also other glycosylation pathways, for instance O-glycosylation. During O-glycosylation an O-glycan is covalently bond to the hydroxyl-group of a Ser or Thr residue of the polypeptide chain via N-acetylglucosamine. It mainly takes place in the Golgi apparatus. Besides, there are also other glycosylation variants such as glycosylphosphatidylinositol anchor (GPI-Anchor) glycosylation or glycosphingolipid glycosylation or any combinations thereof (Varki and Kornfeld 2015). More than 700 proteins are involved in the diverse glycosylation pathways. Defects in the glycosylation pathway disturb the function of those proteins, lead to their mis-localization and may ultimately result in CDG (Stanley, Taniguchi, and Aebi 2015b).

1.2 A brief overview of congenital disorders of glycosylation (CDG)

CDG which is short for “congenital disorders of glycosylation” (before 1999 it was called “carbohydrate deficient glycoconjugate syndrome”) are genetic diseases due to disturbance in the N- and/or O- glycans and were first reported in the 1980s by Jaeken (J Jaeken et al. 1980). In the recent years several different novel forms have been discovered and the number is still rising (Grünewald, Matthijs, and Jaeken 2002). Nowadays more than 100 subtypes of CDG are known: deficiencies in N-glycosylation, defects in protein O-glycosylation, defects in glycosphingolipid and in glycosylphosphatidylinositol anchor glycosylation and defects in multiple glycosylation and other glycosylation pathways. Interestingly, there are no known defects in C-glycosylation (Francisco et al. 2018). CDG were originally divided into two main groups: (i) CDG Type-I, where the LLO precursor is disrupted and (ii) CDG Type-II, where the processing or the assembly is disrupted (Freeze and Schachter 2009). As many additional forms of CDG have been identified, the classification has been revised (see below).

CDG are rare diseases, primarily because most of the embryos do not survive until they are born, if they have defects of a whole glycosylation step (corroborating the importance of protein glycosylation). Those who survive are usually hypomorphic and have at least some activity in the pathways. 20% of the patients do not live past their fifth year (Cylwik et al. 2013). Due to the variability of CDG etiology, the clinical manifestations also have a broad diversity and depend on which of the over 100 types it is. The most common clinical manifestations affect the central and the peripheral nervous system, resulting in development delay, seizure, ataxia, but may also lead to defects in the gastrointestinal tract and the hepatic system or endocrine abnormalities. There are different forms of skeletal manifestations as well (Coman et al. 2008).

1.3 Most common types of CDG

As mentioned above the CDG nomenclature has been revised from CDG-I and CDG-II to the gene name followed by CDG and one of the two types e.g. PMM2-CDG IIa. In this work I will focus on N-glycosylation defects. All other defects listed in Supplementary table 1.

Until 2013, 40 different defects in the N-glycosylation pathway and there are a lot of new discoveries. They lead to a various number of syndromes. They still got divided into assembly defects which are CDG-Type I and processing defects which are CDG-

Type II (Jaak Jaeken and Péanne 2017). Below I will focus on the most important defects in N-glycan synthesis.

1.3.1 MPI-CDG (CDG Ib)

MPI-CDG is a deficiency of the phosphomannose isomerase, also known as mannosephosphate-6-isomerase which is responsible of the conversion of fucose-6-phsophate (Fuc-6-P) to mannose-6-phosphate (Man-6-P). This catalyzation step takes place in cytoplasm (Freeze and Schachter 2009).

Mannose is one of the basic monosaccharides involved in 14 LLO precursors. It is encoded by the MPI gene where a lot of different mutations have been found so far. The total loss of the MPI genes in mice is lethal during embryonic phase because of the “honeybee effect”. It leads to a decrease of the Man-6-P pool which results in glycosylated proteins lacking those sugar chains or in an inactive N-glycosylation sequon (the amino acid sequence, harboring the glycan-linked Asn). It is one of the few CDG types that can be treated by oral mannose administration (Cylwik et al. 2013) (Freeze and Schachter 2009).

In contrast to other CDG, patients have no neurological symptoms. It mainly affects the liver and gastrointestinal tract. The clinical manifestations are vomiting, diarrhea, gastrointestinal bleeding, protein-losing enteropathy and hepatomegaly. In more severe cases there are also hypoglycemia, coagulopathy along with thrombotic events (Goreta, Dabelic, and Dumic 2012a).

1.3.2 PMM2-CDG (CDG Ia)

The phosphomannomutase 2 transforms Man-6-P to Man-1-P (mannose-1-phosphate). It is the most prevalent CDG type, with about 800 people affected (Freeze and Schachter 2009). Through molecular analyses, over 100 mutations in 8 exons of the PMM2 gene have been identified. However, there are also mutations in different variants all over the PMM2 gene (Kjaergaard 2004). Consequently, a lot of different clinical presentations are observed that range from early death to severe infections, liver insufficiency or cardiomyopathy. PMM2 at birth could cause (usually critical) multi-organ diseases, also characterized by a dysfunction of the central and peripheral nervous system. Mutual features are also inverted nipples, unusual subcutan fat, thin upper lip and frequently muscular hypotonia (de Lonlay et al. 2001).

For diagnosis, IEF or capillary zone electrophoresis of transferrin, a blood glycoprotein can be used. In addition to the transferrin pattern, there are also differences found like an increase of serum transaminase hypoalbuminemia, hypocholesterolemia, and tubular proteinuria (Jaak Jaeken 2010).

1.3.3 ALG6-CDG (CDG Ic)

The Alg6 gene encodes the glycosyltransferase I, which is involved in the formation of the LLO precursor of N-linked glycosylation. Mutation of Alg6 leads to a defect in an attachment of the first of three glucose molecules to the precursor. It is the second most common N-glycosylation deficiency with more than 30 patients. The defect causes an accumulation of a nonglycosylated LLO precursor in fibroblasts, which furthermore gets weakly transferred to the polypeptide chain. Patients show moderate psychomotor retardation, muscular hypotonia, strabism and seizure (Grünewald et al. 2000) (L. Sun et al. 2005) (J. Jaeken 2013).

1.3.4 ALG3-CDG

Alg3 is a mannosyltransferases that catalyzes the attachment of the sixth mannose residue to the LLO precursor in the lumen of the ER. The defects arise due to mutations in the Alg3 gene which encodes an α-1,3-mannosyltransferase (Freeze and Schachter 2009). The deficiency causes a shortened version of the LLO precursor: instead of nine mannose residues it consists of only four. Since this variant is no preferable substrate for the OST complex, LLO precursors accumulate in the cell. Patients with defects in these gene show psychomotor retardation, microcephaly, coloboma of the iris, atrophy of the optic nerve and brain and corpus callosum (Korner et al. 1999) (Rimella-Le-Huu et al. 2008).

1.3.5 COG7-CDG (CDG II)

It was the first discovered defect in the COG-complex. COG7 deficiency causes disruption of the trafficking of multiple glycosyltransferases and nucleotide sugar transport. This defect influences N-glycans as well as O-glycans and also glycosaminoglycans. In most of the patients, they found a homozygous intronic splice site mutation in the COG7 gene. Patients suffer from growth retardation, progressive, severe microcephaly, hypotonia, adducted thumbs, feeding problems by gastrointestinal pseudo-obstruction, failure to thrive, cardiac anomalies, wrinkled

skin and episodes of extreme hyperthermia and it could also end lethal in early stages (X. Wu et al. 2004) (Spaapen et al. 2005) (Morava et al. 2007) (Ng et al. 2007).

1.3.6 Other glycosylation defects

During O-glycosylation a glycan is linked to the OH-group of a Ser or Thr. There can be defects in the O-glycosylation pathway, which can cause the Walker-Warburg syndrome, but compared to N-glycan defects those are even rarer. O-glycan defects often are tissue-specific and accompanied by N-glycan defects. Defects in the GPI-anchor are an emerging CDG group lately. They are characterized by intellectual disability and biochemically by hypophosphatasia. Hypophosphatasia reflects the inability of an ectoenzyme, e.g. alkaline phosphatase to anchor to the membrane. It also plays a role in T-cell antigen anchoring (Goreta, Dabelic, and Dumic 2012b) (Francisco et al. 2018) (Al Teneiji et al. 2017) (Witters and Morava 2016).

1.4 Laboratory diagnostics and symptoms of CDG

Due to the high variety of those CDG types and symptoms and the similarity to other diseases, diagnosis of CDG is very critical. Unfortunately, no specific tests are available yet, even if they would be really helpful. Nowadays, four different techniques are used: glycan analysis, enzyme activity and metabolite measurement, classical biochemical analysis and molecular diagnostics (PCR + sequencing). There is no official procedure that is recommended by any professional organization. According to Jaeken there are some general guidelines to follow: Isoelectric focusing (IEF) of serum transferrin (Tf) or alternatively, of apolipoprotein C-III (ApoC-III), should be the first step of diagnostics (Jaak Jaeken 2010). This can help discriminating between N- or O-glycan defects. Serum transferrin has different isoform patterns, in CDG they show abnormalities. In addition, the Tf patterns differ in CDG-I and CDG-II (Theodore and Morava 2011).

Some CDG types cannot be detected with IEF. Therefore, high pressure liqid chromatography (HPLC), capillary zone electrophoresis and different forms of mass spectrometry (MS) have been utilized for diagnostics (Carchon et al. 2004) (Goreta, Dabelic, and Dumic 2012a). Furthermore, there are also biochemical tests to provide diagnostics of certain CDG types (Marquardt and Denecke 2003).

The severity of any particular CDG is difficult to assess by molecular diagnostics, and there are often 100 mutations related to particular types. Thus, there are a lot of different techniques that can be used. One of those is single stranded conformational polymorphism analysis. Restriction fragment length polymorphism (RFLP) or real time PCR are also used. Sometimes it also makes sense to sequence the whole genetic locus, including promotors exons and introns. These molecular diagnostics are often used in addition to the other diagnostic tools to identify the mutations (Lefeber, Morava, and Jaeken 2011) (Goreta, Dabelic, and Dumic 2012a).

Prenatal diagnosis is also available and possible, especially for individuals with a family CDG background or carriers of a recessive CDG gene. Unfortunately, prenatal diagnostics have been applied only for a few CDG types and is best established for PMM2-CDG. They often analyze particular enzyme activities in fibroblasts, leucocytes or lymphocytes. It is also possible to screen specific genes for mutations (Matthijs, Schollen, and Van Schaftingen 2004). False positive result on those enzyme assays are possible due to low levels of the enzymes in the fetus (Matthijs, Schollen, and Van Schaftingen 2004).

1.5 Treatment strategies

Hitherto, only a few CDG treatment options are available. Therefore, a better understanding of the molecular etiology and finding novel treatment options are urgently needed. Furthermore, there is also a lack of cellular model systems to test potential therapeutic drugs. Most common cellular models are patient derived fibroblasts, but most of the time those cells are not representative for those who are effected by the different CDG types (Thiesler et al. 2016).

In CDG, dietary therapy, in particular monosaccharide supplementation, is an important intervention. The first treatable CDG was MPI-CDG with a high mannose supplementation. Nearly all symptoms can be ameliorated with mannoses supplementation except for liver fibrosis or bile duct abnormality. However, it does not correct the overall glycosylation profile, additionally it sometimes shows side effects (Liem et al. 2008) (Damen et al. 2004). Another CDG deficiency that can be partly treated is SLC35-CDG with L-fucose supplementation, although the mechanism is not fully understood. Unfortunately, monosaccharide supplementation can only help selected CDG patients. Thus, additional treatment options are of great interest.

Nowadays there are some new therapeutic strategies and because only a few CDG defects can be compensated with food supplementation, most of the defects need other strategies. Pharmacological chaperons, which help to reinstate the equilibrium between unfolded and folded proteins are one of the newer methods (S. Brasil et al. 2018). In healthy persons there is a defined intracellular equilibrium between folded and unfolded proteins, defects in CDG can shift this equilibrium to more unfolded proteins by inducing destabilization (Gámez et al. 2018). Pharmacological chaperons can assist protein folding, because they can bind to the active site or the allosteric sites of the proteins. This could represent an option for PMM2-CDG patients, because that defect causes a destabilization in protein folding (Yuste-Checa et al. 2017).

Another treatment option could be antisense therapy. It is used in defects disrupting the splice site mutations which than lead to pseudo-exons. With antisense therapy using morpholine oligonucleotides a normal splicing profile within 24 hours post transfection could be reestablished (S. Brasil et al. 2018) (Vega et al. 2009). CDG patients are also considered as possible candidates for gene therapy. These therapies consist of a successful transfer and activation of a fully functional copy of an aberrant gene, which, however, also requires a safe vehicle for the gene copy. Adenoviral-associated vectors are commonly used as vehicle because they are very safe compared to other vehicle options. In different disease models and also in patient cells there was a rescued phenotype found when a copy of the normal gene was introduced (Ng et al. 2016) (Bryson et al. 2017). Since CDG mutations affect a variety of tissues and cell types in parallel, the use of viral vectors (including those used for CRISPR/Cas9 mediated gene editing) is limited. Thus, there are often no other options than organ transplantations due to the severe symptoms. CDG can affect the heart and liver and in this case, organ transplantation can help to treat he defects. Besides those two transplantations options, cell transplantations such as hematopoietic stem cell transplantation from cord blood and bone marrow could be considered as treatment options for some CDGs (Janssen et al. 2014) (Jansen et al. 2016) (Kapusta et al. 2013) (Klcovansky, Mørkrid, and Möller 2016) (Stray-Pedersen et al. 2014) (Patiroglu et al. 2015).

Research advances in the last few years opened some new opportunities and strategies in the therapy of CDG patients. Nevertheless, there is no overall solution or strategy for a therapy because of the complexity of the defects in CDG.

1.6 Yeast as a model organism

Saccharomyces cerevisiae is one of the most important model organisms to study mechanisms of central, conserved, cellular and molecular eukaryotic processes even though it is one of the simplest eukaryotes. Moreover, *S. cerevisiae* was the first eukaryote with a fully sequenced genome (Goffeau et al. 1996). Many genes of *S. cerevisiae* have human orthologs and can even be replaced by them (Kachroo et al. 2015). Moreover, yeast is a very inexpensive, non-pathogenic organism with efficient methods for genetic modifications. Yeast also exhibits hallmarks of programmed cell death, which make it a very suitable model organism for studying human disease. This includes Alzheimer's disease, Parkinson's disease, Huntington's disease or cancer research (D Botstein, Chervitz, and Cherry 1997) (David Botstein and Fink 2011) (Frank Madeo et al. 2004a) (Mager and Winderickx 2005). Due to the homology between human and yeast enzymes, there are also some models that are used for studying CDG in yeast. Therefore, different mutants are available such as Alg1 mutant, Alg6 mutants or Alr1 mutants which are all linked to N-glycosylation defects. (Sandra Brasil et al. 2018). In addition mutation of *WBP1*, the homolog of human OST48, which is the beta subunit of the OST complex, leads to a CDG phenotype (Knauer and Lehle 1994).

1.6.1 Yeast oligosaccherlytransferase (OST)

In *Saccharomyces cerevisiae* the OST consists of eight different proteins, i.e. either Ost3 or Ost6 plus seven shared components: Swp1, Ost1, Ost2, Ost4 and Ost5, Stt3, and Wbp1. The yeast OST and its subunits have homologous proteins in human (Silberstein and Gilmore 1996) (Knauer and Lehle 1999). All of them are integral membrane proteins of the ER and the holoenzyme interacts with the Sec61p pore complex, which is involved in the protein import in the ER (Figure 3 A) (Chavan, Yan, and Lennarz 2005). Overall the OST is divided into 3 subcomplexes: (i) OST1-OST5 subcomplex, where OST1 stabilizes the glycosylated protein and OST5 is an accessory factor for OST1 (Yan, Prestwich, and Lennarz 1999) (Bai et al. 2018). (ii) OST2-Swp1-Wbp1 the function of which is poorly understood. Notably, Wbp1 has a domain, a so called GIFT domain, that could be probably involved in the LLO binding (Beatson and Ponting 2004). Additionally, it has been shown that Swp1 as well as Wbp1 are involved in the cross linking process of LLO (Pathak, Hendrickson, and Imperiali 1995a) (Bause et al. 1997).

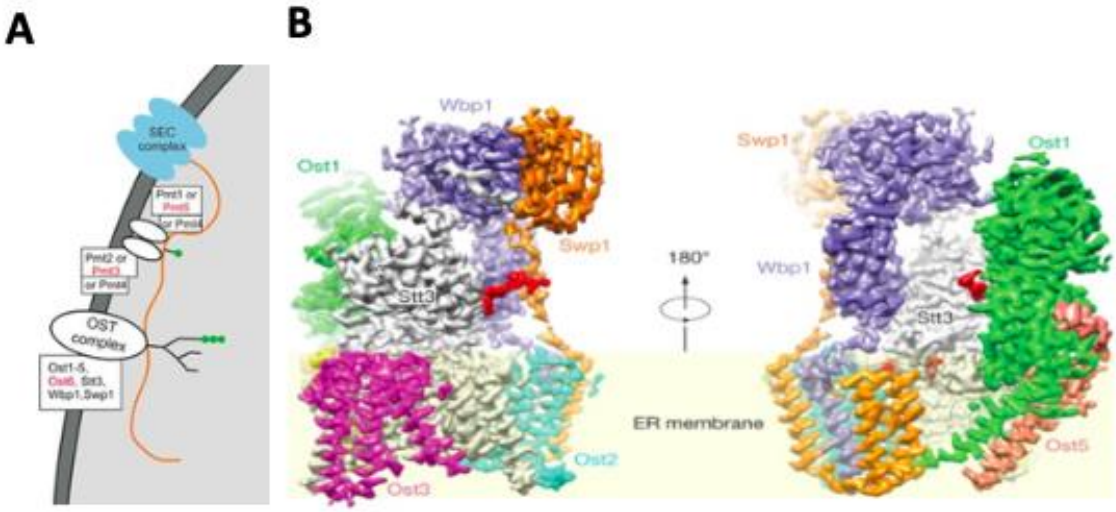


Figure 3: Scheme of the OST complex in the ER membrane. A) On the ER inside (lighter grey) the SEC complex is indicated and the N-glycan-precursor (green dots). It further indicates how the N-glycan precursor got synthesized and the involvement of the OST-complex. **B)** Cryo-electron microscopy map of the OST shown in front and back views. The different subunits are indicated in different color. (Figure and figure legend by (Bai et al. 2018)).

(iii) Last but not least the Stt3-OST4-OST3 subcomplex, where OST4 stabilizes Stt3 and Stt3 and OST3 form a vertical groove together**.** (Figure 3 B) This groove could be the possible docking site for the LLO (Mohorko, Glockshuber, and Aebi 2011). The proposed model for the OST translocon interface is as follows: First the nascent protein, which emerges from the Sec61 complex, is bound by OST3. It further passes the catalytic site of the Stt3 for N-glycosylation. OST1 maintains the stabilization of the glycosylated peptide (Bai et al. 2018).

1.6.2 Yeast Wbp1p

The yeast Wbp1 (Wheat germ agglutinin-binding protein 1) is a 45 kDa protein that is essential for yeast growth and a crucial part of the OST and homologue of the human OST48 (te Heesen et al. 1991). It has a motif that functions as an ER retrieval signal and is also proposed to be essential for binding the LLO (Gaynor et al. 1994) (Pathak, Hendrickson, and Imperiali 1995b). Wbp1-depleted *S. cerevisiae* cell show under-glycosylated glycoproteins, such as carboxypeptidase Y (CPY) and it is essential for the OST activity (te Heesen et al. 1992). Wbp1 deficiency further leads to apoptosis hallmarks in yeast including elevated ROS levels (Hauptmann et al. 2006).

1.7 Programmed cell death

Programmed cell death (PCD) is an important physiological process in multicellular organism and has a central role during development, maintenance of tissue homeostasis, removal of cells with mutations, or virus-infected cells. The phenomenon of PCD was first described by Lockshin and Williams 1965, where they observed cell death that follows locally and temporally controlled events in a silkworm (Lockshin and Williams 1965). Nowadays, we know different forms of PCD, which are classified into apoptosis, programmed necrosis and autophagic cell death, which have partly overlapping pathways and executor proteins can occur in a caspase-dependent or independent fashion. Any dysregulation in PCD can cause a variety of diseases, such as cancer, autoimmune disorders or neurodegenerative disorders (Kroemer et al. 2005) (Galluzzi et al. 2012) (Broker, Kruyt, and Giaccone 2005).

Apoptosis is a non-inflammatory form of PCD, and apoptotic cells are typically shrinking and show a condensed chromatin. The plasma membrane is blebbing and apoptotic bodies start to build. Apoptotic bodies are filled with cytoplasm and packed with organelles, the integrity of which is still maintained. Those bodies are quickly phagocytosed by surrounding macrophages, which prevents inflammation. In contrast, necrosis is characterized by cellular swelling until the cell wall integrity can no longer be sustained, and the cytoplasmic content is released into the surrounding tissue. This typically results in the recruitment of inflammatory cells (Savill and Fadok 2000) (Elmore 2007).

In unicellular organisms like *S. cerevisiae* PCD is advantageous too, namely, to eliminate certain cells in a population to ensure the survival of the population and protection of the genetic material, independent of a single cells fate (Kametaka et al. 1998). Furthermore, different forms of PCD are found in yeast with conserved pathways. Even similar key factors of cellular life and death decisions have been identified in *S. cerevisiae*. Similar to the intrinsic pathway of apoptosis in mammals, mitochondria are involved in yeast PCD (F Madeo, Fröhlich, and Fröhlich 1997) (Frank Madeo et al. 2004b) (Didac Carmona-Gutierrez and Büttner 2014). Interestingly, many pharmacological agents that have been shown to modulate PCD in mammals influence different forms of cell death in yeast, corroborating the use of yeast to understand basic PCD principles (Eisenberg et al. 2007) (D Carmona-Gutierrez et al. 2010) (Carmona-Gutiérrez et al. 2011).

1.8 Yeast target of rapamycin (TOR)

TOR is a serine/threonine protein kinase and is part of the phosphatidylinositol kinase related protein kinase family. It was first discovered in *Saccharomyces cerevisiae* and has two different isoforms, TOR1 and TOR2 (Mohorko, Glockshuber, and Aebi 2011), which, in conjunction with other proteins, can form different TOR complexes (TORC1 and TORC2). In mammals, two different protein complexes (mTORC1 and mTORC2) with different regulatory roles have been described as well (Brown et al. 1994) (McCormick, Tsai, and Kennedy 2011). TOR can be inhibited by rapamycin, mainly the TOR1 complex but through long term exposure also TOR2. In detail, rapamycin binds to Fk506-sensitive proline rotamase (Fpr1 in yeast, FKBP12 in mammals), and the forming complex binds specifically inhibits TOR activity (Heitman et al. 1991) (Shaw et al. 1988).

The TOR pathway receive signal either directly or indirectly and from various different effectors, such as nutrients, growth factors, oxygen, mediators of energy balance and a lot of other environmental factors. Those signals influence different part of the TOR signaling pathway and lead either to activation or inhibition of the TOR-kinase (González and Hall 2017) (Inoue and Nomura 2018).

In yeast, TOR regulates growth related pathways and is therefore responsible for the balance between macromolecular biosynthesis and turnover. TORC1 promotes in parts the biosynthesis of those macromolecular biosynthesis through translational activation and ribosome biogenesis. Simultaneously it also represses turnover processes such as autophagy and also inhibits stress response. During poor nutrient conditions, TORC1 is inhibited and as part of that, turnover processes start such as TCA cycle and autophagy are upregulated, and the stress response is switched on (Eltschinger and Loewith 2016) (González and Hall 2017) (Wei and Zheng 2011).

During nutrient availability TORC1 phosphorylates Sch9, which is the yeast homologue of serine-6-kinase 1 (S6K1). Sch9 regulates the transcription of ribosomal biogenesis and also the initiation of protein translation (Urban et al. 2007). Additionally, TORC1 can regulate Fhl1, Sfp1, and Maf1 which are responsible for regulating the transcription of ribosome biogenesis factors (Martin, Soulard, and Hall 2004) (Jorgensen et al. 2004). When TORC1 is inhibited, Maf1 is dephosphorylated and enters the nucleolus inhibiting ribosomal biogenesis by associating with Polymerase III (Wei, Tsang, and Zheng 2009). During starvation and TORC1 inhibition, ATG13 is dephosphorylated and in this state, it can bind to Atg1. The Atg13-Atg1 complex then starts the

recruitment of other autophagic proteins and thereby start the formation of autophagosomes (Noda and Ohsumi 1998) (Wei, Tsang, and Zheng 2009). TORC1 also regulates the metabolism during nutrient uptake trough the regulation on nutrient transporters, different high affinity permeases and also on transcriptional level via Gln3 and Gat1, which are responsible for the NCR (nitrogen catabolite repression) genes. In lack of preferred nitrogen sources, those genes get activated. In addition, Gln3 is regulated by the phosphatase PP2A (Carvalho and Zheng 2003) (Kuruvilla, Shamji, and Schreiber 2001). Rtg1/Rtg3 form another transcription factor, which regulates genes of the TCA cycle. TORC1 regulates Rtg2 and Mks1 which further regulate the Rtg1/Rtg3 transcription factor (Komeili et al. 2000) (Magasanik and Kaiser 2002).

Under starvation, TORC1 is inhibited which further induces stress response genes, mainly regulated by Msn2 and Msn4. Glucose depletion or rapamycin treatment leads to TORC1 inhibition and Msn2/Msn4 dephosphorylation and translocation in the nucleus. This also involves Rim15, a kinase that is not only involved in transcriptional induction of Msn2/Msn4 it is also required for G0 entry and lifespan extension. Rim15 is further regulated by Sch9 (Beck and Hall 1999) (Mayordomo, Estruch, and Sanz 2002) (Pedruzzi et al. 2003a) (Swinnen et al. 2006).

How is TOR exactly regulated? It has the ability to sense environmental nitrogen and carbon sources and can sense the intracellular amino acid availability through its association to lysosomes (vacuole in yeast) (Chantranupong et al. 2014). However, in yeast little is known about nutrient sensing and transmitting the information to TOR. What is known is that in both mammalian and yeast cells, glucose and amino acid transporter are required for TOR regulation. In yeast, cell growth regulation requires Mep2, an ammonium transporter, Ssy1 an amino acid permease and Snf3 a glucose sensor (Ozcan and Johnston 1999) (Iraqui et al. 1999) (Wei and Zheng 2011). It also needs a vacuolar compartment for sensing amino acid availability. In yeast cells, EGO (exit from rapamycin-induced cell arrest), a protein complex consisting of Ego1, Ego3, Gtr1 and Gtr2 might fulfill this role. EGO could be transmitting critical nutrient availability to TOR. In sum, the extracellular nutrient availability is reflected by the intracellular nutrient transports, which are both sensed by the nutrient transporters and EGO and likely, other unknown mechanisms (Kogan et al. 2010) (T. Zhang et al. 2012) (Binda et al. 2009). Upstream effectors and diverse downstream targets of TOR are summarized in Figure 4.

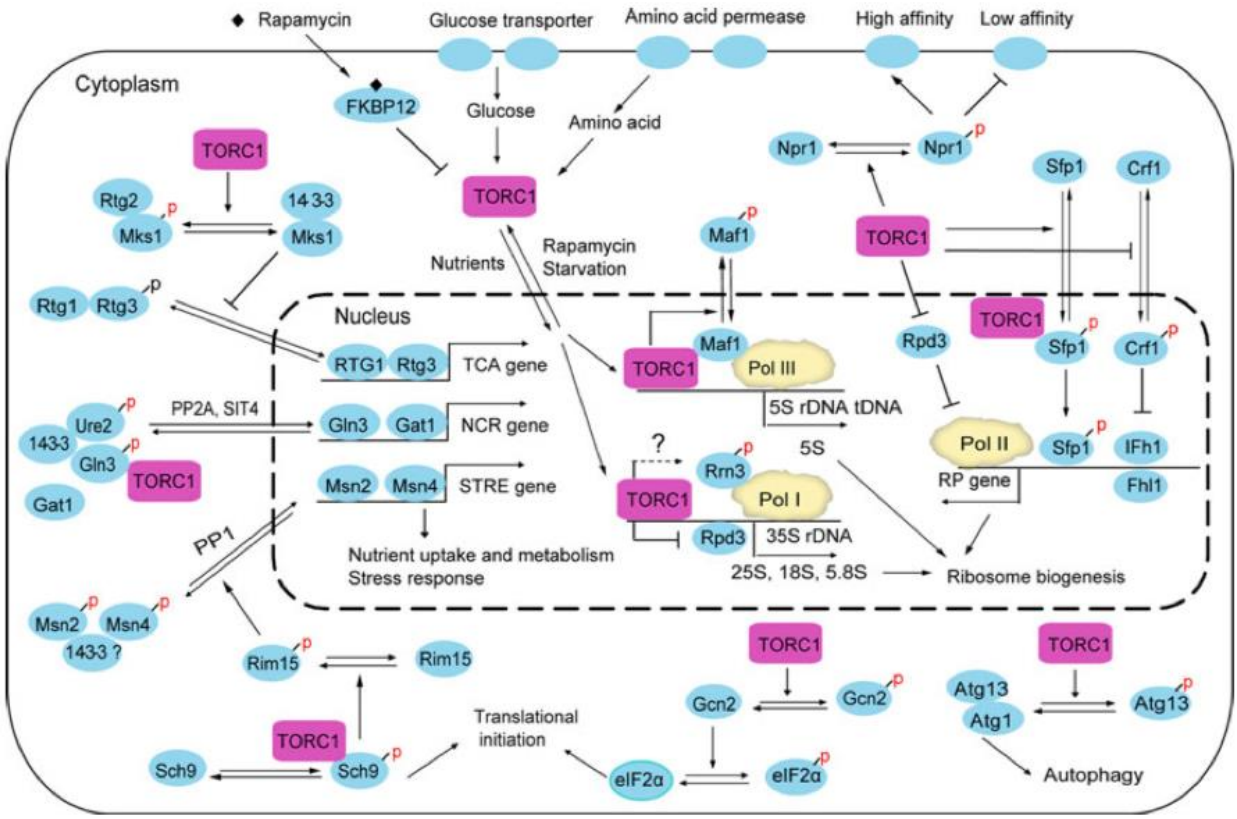


Figure 4: TORC1 has diverse downstream targets in yeast cells. In response to nutrient signaling TORC1 activates various different pathways. During cell growth it stimulates anabolic processes such as ribosome biogenesis via Maf1 phosphorylation or Sch9 activation. Active TORC1 promotes protein and nucleotide synthesis on different levels, and catabolic processes such as autophagy, TCA genes, stress response and NCR genes are inhibited. During starvation or rapamycin treatment TORC is inactivated. Now autophagy is switched on by rapid dephosphorylation of Atg13 or activation of stress response by activating the transcription factors Msn2 and Msn4 (Figure and figure legend by (Wei and Zheng 2011)).

1.9 Rapamycin

Rapamycin is a macrolide natural component produced by different actinomycetes. It was first discovered in the soil bacteria *Streptomyces rapamycinicus* on the Eastern Island, also called Rapa Nui (Vézina, Kudelski, and Sehgal 1975). Rapamycin inhibits TOR by forming a complex with the FK506-binding protein (FKBP12, Fpr1 in yeast), acting as an allosteric inhibitor. Rapamycin treatment activates autophagy and inhibits cell growth, which makes it an possible anti-cancer agent (Law 2005). Primarily it was described as an antifungal agent, later, it was identified as a immunosuppressive, that also can inhibit the T-cell proliferation and inhibits protein translation by suppressing the amino acid incorporation (Martel, Klicius, and Galet 1977) (Dumont et al. 1990) (Singh, Sun, and Vézina 1979). Rapamycin also has antitumor activity,

neuroprotective/neurodegenerative and lifespan extension activities, which was not only observed in yeast, but also in mice. It is not known yet, if rapamycin has similar beneficial effects in humans (Houchens et al. 1983) (Pan et al. 2008) (Malagelada et al. 2010) (Harrison et al. 2009).

1.10 Unfolded protein response (UPR)

When the ER responds to ER stress, which means unfolded or misfolded or mis-localized proteins are accumulating in the lumen of the ER it activates an intracellular signaling pathway, the so-called unfolded protein response (UPR). ER stress can be caused by environmental influence like starvation, virus infections or also heat. UPR further leads to extensive transcriptional response, which adjusts the protein folding as need (Kozutsumi et al. 1988) (J S Cox and Walter 1996) (J. Wu and Kaufman 2006). Of note, pharmacological induction of UPR is typically achieved by tunicamycin treatment, an inhibitor of GlcNAc phosphotransferase (GPT), which is involved in N-glycosylation (J S Cox and Walter 1996) UPR and its signaling components were first discovered in yeast. In principle it is based on two proteins, one signaling protein in the ER membrane, the transmembrane signaling protein Ire1 (inositol-requiring enzyme 1), which is also the sensor of the ER stress, and its downstream effector protein Hac1, a transcriptional activator. Hac1 activates genes that code for proteins involved in protein folding and coping with ER stress (Jeffery S. Cox, Shamu, and Walter 1993) (Mori et al. 1996). Nowadays it is understood that in higher eukaryotes the signaling pathway is not as simple, but instead has parallel and cross-wired circuits. Also for yeast it became clear that it is more a signaling network than an isolated pathway (Bernales, Papa, and Walter 2006) (Leber, Bernales, and Walter 2004). The UPR can lead to apoptosis, if the ER stress persists. Disruption in the ER-UPR signaling or in the homeostasis can lead to several human diseases such as Alzheimer´s disease, cancer or diabetes (Wang et al. 2009).

Recently there is more and more evidence that the UPR signaling network and TOR signaling network have some intersections. Late studies show that there is a crosslink between IGF-1 signaling, the ER chaperone GRP78 (glucose regulated protein 78) and mTOR/AKT/PI3K pathway in mice (Pfaffenbach et al. 2012). Furthermore, mTORC1 alone can activate UPR through IRE1 signaling during ROS stress. In tumor cells, two ER-stress sensors can be activated though mTORC1 signaling. (Kato, Katoh, and Kitamura 2013) (Urra and Hetz 2014). There is growing evidence that UPR

could be downstream of mTORC1 because of its sensitivity to nutrient and growth signaling (Appenzeller-Herzog and Hall 2012).

1.11 *Drosophila melanogaster* as a model organism

Drosophila melanogaster is an established and often used model organism. Not only has it similar genetic and molecular structure as humans, but also allows easy handling in the lab due to its short generation time and a compact fully sequenced genome (Adams et al. 2000). The use of flies is inexpensive and ethically uncritical, due to their relatively short life span (approx. 2-3 months), rapid screening of substances or genetic modifications in a multicellular context are possible. Since flies have been used for nearly a century as model organisms, the have a consolidated role in various different research fields, such as aging, neurodegeneration and other human diseases. Additionally, they have a numerous amount of behavioral and developmental traits that can be studied (Helfand and Rogina 2003) (Y. Sun et al. 2013) (Jennings 2011).

Another characteristic of *Drosophila melanogaster* is the four-stage life cycle. The mother flies lay eggs directly onto the food, up to 100 eggs per day depending on the age of the mother fly. The eggs develop to larvae, which go through three different instars before becoming a pupae. Out of its adult flies eclose. At standard conditions (25°C, 12-hour light/dark cycle and approximately 70% humidity) the full development cycle takes less than two weeks. By reducing the temperature the reproductions cycle can be extended up to 28 days (Busson 1993) (Brand et.al 1993) (Stocker and Gallant 2008).

To study different human diseases in fly, genome wide RNAi libraries based on the Gal4/UAS system are available. It allows directed gene expression in *Drosophila* using the yeast transcription factor Gal4 and a specific upstream activating sequence (UAS). Gal4 can be placed under the control of a tissue-specific promotor, called the Gal4-driver line. This line can be crossed with a line carrying the UAS followed by the gene of interest for overexpression studies or an RNAi construct for RNA interference in the F1 generation (Brand and Perrimon 1993) (Duffy 2002). *Drosophila* CDG disease model are available that are based on this principle, including PMM2 mutation lines. There are also RNAi lines available with genes of interest that are part of the glycosylation pathway, but as disease model they are not so well established yet (Parkinson et al. 2016).

2. Materials

2.1. Laboratory equipment

Special equipment used during this study is listed in Table 1.

Table 1: Special equipment used during this work.

Equipment	Company
BD FACSAriaTM Flow Cytometer	BD Bioscience, USA
PIPETMAN ClassicTM	Gilson, Inc., USA
Cell Counter CASY	Schärfe System, Germany
Centrifuge 5427 R	Eppendorf AG, Germany
Centrifuge 5810 R	Eppendorf AG, Germany
Photometer	Hitachi
ChemiDocTM Touch	Bio-Rad Laboratories GesmbH
FACS plates	Greiner
Freezer (-80°C)	Forma Scientific and Sanyo
Fridge (-20°C)	Liebherr
TECAN microplate reader	TECAN (Austria)
Vials large for flies	K-TK (Germany)
Vials small for flies	K-TK (Germany)

2.2 Strains and plasmids

For all yeast experiments *Saccharomyces cerevisiae* strains were used, which have been received from Euroscarf. All the yeast strains for this work were generated by transformation using the lithium acetate method and selected on synthetic minimal media supplemented with amino acids required for survival, are listed in Table 2, also all *E. coli* strains used are listed in this table. All *Drosophila melanogaster* lines used during this work are listed in Table 3. All Plasmids used during this work are listed in Table 4.

Table 2: *Saccharomyces cerevisiae* strains used during this study:

Strain	Genotype	Origin
Saccharomyces cerevisiae strains		
Wt (BY4741)	MATa ura3-0 his3-1 leu2-0 met15-0	Euroscarf
BY4742 GFP-Atg8	MATalpha ura3-0 his3-1 leu2-0 lys2-0 NatNT2:PAtg8-GFP-*ATG8*	Andreas Zimmermann
Tet$_{OFF}$-WT	BY4741 URA3::CMV-tTA	This work
Tet$_{OFF}$-WBP1	BY4741 URA3::CMV-tTA KanMX:tet-O2-*WBP1*	This work
Tet$_{OFF}$-WBP1 GFP-Atg8[a]	*Tet$_{OFF}$-WBP1* NatNT2:PAtg8-GFP-*ATG8*	This work
Tet$_{OFF}$-WBP1 UPR[a]	*Tet$_{OFF}$-WBP1* [pMCZ-Y]	This work
Tet$_{OFF}$-WBP1 ΔPP2A (pph21/pph22)[a]	*Tet$_{OFF}$-WBP1 pph21*::NatNT2 *pph22*::*LEU2*	This work
Tet$_{OFF}$-WBP1 Δsch9[a]	*Tet$_{OFF}$-WBP1 sch9*::*LEU2*	This work
Tet$_{OFF}$-WBP1 Δatg1[a]	*Tet$_{OFF}$-WBP1 atg1*::*LEU2*	This work
Tet$_{OFF}$-WBP1 Δatg6[a]	*Tet$_{OFF}$-WBP1 atg6*::*LEU2*	This work
Tet$_{OFF}$-WBP1 Δmsn2/msn4[a]	*Tet$_{OFF}$-WBP1 msn2*::NatNT2 *msn4*::*LEU2*	This work
Tet$_{OFF}$-WBP1 Δrim15[a]	*Tet$_{OFF}$-WBP1 rim15*::*LEU2*	This work
Tet$_{OFF}$-WBP1 Δmaf1[a]	*Tet$_{OFF}$-WBP1 maf1*::NatNT2	This work
Tet$_{OFF}$-WBP1 Δhcm1[a]	*Tet$_{OFF}$-WBP1 hcm1*::NatNT2	This work
Tet$_{OFF}$-WBP1 Δdot6[a]	*Tet$_{OFF}$-WBP1 dot6*::NatNT2	This work
Tet$_{OFF}$-WBP1 Δrtg2[a]	*Tet$_{OFF}$-WBP1 rtg2*::*LEU2*	This work
E. coli strains		
E. coli XL1		Agilent

[a] corresponding wildtype controls were generated in the *Tet$_{OFF}$-WT background*

Table 3: *Drosophila melanogaster* lines used during this work

Lines	Genotype	Origin
w^{1118}		Bloomington (3605)
Alg6 RNAi	GD line, Chromosome 2, no Off-targets, mini-white linked to UAS-RNAi	v2782
PMM2 RNAi	Chromosome 3, 1 Off-target: CG12278, mini-white linked to UAS-RNAi	v39715
Cog7 RNAi	one Off-Target: CG7707, Chromosome 3, mini-white linked to UAS-RNAi	v39926

da_Gal4	w*;+;daGal4;+

Table 4:Plasmids used for UPR expression and knockout cassettes during this work.

Plasmids	Description	Origin
pUG73	*Kluyveromyces LEU2* marker	Euroscarf
pFA6a-NatNT2	NatNT2-Marker	Euroscarf
UPR-Plasmid	[pMCZ-Y] (UPRE-lacZ, URA3)	Andreas Zimmermann

2.3 Primer and oligonucleotides

Primer listed in table 5 are all obtained from MWG Biotech (Germany). The lyophilisated primer were all dissolved in Fresenius ddH_2O to a concentration of 100 pmol/µg.

Table 5: Primers used during this work (e.g. knockout primers for pUG73 and pFA6a-NatNT2 and control primers)

Gene	Type	Sequence (5´-3´)	Origin
Pph21	KO fwd	AAAGAGGGATATAAATTATCGCATAAAACAATAA ACAAAAAGAAAACAGCTGAAGCTTCGTACGC	A. Zimmermann
	KO rev	AGAAAAGTGAATATATATCTATATAGATGCATAT ATGTATACATACGCATAGGCCACTAGTGGATCT G	A. Zimmermann
	control	TTTCCGGATTACATTAGTTCGG	A. Zimmermann
Pph22	KO fwd	GAATTTTATATTATTGGCACTTCTGTATAACTGG CTTTCATTCGAAAAAACGTACGCTGCAGGTCGA C	A. Zimmermann
	KO rev	TATGTTGGAATGAAATAGCGTAGTAAGGATAAA GGTGTAATAGATATATAATCGATGAATTCGAGC TCG	A. Zimmermann
	control	TACATCCAGGAATAGAGTCCAC	A. Zimmermann
Sch9	KO fwd	GACCAACACATGGATGACGAATTTGTCAGTGGA AGA…	C. Ruckenstuhl
	KO rev	AAGAAAAGGAAAAGAAGAGGAAGGGCAAGAGG AGCG…A.	C. Ruckenstuhl
	control	CGATAACGGTTCTTTCTGCATAT	C. Ruckenstuhl
Atg1	KO fwd	ATATTTTCAAATCTCTTTTACAACACCAGACGAG AAATTAAGAAAcagctgaagcttcgtacgc	C. Ruckenstuhl
	KO rev	TAGCAGGTCATTTGTACTTAATAAGAAAACCATA TTATGCATCACgcataggccactagtggatc	C. Ruckenstuhl
	control	CGCTCGGCTCTGATTTCT	C. Ruckenstuhl
Atg6	KO fwd	GTCACTGTTTTCGCAAAGACTCCCAGACACGG GCATTAAAAGCTGAAGCTTCGTACGC	C. Ruckenstuhl
	KO rev	TTTTCCCTTTATCACATTTATGAAAAAATGCATTT ATATGAACTACGCATAGGCCACTAGTGGATCTG	C. Ruckenstuhl
	control		C. Ruckenstuhl
Msn2	KO fwd	TTTTCTTTCTTTTTTCAACTTTTATTGCTCATAGA AGAACTAGATCTAAACAGCTGAAGCTTCGTACG C	A. Zimmermann

	KO rev	CAGAATTATCTTATGAAGAAAGATCTATCGAATT AAAAAAATGGGGTCTAGCATAGGCCACTAGTG GATCTG	A. Zimmermann
	control	GTTGTTTCCAGCGAAAGAGAC	A. Zimmermann
Msn4	KO fwd	CCTTTATCAGTTCGGCTTTTTTTTCTTTTCTTCTT ATTAAAAACAATATACGTACGCTGCAGGTCGAC	A. Zimmermann
	KO rev	TGTCATACCGTAGCTTGTCTTGCTTTTATTTGCT TTTGACCTTATTTTTTATCGATGAATTCGAGCTC G	A. Zimmermann
	control	TTCTCCCACGAGGTTTCACTG	A. Zimmermann
Rim15	KO fwd	CTTGCCTCATTTGATAGAATAGATAAGCCCAGT AGAGGAAGACAGcagctgaagcttcgtacgc	C. Ruckenstuhl
	KO rev	AATTATCTTTATCTTAAAATTTATCAGTGCGTTTC ATCAGAATCGgcataggccactagtggatc	C. Ruckenstuhl
	control	GCTGAGCCACTTTGCCT	C. Ruckenstuhl
Maf1	KO fwd	TTAACCGCTCATTACTCCAAACGGATTTTTTTGC CTAAAGAATCACGACAATGCGTACGCTGCAGGT CGAC	T. Eisenberg
	KO rev	ATAGGTGTAAGACAAGGAAAATTCACAAATTAA AGTTTAAAACTAATCGATGAATTCGAGCTCG	T. Eisenberg
	control	GTATTCGGTCCTCATTATATCG	T. Eisenberg
Hcm1	KO fwd	ATTTCGAAGCAAAAAAAAAAAAAATCAACAAAAA TTCCAACTTTTCAATGCGTACGCTGCAGGTCGA C	A. Zimmermann
	KO rev	TTAGTTTTCGGTGAGGAAAAGAAAATGAAAAAA AAAAAAAAAAAAAGTCAATCGATGAATTCGAGC TCG	A. Zimmermann
	control	GACCACTTTCCCATTTTGGTC	A. Zimmermann
Dot6	KO fwd	ATAGCTTCCGTGCACGTTCCAGTCTTCCCTCCC TTCTCTGCTCCGTGATGCGTACGCTGCAGGTC GAC	A. Zimmermann
	KO rev	GTTGATATTTTTTTATTTTTATTTTTTTTTCATTTT AAGTTTTCCCCTCAATCGATGAATTCGAGCTCG	A. Zimmermann
	control	TGCAGCGACGCCACAAAAACG	A. Zimmermann
Rtg2	KO fwd	No sequence available!	C. Ruckenstuhl
	KO rev	No sequence available!	C. Ruckenstuhl
	control	No sequence available!	C. Ruckenstuhl
LeuB	Control pUG	AGTTATCCTTGGATTTGG	C. Ruckenstuhl
pFA6a-NatNT2	Control pFA6a-NatNT2	GTCGACCTGCAGCGTACG	A. Zimmermann

2.4 Chemicals, kits and antibodies

All fine chemicals used in that work are produced by following companies: ROTH (Karlsruhe), FLUKA Chemie (Swiss), und SIGMA (USA). The constitute for culture media, such as yeast extract, bacto-pepton and yeast nitrogene base with or without amino acids were obtained from BD (USA). Amino acids are from Serva (Germany). Molecular weight standards for gel electrophorese and enzymes are received from

Thermo Fisher. Antibodies were obtained from Ustate (Millipore), Abcam, Sigma. (Tab. 6).

Table 6: Chemicals, enzymes, kits and antibodies used in this work.

Chemicals, kits and antibodies	**Company**
Kits	
GeneJET™ Plasmid Miniprep Kit	Fermentas
GeneJET™ Gel Extraction Kit	Fermentas
GeneJET™ PCR Purification Kit	Fermentas
Chemicals/enzymes	
Restriction enzymes	Fermentas
Primer	MWG Biotech, Germany
Taq Polymerase	Finnzymes
Lambda DNA/EcoRI+HindIII Marker	Fermentas
PI	Roche, Germany
Amino Acids	AppliChem, Germany
Sorbitol	Fluka BioChemika
NaOH	ROTH, Germany
Sodium dodecylsulfat	Serva
Glycerol	Sigma Aldrich
Tris	MP Biomedicals
β-mercaptoethanol	ROTH, Germany
K2HPO4	ROTH, Germany
KH2PO4	ROTH, Germany
EDTA	Calbiochem
Proteinase Inhibitor	Roche, Germany
ECL	BioRad/selfmade
MES	ROTH, Germany
Acrylamid	ROTH, Germany
Milk powder	BioRad
PVDF Membrane	Millipore, USA
Page RulerTM Prestained Protein Ladder	Fermentas
Doxycycline	
Rapamycin	LC Laboratories
primary antibodies	

α-CPY	Sigma
diluted: 1:1000	
α -GAPDH	ThermoFischer Scientific
diluted: 1:10000	
secondary antibodies	
α-mouse POD	Sigma
diluted: 1:10000	

2.5. Rapamycin & doxycycline administration and PI-staining

Rapamycin was obtained from LC Laboratories and dissolved in DMSO in a stock concentration of 1.1 mM for addition in yeast media. For usage in fly food, rapamycin was dissolved in ethanol in a stock concentration of 2 mM.

Doxycycline was obtained from Sigma and dissolved in Fresenius ddH_2O in a stock concentration of 10 mg/ml.

For PI staining and FACS measurement, PBS was prepared as 10x stock and stored at room temperature. Propidium iodide (PI) was prepared at 1000x stock and stored at -20°C. before used PBS and PI were diluted with ddH_2O to 1x concentration (Table 7).

Table 7: Substances and Solutions used for Pi staining and FACS measurement.

Solution	Composition
Phosphate buffered saline (PBS)	25 mM potassium phosphate buffer (pH 7,0) 0,9% NaCl (w/v), ddH2O
1000x Propidium iodide (PI)	100 µg/ml in ddH2O, Sigma-Aldrich (USA)

2.6 Culture media

2.6.1 Preparation of culture media

For the preparation of the liquid and plate cultivation media, double distilled water was used. For the plate cultivation media, 2% of agar was additionally added. The ingredients were obtained from BD (USA) and AppliChem (Germany). The autoclaving was performed either with Systec autoclave (program 8) or CertoClav. The antibiotics were added after autoclaving the media and before use. 10x amino acids stocks which were used for minimal media were autoclaved, stored at -20°C and added to the media before usage or pouring plates.

2.6.2. Growth media for cultivation of *E. coli*

E. coli strains were cultivated in Luria Bertani medium (LB). For the selection of *E. coli* strains carrying the plasmids, 100 µg/ml of Ampicillin was added to the media before the cultivation of the *E. coli* culture. Composition of the LB medium is listed in Table 8.

2.6.3. Growth media for cultivation of *S. cerevisiae*

S. cerevisiae strains were cultivated in full media or minimal media containing glucose as carbon source. Full media contained all the amino acids, while minimal media were lacking one, (leucin) depending on the plasmids yeast is carrying. The media used for cultivation are listed in Table 8.

Table 8: Media for growth of *Saccharomyces cerevisiae* and *E. coli* and its composition used during this work.

Media	**Composition**
***Saccharomyces cerevisiae* media**	
YPD (Yeast peptone dextrose) full medium	1% Yeast extract (BD) 2% Bacto peptone (BD) 4% Glucose As above + 2% agar
Clonnat YPD agar plates	As above + 250 µg/ml (added when cooled down, before pouring)
SMD/SMG (synthetic minimal dextrose/galactose) minimal medium	0,17% yeast nitrogen base (BD) 0,5% ammonium sulfate (Roth) 2% glucose, galactose 80 mg/l histidine 200 mg/l leucine 300 mg/l uracil 30 mg/l adenine 30 mg/l all other amino acids
SM agar plates	As above + 2% agar
E. coli media	

LB + ampicillin (liquid) (lysogeny broth) full medium	0,5% Yeast extract (BD) 1% Bacto tryptone (BD) 0,5% NaCl (Roth) 100 µg/ml Ampicillin
LB + ampicillin (solid plate)	As above + 2% agar

2.6.4. Food for cultivation of *Drosophila melanogaster*

All food recipes used during the work with *D. melanogaster* are listed in Table 8.

Table 9: adapted food recipes for Drosophila melanogaster, its composition and literature.

Media	**Composition**	**Literature**
1x SYA 100 g	baker's yeast (Lesaffre, France) 50 g sucrose (Roth, Germany) 10 g agar 3 ml propionic acidA 30 ml nipagin solutionB add ddH2O to above to make 1 liter	Bass et al. 2007
1,5x SYA (0,6% agar) (crossing food)	As above except: 150 g baker's yeast 6 g agar	Bass et al. 2007
Standard Bloomington medium with minor modifications	4,2 g agar 85,3 g sugar beet syrup (Backhof, Germany) 7,5 g baker's yeast 8,3 g soy meal (Nestelberger, Austria) 67 g corn meal (Nestelberger, Austria) 1,3 g nipagin (in 4,2 ml EtOH) 5,25 ml propionic acid add ddH2O to above to make 1 liter	

2.6. Buffers and Solutions

For all buffers and solutions double-deionized water was used. The mentioned pH-value is adverted to room temperature.

2.7 Agarose gel electrophoresis

Table 10: Solution and buffers used for agarose gel electrophoresis

Solution	Components
TAE	40mM Tris/Acetat 1mM EDTA, pH 8,0
Loading buffer	50ml 87% glycerine with a little bit of bromophenol blue
Agarose gel	1% Agarose in TAE 0,001% ethidiumbromid

2.8 SDS-PAGE and Immunoblotting

Table 11: Composition of solutions and buffers used for SDS-PAGE and immunoblotting

Solution	Components
Electrophoresis buffer	25mM Tris/HCl 192mM glycine 0,2% SDS, pH 8,3
Stacking gel	250mM Tris/HCl, pH 6,8 0,2% SDS 5% acrylamide 0,13% N,N'-methylenebisacrylamide 0,1% Ammoniumperoxo-disulphate 0,01% N,N,N',N'-tetramethylethylendiamin
Running gel	250mM Tris/HCl, pH 8,8 0,2% SDS 12,5% acrylamide 0,13% N,N'-methylenebisacrylamide 0,1% Ammoniumperoxo-disulphate 0,01% N,N,N',N'-tetramethylethylendiamin

Blotting buffer	20mM Tris 150mM glycine 0,05% SDS 20% methanol
TBS(-T)	10mM Tris/HCl 150mM NaCl, pH 7,6 (0,02% Triton-X 100)
Blocking solution	5% milk powder in 1x TBS
ECL reagents	50% ECL solution 50% ECL solution
Stripping buffer (50ml)	6,25ml of 0,5M Tris/HCl, pH 6,7 10ml of 10% SDS 357µl β-mercaptoethanol 33,4ml ddH2O

2.9 Yeast knockout and plasmid transformation

Table 12: Solutions used for yeast knockout and plasmid transformation during this work

Solution	Components
TE/Lithium acetate	10mM Tris/HCl, pH 7,5 1mM EDTA, pH 8,0 100mM Lithium acetate
PEG solution	10mM Tris/HCl, pH 7,5 1mM EDTA, pH 8,0 100mM Lithium acetate 50% Polyethylene glycol 3350
Carrier-DNA (ssDNA)	Herring sperm DNA (10 mg/ml)
DMSO	Roth, ≥ 99,8% purity

SORB	100 mM lithium acetate 10 mM Tris/HCl (pH 8,0) 1 mM EDTA/NaOH (pH 8,0) 1 M sorbitol

2.10 Yeast colony and knockout PCR

Knockout cassettes were generated by PCR all compositions used for the two different knockout PCR are listed in table 13. The PCR programs used for knockout PCR´s are listed in table 14. All knockout transformations were controlled by colony PCR. Composition of PCR and PCR program are listed in table 13 and 14.

Table 13: compositions used for knockout PCR and colony PCR during this work

Volume (µl)	**Component**
PCR-compositions pFA6a-NatNT2	
1 (=100 µg/ml cassette plasmid)	template
0,5	Phusion Hot Start (5 U/µl)
5	Buffer 2 (10x)
8,75	dNTPs (2 mM)
0,32	Each primer (100 pmol/µl)
34,11	ddH2O (Fresenius)
PCR-compositions pUG73	
6 (=100ng/µl cassette plasmid)	template
0,15	Taq-Polymerase (5 U/µl)
3	Thermo-Pol Buffer (10x)
3	dNTPs (2 mM)
1,5	Each primer (100 pmol/µl)
15	ddH2O (Fresenius)
PCR-compositions colony	
5	template
0,125	Taq-Polymerase (5 U/µl)
2,5	Thermo-Pol Buffer (10x)
2,5	dNTPs (2 mM)
0,5	Each primer (100 pmol/µl)
9,875	ddH2O (Fresenius)

Table 14: PCR programs to generate knockout cassettes and colony PCR program to verify them.

Temperature (°C)	Time (min:sec)	stage	
PCR-Program pFA6a-NatNT2			
97	4	Initial denaturation	
97	2	Denaturation	
54	0:30	Annealing	10x
68	2:40	Elongation	
97	1	Denaturation	
54	0:30	Annealing	20x
68	3:00	Elongation	
68	15	Final elongation	
4	∞		
PCR-Program pUG73			
95	5	Initial denaturation	
95	0:30	Denaturation	
50-55	0:30	Annealing	35x
72	3	Elongation	
72	5	Final elongation	
4	∞		
PCR-Program colony			
98°C	0:30	Initial denaturation	
98°C	0:10	Denaturation	
60°C	0:30	Annealing	35x
72°C	1	Elongation	
72°C	2	Final elongation	
4°C	∞		

2.11 Cell count measurement (CASY)

For the determination of cell counts with the CASY cell counter system, CASYton™ (0,9% NaCl; 0,1 mM EDTA) was sterilized by filtration right before use. CASYton™ was stored as a 10x stock.

2.12 Beta -Galactosidase Assay

All solution and buffers used during β -Galactosidase assay are listed in table 13

Table 15: Solutions and components used during this work for B-Galactosidase assay.

Solution	Components
Breaking Buffer	Roche protease inhibitor
	20% Glycerol
	100mM Tris/HCl pH=8.0
	ddH_2O

Z buffer for β-galactosidase assay	60mM Na_2HPO_4 40mM NaH_2PO_4 10mM KCl 1mM $MgSO_4$ 50mM β-mercaptoethanol
ONPG	4mg/mL in Z-Buffer without β-mercaptoethanol
Na_2CO_3	1M
Acid washed glass beads	

3. Methods

3.1 Microbial methods

3.1.1 Preparing the growth media and incubation

For preparing growth media only ddH_2O was used. For synthetic media all ingredients were mixed together, except from amino acid mix, they were prepared and autoclaved separately as a 10x stock added afterwards. Complex full media (YPD, LB) were mixed together before autoclaving. Sterilization was performed at 121°C for 25 minutes.

Yeast strains in liquid culture were incubated at 28°C, *E. coli* at 37°C, both under shaking conditions. Plasmid containing yeast strains were grown in synthetic media lacking histidine or leucin. 100 µg/ml ampicillin was added to LB media for plasmid containing *E. coli*. Agar plates with yeast were incubated for 2-4 days at 28°C, depending on the media composition and the strain. *E. coli* plates were incubated for one day at 37°C. Plates were stored at 4°C for up to two weeks.

3.1.2 Long time storage

Saccharomyces cerevisiae and *E. coli* strains were stored in 2 ml cryotubes at -80°C. For the storage 750 µL ONC were mixed with 750 µL 50% glycerol. Before reusing stored strains, sterile pipette tips were used to inoculate agar plates without thawing the cultures.

3.1.3 *E. coli* XL-1 transformation

Transformation of *E. coli* cells with plasmid DNA was performed after the electroporation protocol. Therefor DNA was purified by dialysis on a swimming nitrocellulose membrane before the electroporation. For the electroporation 40 µl of competent *E. coli* cells were combined with 2-4 µl Plasmid DNA in the cuvettes. The transformation was performed with an Eppendorf® Multiporator system using 2500 V, exhibiting a time constant of at least 4 ms. After the electric shock 1 ml of LB-media was added to the suspension and transformed to an Eppendorf tube. Then incubated at 37°C for 30 minutes to allow gene expression. The cell suspension was plated onto LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C

3.1.4 Yeast knockout generation and plasmid transformation

A protocol by Knop et al. was used for knocking out genes in *S. cerevisiae*. To do so, an ONC was used to inoculate fresh media to a start OD_{600} of 0,2. After reaching a cell density of approximately OD^{600} of 0,6 (5 to 6 hours of incubation) the cells were harvested and washed first with ddH_2O and afterwards with SORB. The cells were resuspended in 360 µl SORB and mixed with 40 µl carrier DNA. The suspension was stored at -80°C in 50 µl aliquots. Thawed cells were used for the transformation and consequently mixed with the desired (linear) DNA (2-6µl of knockout cassette for knockout transformations) and 300µl PEG. The suspension was incubated at room temperature for 30 minutes then 40µl DMSO was added. Afterwards cells were heat-shocked at 42°C for 10 minutes, harvested and resuspended in 50µl ddH2O. The cells were plated on non-selective YPD in the case of NatNT2 knockout cassette, and incubated for one day at 28°C and replica-plated on selective media (YPD agar plates with 100 µg/ml clonnat). For the pUG73 with the Leu-Marker they were already plated on SMD-Leu plates. After two days a number of clones, which could grow on the selective media, were tested for the correct integration of the knockout cassette with a colony PCR. For plasmid transformation the same protocol as well, but only 2 µl Plasmid were used and they were already plated on selective media.

3.1.5 Yeast colony PCR

Yeast knockout and plasmid transformations were controlled by performing colony PCRs. Composition and PCR programs for the yeast colony PCRs are depicted in Tab. 16 and Tab. 17. To verify the right integration of the NatNT2 or LEU2 cassette, a gene-

specific primer forward primer and cassette specific reversed one were used. For colony PCR a special DNA-extraction protocol after was used. One yeast colony was picked from the plate and suspended in a 100µl solution of 200mM LiOAc and 1% SDS. Afterwards incubated for 5 min at 70°C, then 300µl 100% ethanol was added and the whole solution vortexed. Centrifugation for 3 min and full speed. Supernatant was decanted and pellet washed with 70% ethanol. After air-drying Pellets were dissolved in 50 µl Fresenius H_2O and 5 µl used for PCR.

3.1.6 Ethanol precipitation

For ethanol precipitation the volume of the sample was measured and an equal amount of 5M ammonium acetate was added to adjust the salt concentration to a final concentration of 2.0-2.5 M. Afterwards 2-2.5 volumes of 100% EtOH were added at room temperature followed by an incubation of 30 min. After centrifugation at maximum speed for 15min supernatant was removed and 1 ml ice-cold 70% EtOH added to wash the pellet. After centrifugation for 2min at maximum speed, the supernatant was decanted and pellet air-dried. Afterward the pellet was dissolved in 20 µl Fresenius H_2O.

3.1.7 Chronological yeast agings

All chronological yeast agings in this work were performed in 10 ml culture per 100 ml flasks with baffles. The flasks were incubated at 28°C under constant shaking (145 rpm). 5 ml ONC in appropriate media were used to inoculate 10–50 ml media in flasks to a start OD_{600} of 0,05. Doxycycline and rapamycin were added. The flasks were incubated under shaking conditions at 28°C.

3.1.8 Addition of rapamycin and doxycycline

Doxycycline as well as rapamycin was added to the flasks before or right after inoculation. Doxycycline was dissolved in Fresenius H_2O and added in a final concentration of 100 ng/µl. Rapamycin was dissolved in DMSO and added in a final concentration of 40 nM if not stated otherwise, whereas the control flasks got the same amount of DMSO.

3.1.9 Cell counting and survival plating

The survival rate of liquid, chronologically aging yeast culture was determined with a clonogenicity assay. Aliquots of the culture were diluted 1:100 in ddH_2O, further diluted 1:100 in 10 ml sterile (filtrated) CASYton and measured with a CASY (detection limit of 1,5–15 µm). The background signal was kept at least two powers lower than the measured concentration of yeast cells. Two measuring cycles with 200 µl were performed for each sample. 500 cells were plated on YPD plates, based on the cell counts obtained by the CASY measurement. For plating a 1:10,000 dilution of the initial culture was used (out of the same dilution used for the measurement). The plates were incubated at 28°C for two days.

3.1.10 PI staining and flow cytometry

At different time points 30 µl of liquid cell culture were transferred into 96-well FACS plates and centrifuged for 5 min at 4500 rpm. The pellets were resuspended in 100 µl PI solution (in 1x PBS) and incubated for 5 min. Centrifugation was repeated and the pellets resuspended in 100 µl 1x PBS. 30,000 cells were analyzed with the flow cytometer BD LSRII Fortessa, equipped with a high-throughput sampler. Flow rate was set to 2 µl/s and 30 µl sample were analyzed. Mixing volume was set to 50 µl, mixing speed to 180 µl/sec and number of mixes set to 2. Washing was carried out with 400 µL. PI was measured with the PerCP-Cy5.5 channel (excitation wavelength: 488 nm, emission: 695 nm).

3.1.11 Protein extraction and western blots

Unless otherwise stated, 3 OD units of cells were harvested at indicated time points. Protein extraction was performed by resuspending the cell pellet in 150 µl lysis buffer (1.85 M NaOH, 7.5% β-mercaptoethanol), incubation for 10 min on ice, protein precipitation by adding 150 µl 55% trichloroacetic acid for 10 min on ice, centrifugation at 10,000 × g for 10 min at 4°C and solubilization of the resulting pellet in 150 µl FSB (62.5 mM Tris/HCl pH 6.8, 15 mM DTT, 10% glycerol, 2% SDS, 0.002% bromophenol blue) followed by denaturation at 95°C for 5 min, and centrifugation for 2 min at 10,000 × g . For each sample, 0.02 OD units were loaded on 12.5% polyacrylamide/TRIS-Glycine/SDS gels. Proteins were transferred to PVDF membranes (Roth) using CAPS buffer (10 mM CAPS; 10% methanol; pH 11). Membranes were blocked for one hour with TBS (10 mM Tris/ HCl; pH 7.6, 150 mM

NaCl) containing 3% dry, non-fat milk powder (Roth) and then antibody was on the blots over night at 4°C. Detection was carried out using Chemidoc (BioRad) and Clarity™ ECL (BioRad).

3.1.12 Agarose gel electrophoresis

1% agarose gels were used for electrophoresis. Agarose was melted in 1x TAE in a microwave oven. 0.001% ethidium bromide was added to the cooled gel after pouring it into a gel tray, but before polymerization. DNA samples were treated with 6x DNA loading dye before loading onto the gel. Usually a λ/*Eco*RI+*Hin*dIII DNA marker or a 1 kbp ladder was used to determine the length. 0,5x TAE was used as running buffer. The electrophoresis was carried out at 80 V for small and 120 V for large gels, respectively. A UV transilluminator was used for DNA visualization.

3.1.13 Purification of the PCR product

PCR products, if needed, were cleaned with the GeneJet PCR Purification Kit from Thermo Scientific. The manufacturer's protocol was used.

3.1.14 Plasmid isolation

Plasmids from *E. coli* were isolated using the GeneJet Plasmid Miniprep Kit from Thermo Scientific. The manufacturer's protocol was used.

3.1.15 Beta - Galactosidase Assay

5 ml ONC in appropriate media were used to inoculate 10–45 ml media in flasks to a start OD_{600} of 0,05 and adding of doxycycline and rapamycin. The flasks were incubated under shaking conditions at 28°C. After 5-6 hours cells were harvested to a OD_{600} of 1,5 and then resuspended in 300µl breaking buffer (4°C) In a 96-deep-well plate glass beads were added and then the cell suspension was added. That was vortexed for 30 sec and rested for 30 sec on ice 3 times. Afterwards centrifugation on 4°C for 5 min and 4500 rpm. 50 µl were carefully transferred to a new 96 well plate and 150 µl Z-buffer added. The reaction was started by addition of 50 µl ONPG and the reaction monitored at A_{420} (Tecan Photometer, 405/10 nm filter).

For calculation of Miller Units/µg enzyme a Bradford Assay was done. 300 µl Bradford reagent were added in a 96-well plate and 2-5 µl of the cell extracts were added The

A_{595} was measured (Tecan Photometer, 600 nm filter). A protein standard curve was made with BSA to determine the µg/µl protein concentration.

3.2 Fly methods

3.2.1 Food preparation and addition of rapamycin

Food for *Drosophila melanogaster* was prepared according to instructions obtained by the Bloomington center. Only ddH_2O was used for the food and mixed with agar prior to cooking. After an initial boiling phase all substances were added one by one and brought to a full boil. After every step the mixture was kept boiling for 5–10 minutes before adding the next ingredient. Propionic acid and nipagin were added once the food had cooled down to 60°C. Rapamycin was added below 40°C to exclude heat inactivation. Food was poured into vials in a semi-defined way.

3.2.2 Developmental assay

For developmental assay virgin females from a daughterless-GAL4 line (ubiquitous, relatively weak, but early inducer) were collected and crossed with male RNAi lines or control males. The flies were either kept on food without rapamycin or on food with 25 µM rapamycin for 48 h. In the vials 30 virgin females and 15 males were kept together. Then 3 females were transferred into a new vial without rapamycin or with 2 µM rapamycin for 24 h. At this timepoint eggs were counted. After another 24 h larvae were counted. All eggs laid by the females were GAL4/RNAi (GAL4/+ for the control), so they all had RNAi for the targeted gene. Rapamycin was dissolved in ethanol and ethanol was added in the same amount as rapamycin to the control food.

3.3 Statistics

Statistical analysis were performed with Graphpad Prism 7. If not stated otherwise, 2-way ANOVA was used for the analysis and “n” indicates independent experiments. Multiple comparisons were taken account of by applying a post-hoc Bonferroni correction.

4. Preliminary Data and Aim of the project

Preliminary experiments showed that shifting the temperature-sensitive mutant *wbp1-1*, a glycosylation defective yeast model from 25°C to 37°C, leads to an upregulation of autophagy related (ATG) genes, compared to the wildtype at 37°C (Hauptmann *et al*, unpublished data). Further investigations uncovered that upregulation of ATGs might compensate an autophagic blockage rather than mediate autophagic cell death since deletion of ATGs did neither prevent cell death, nor ROS accumulation (Figure 5 A,B) and preliminary data indicated that autophagic flux might be inhibited (data not shown). Consequently, the autophagy inducer rapamycin inhibited cell death and ROS accumulation in protein glycosylation defective cells (Figure 5 C,D) (Moitzi 2012).

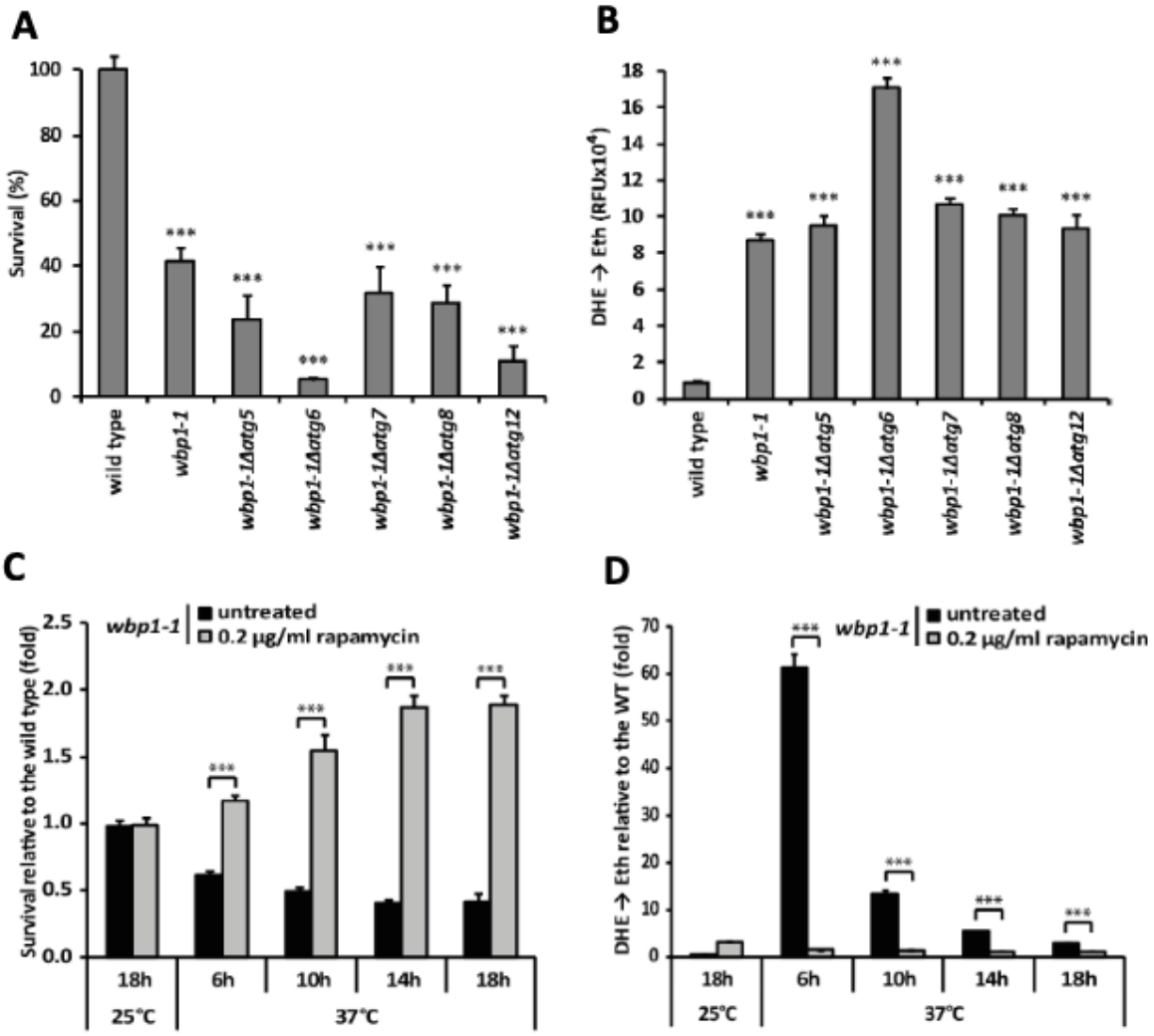


Figure 5: Autophagic cell death does not play a role in N-glycosylation defective yeast cells, but autophagic flux may be blocked. Rapamycin does reinstall the autophagic blockage. A) Survival of wbp1-1 temperature sensitive mutant and several atg knock out strains compared to the wild type strain.

Incubated 14 hours on 37°C B) quantification of ROS accumulation using DHE to Ethidium conversion. Comparison of wildtype, wbp1-1 and atg knockouts after incubation of 14 hours on 37°C. C) survival rate relative to the wild type compared before and after treatment with rapamycin and also shifted from 25°C to 37°C. D) On the same treated cells ROS accumulation was measured by DHE to ethidium conversion. (Figure and Figure legend are from (Moitzi 2012))

Control experiments revealed that the observed autophagic blockage might be caused by the temperature shift to 37°C. This was further substantiated in wildtype cells, which showed an inhibition of vacuolar localization of GFP-Atg8, even after a short (2h) temperature shift (Figure 6). Similar results were obtained in viable yeast deletion mutants of the N-glycosylation pathway, which did not exhibit an apparent autophagy defect (data not shown).

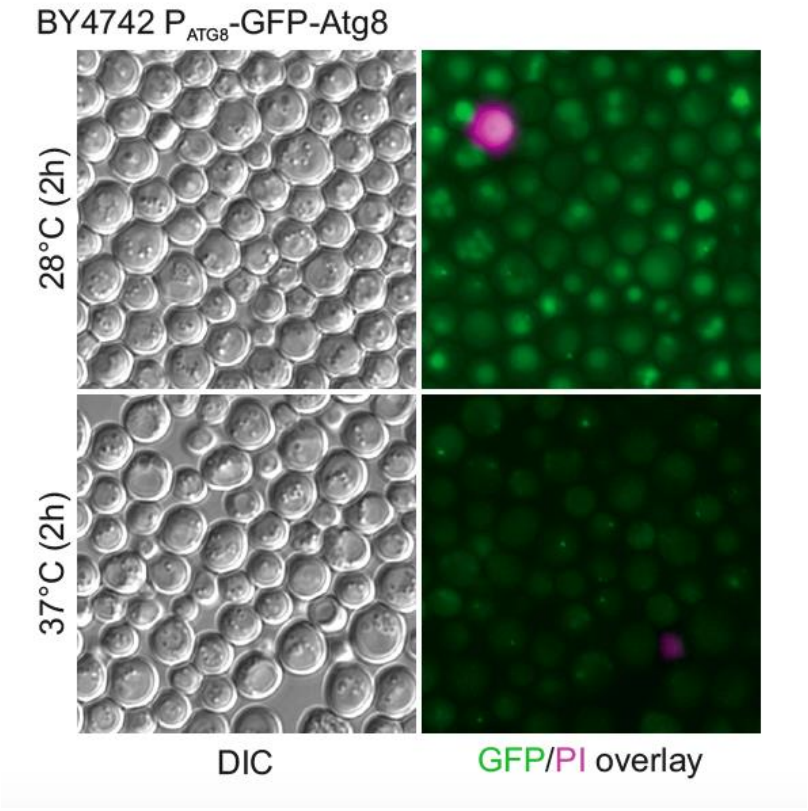


Figure 6: Autophagy in wildtype cells is inhibited at 37°C. Fluorescence microscopy of BY4742 harboring GFP- Atg8 under the control of the endogenous Atg8 promoter, incubated 2 hours at 28°C or 37°C. Propidium iodide (PI) served to stain dead cells.

In light of these results, we decided to investigate the beneficial role of rapamycin treatment in an alternative system of the defective glycosylation yeast-strain. Importantly, we wanted to find out whether autophagy played a role in the improvement of survival by rapamycin, and if not, which other pathways might contribute to rapamycin-mediated rescue. We also hypothesized that the unfolded protein response

(UPR) could be increased due to misfolded or mis-localized proteins. Therefore, we wanted to measure the UPR activity using an beta-galactosidase-based reporter assay. Parallel we tested downstream mediators of TOR (target of rapamycin). Of note, preliminary data suggested that rapamycin did not restore protein glycosylation, as CPY glycosylation remained unaffected by rapamycin treatment (Moitzi 2012). Additionally, we wanted to confirm the beneficial effects of rapamycin in a multicellular CDG disease model.

5. Results

5.1 Rapamycin improves survival in a conditional WBP1 knockdown model

To uncouple the glycosylation defect from a simultaneous heat stress, we decided to use a conditional knockdown system to induce glycosylation defects. Therefore, we employed a tet_{off} system, which allows switching on and off the expression of the protein of interest, in our case Wbp1 protein, using doxycycline. A genomically encoded tetracycline-controlled transactivator (tTA), which consists of the Tet repressor protein (TetR) fused to the C-terminal part of the herpes simplex virus VP16 activation domain, allows transcription from a Tetracycline Element (TRE)-containing promoter (tetO2), placed upstream of the *WBP1* ORF. After Doxycycline gets added it binds to the tTA and the tTA can no longer bind to the TRE and the expression of Wbp1 is blocked (M Gossen and Bujard 1992) (Manfred Gossen, Bonin, and Bujard 1993) (U Baron et al. 1995) (Hillen and Berens 1994) (Udo Baron, Gossen, and Bujard 1997).

To test whether our new generated yeast strain still shows the same phenotype as the temperature-sensitive *wbp1-1* strain, we treated tet_{off}-*WBP1* cells with doxycycline and measured cell death rate via PI (propidium iodide) staining and flow cytometry. As controls, tet_{off} cells, which only harbor the tTA but where WBP1 is under the control of the endogenous promoter ("tet_{off}-WT") were used. PI can enter dead cells that lose their plasma membrane integrity and thus serves as a marker for cell death. Indeed, tet_{off}-*WBP1* cells treated with doxycycline exhibited a significant increase of PI-positive cell populations compared to tet_{off}-WT cells (Figure 7 A). Decreased survival was further corroborated by survival plating (Figure 7 B). Importantly, doxycycline had no effect on survival in tet_{off}-WT cells, ruling out off-target effects. Treatment with rapamycin improved survival in the new glycosylation defective yeast strain (Figure 7 C), similar to the effects observed the temperature-sensitive *wbp1-1* mutant (Moitzi 2012). Interestingly, rapamycin was effective at lower concentrations than observed in the temperature-sensitive strain (5-40 nM, Figure 7 C, compared to ~220 nM, see Figure 5 C).

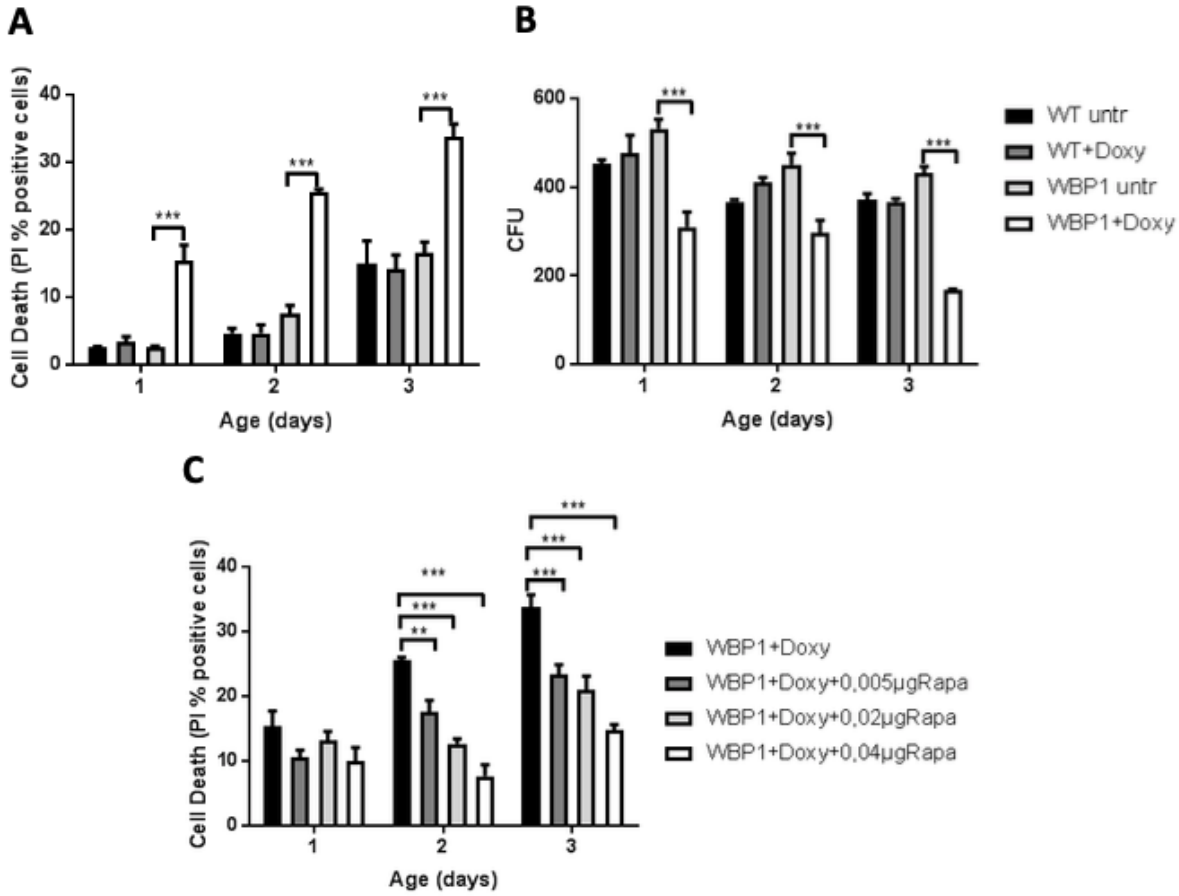


Figure 7: Rapamycin improves survival a conditional glycosylation-defective model. (A-B) tet_{off}-*WBP1* (WBP1) or tet_{off}-WT (WT) cells were inoculated in normal SMD-media (untr.) or media containing 100 ng/ml doxycycline (Doxy) and survival was monitored over three days by PI staining and flow cytometry (A) or survival plating (B). **(C)** Cell death as assessed by PI staining and flow cytometry of tet_{off}-*WBP1* mutant cells, treated with doxycycline (DOXY) alone or additionally with different concentration of rapamycin (Rapa). Different concentration of rapamycin are indicated and all in µg/ml. Data represents means ± s.e.m. *n* = 3 and **, p<0.01; ***, p<0.001.

5.2 Autophagy is not inhibited in glycosylation-defective tet_{off}-*WBP1* cells

To investigate if autophagy is inhibited in the newly generated tet_{off}-*WBP1* mutant strain, we monitored the cellular localization of a GFP-Atg8p fusion protein. GFP-Atg8 is a typical autophagy marker protein, which is transported to the yeast vacuole via autophagy, where it is cleaved by vacuolar proteases to release free GFP (Bridges et al. 2012a). The amount of free GFP can be determined by immunoblotting and is a measure for Autophagic flux. Notably, we did not observe an inhibition of autophagy in doxycycline-treated tet_{off}-*WBP1* cells and after 48h, the amount of free GFP was even increased compared to untreated controls (Figure 8 A-D). Interestingly, while rapamycin treatment led to an increased autophagy in doxycycline-treated tet_{off}-*WBP1*

cells compared to the same cells without rapamycin treatment after 24h, this effect was less prominent after 48h (Figure 8 A-D). In conclusion, autophagy does seem to be defective upon disrupted N-glycosylation.

Importantly, rapamycin treatment failed to reinstate defective N-glycosylation, as demonstrated by immunoblots with a CPY-antibody. CPY is a well characterized model glycoprotein which contains four N-glycan chains. When the protein is underglycosylated, it leads to the appearance of bands with higher mobility compared to the mature protein (mCPY) (Figure 8 A,B). This argues for a role of TORC1 downstream of the glycosylation defect.

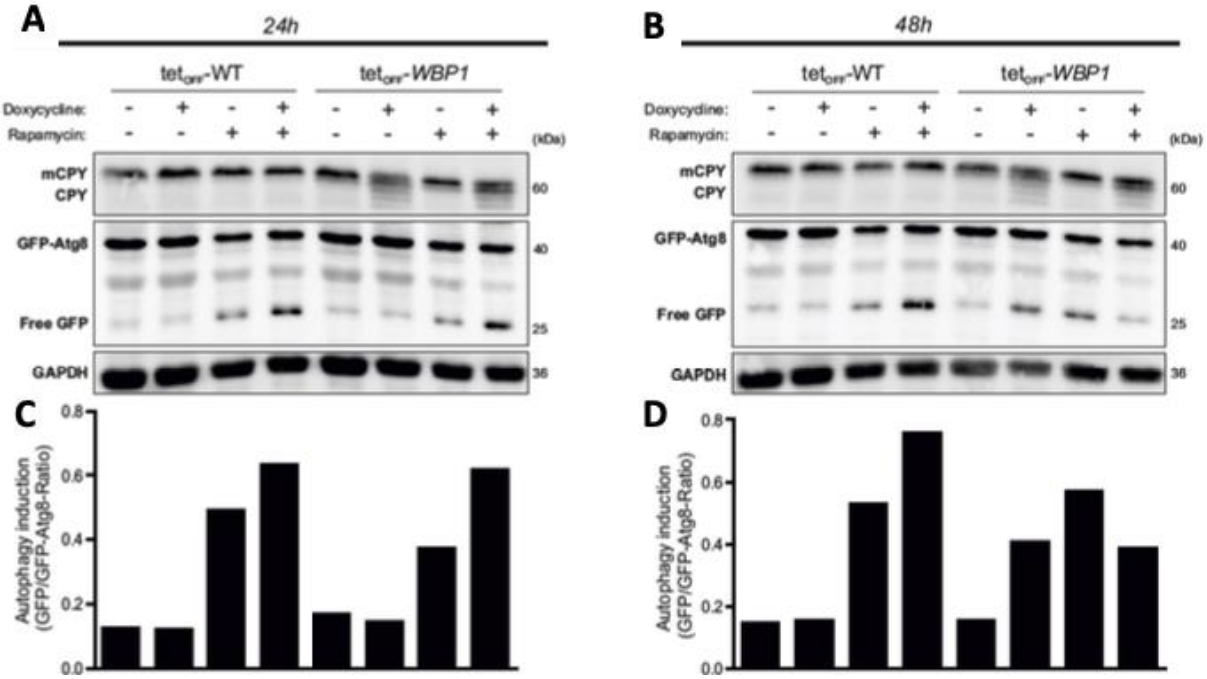


Figure 8: Autophagy is not inhibited in a conditional N-glycosylation-defective model. (A-D) tet_{off}-*WBP1* (WBP1) or tet_{off}-WT (WT) cells harboring GFP-Atg8 under the control of the endogenous Atg8 promoter were inoculated in SC-D media with or without 100 ng/ml doxycycline (Doxy) and rapamycin (40 nM) and autophagic flux was monitored after 24h (A,C) and 48h (B,D) by immunoblot analysis using a GFP antibody. GAPDH served as a loading control. Additionally, a CPY antibody was used to detect glycosylation defects. (C,D) Densitometric quantification of the immunoblot showed in A and B.

5.3 UPR is not increased in glycosylation-defective tet_{off}-*WBP1* cells

Next, we wanted to investigate which other pathways might contribute to rapamycin-mediated rescue. An obvious effect could be the accumulation of misfolded proteins in the ER and the induction of the unfolded protein response (UPR). Of note, prolonged induction of UPR in human cells can lead to cell death, which can be rescued by torin-1 treatment, a specific inhibitor of mTORC1 (Bridges et al. 2012a). We hypothesized

that TORC1 might also act as a mediator of the cell death cascade triggered by prolonged UPR (Figure 9 A).

We measured UPR using a UPR-reporter plasmid, which harbors UPR-responsive elements (UPRE) that control expression of the reporter gene lacZ. Induction of UPR was measured via a β-galactosidase assay, using the UPR-inducer tunicamycin as a control. While tunicamycin treatment strongly increased β-galactosidase expression, could not observe an increase of the UPR in the N-glycosylation-defective tet_{off}-*WBP1* strain compared to the wildtype (Figure 9 B).

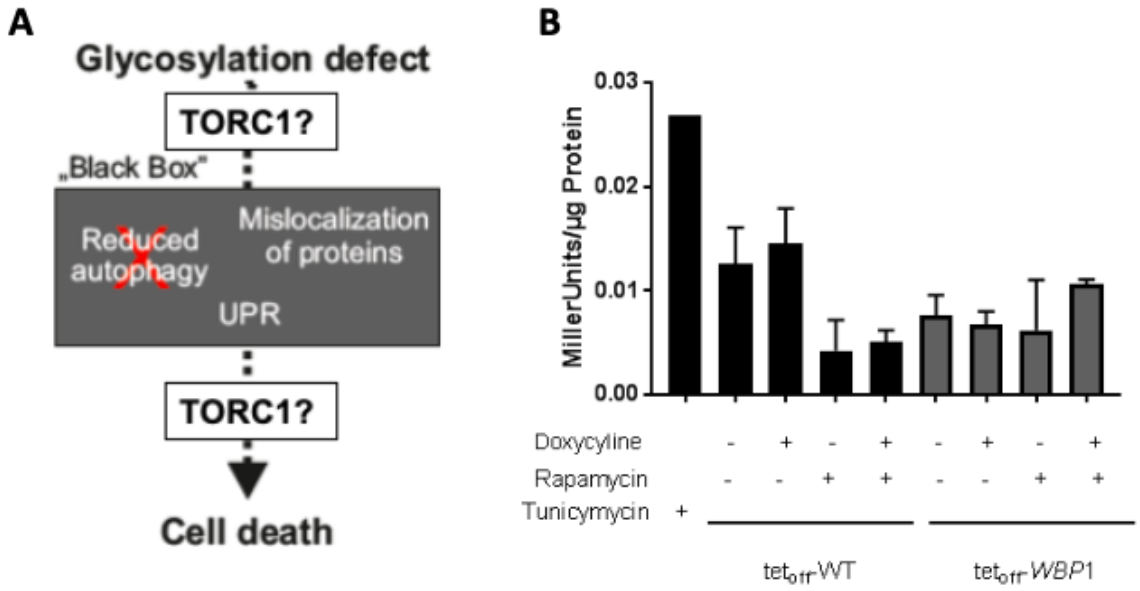


Figure 9: The unfolded protein response is not increased in N-glycosylation defective wbp1. (A) scheme of the involvement of TORC1 and the UPR. **(B)** Calculated Miller Units per µg protein out of the OD_{420} measurements of the β-galactosidase assay and the calculated µg protein from Bradford measurement. As control we used tunicamycin, an ER-stress inducer and commonly used positive control. Data represents means ± s.e.m. *n* = 3.

5.4 *SCH9* deletion in a defective glycosylation model rescues CDG toxicity

Next, we decided to explore the TORC1 signaling network to find downstream mediators of rapamycin (Figure 10). The two major downstream targets of TORC1 are the phosphatase PP2A and yeast homologue of the human serine-6-kinase Sch9. When rapamycin is added, TORC1 is inhibited and Sch9 is no longer phosphorylated and is then inactive too (Urban et al. 2007). PP2A, which is inhibited by active TORC1 and responsible for the regulation of initiation of translational in yeast and is involved in nutrient an growth signaling (Di Como and Arndt 1996) (Jiang and Broach 1999). Hence, we generated knockout strains of both SCH9 and the redundant PP2A subunits

PPH21 and *PPH22* in the background of the N-glycosylation-defective tet$_{off}$-*WBP1* strain as well as in tet$_{off}$-WT cells.

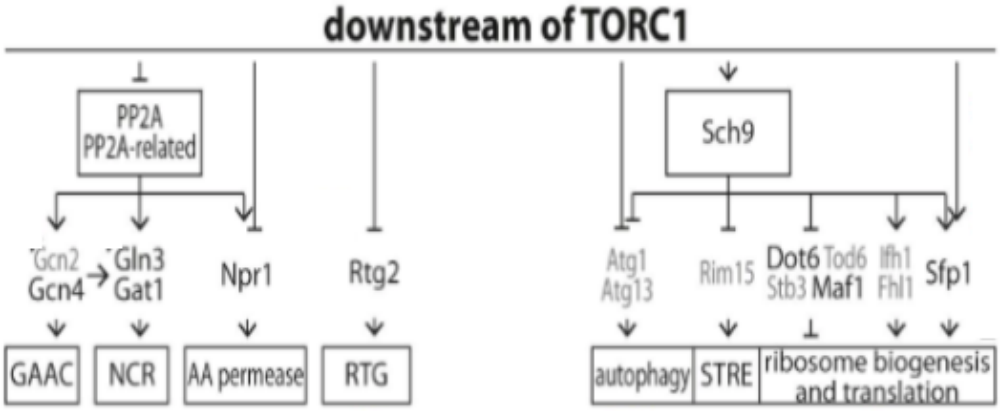


Figure 10: Scheme of the downstream mediators of TORC1. Parts of the downstream network of TORC1 are presented. Arrows indicate the targets activated via the active TORC1 (Figure and figure legend by (Mülleder et al. 2016)**.**

Again, we monitored the cell death rate via PI-staining and flow cytometry as well as glycosylation defects via CPY immunoblotting. Intriguingly, we found that in tet$_{off}$-*WBP1* Δ*sch9* deletion strain, doxycycline failed to increase cell death, while CPY underglycosylation could still be observed (Figure 11 A,B).

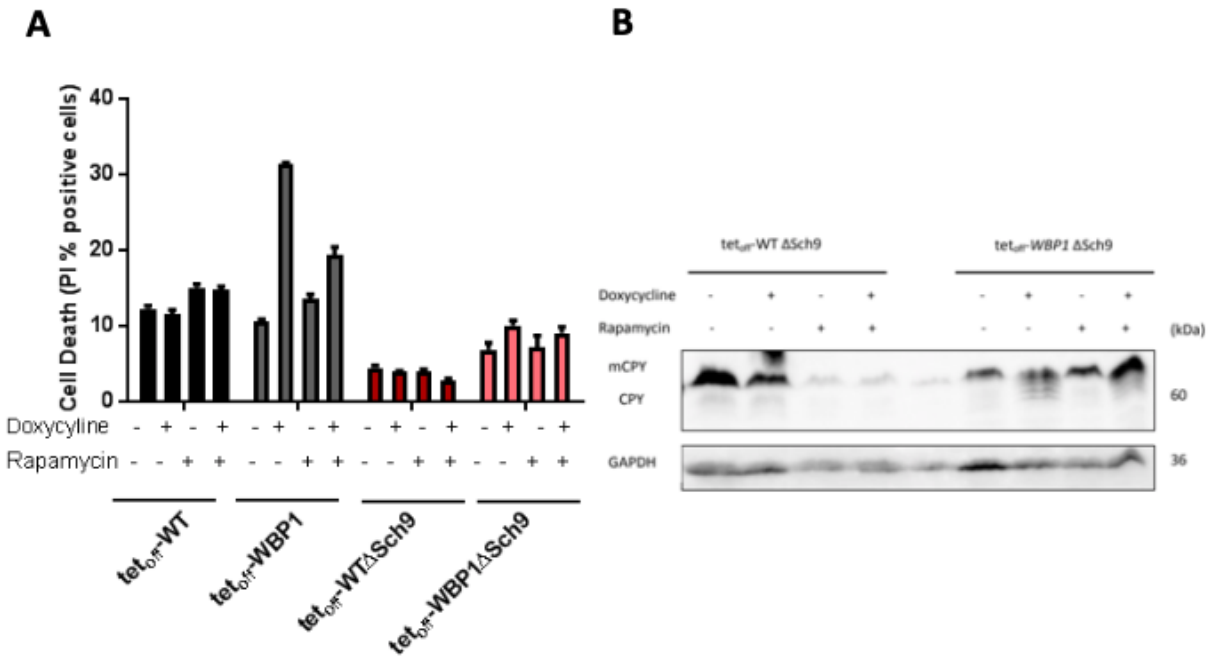


Figure 11: Deletion of the TORC1 target Sch9 rescues CDG toxicity in a glycosylation-defective mutant. (A-B) tet$_{off}$-*WBP1* (WBP1) or tet$_{off}$-WT (WT) cells with or without deletion of *SCH9* were

inoculated in SMD-with or without 100 ng/ml doxycycline (Doxy) and 40 nM rapamycin. The CDG phenotype was assessed by measuring survival 72h after inoculation using PI staining and flow cytometry (A) and immunoblotting of CPY (B) using GAPDH as loading control. (+ = treated, - = untreated) Data represents means ± s.e.m. *n* = 3.

Deletion of PP2A led to increased cell death *per se*, both in the *tet*$_{off}$-*WBP1* and *tet*$_{off}$-WT background (Figure 12 A, compare untreated cells). In these strains doxycycline treatment did not cause further increase of cell death, although the results are difficult to interpret given the high basal cell death rate. In addition, PP2A deletion strains exhibited reduced growth and unusual cellular morphology (data not shown). CPY glycosylation was not altered in PP2A knockout cells (Figure 12 B).

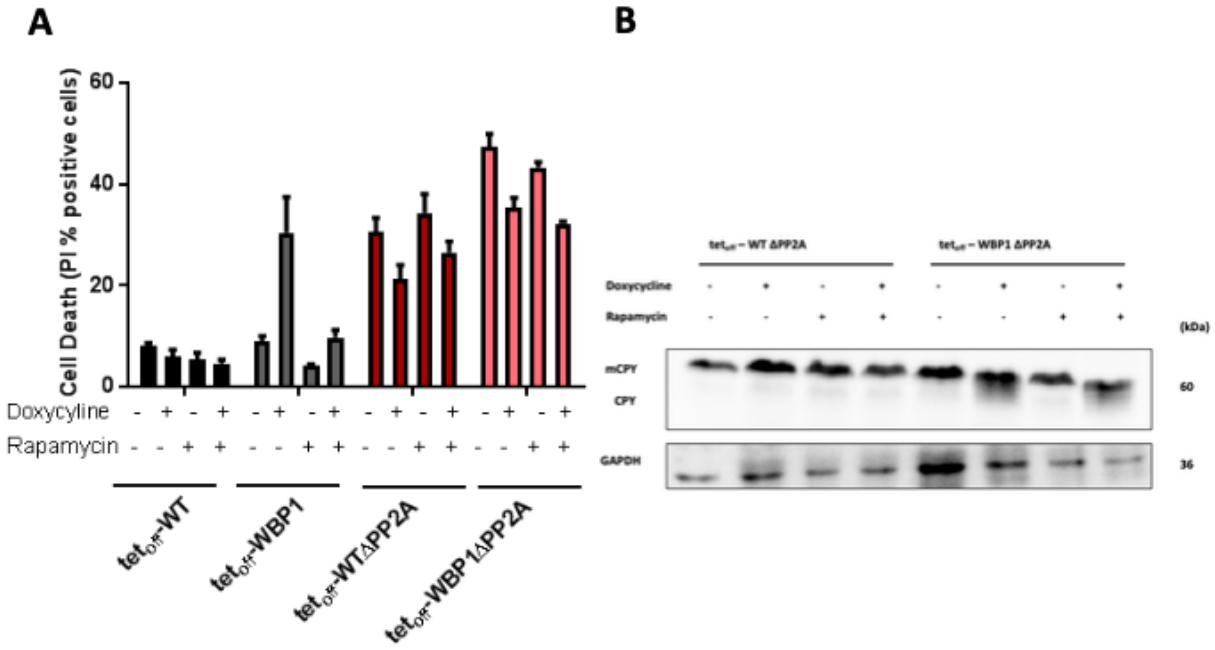


Figure 12: Deletion of PP2A showed slight increase in survival of N-glycosylation defective yeast, but has a decreased survival per se. (A-B) tet$_{off}$-*WBP1* (WBP1) or tet$_{off}$-WT (WT) cells with or without deletion of the PP2A subunits *PPH21* and *PPH22* were inoculated in SMD-with or without 100 ng/ml doxycycline (Doxy) and 40 nM rapamycin. The CDG phenotype was assessed by measuring survival 72h after inoculation using PI staining and flow cytometry (A) and immunoblotting of CPY (B) using GAPDH as loading control. (+ = treated, - = untreated) Data represents means ± s.e.m. *n* = 3.

5.5 The downstream targets of Sch9 have no influence on CDG toxicity

As the Δ*sch9* deletion strain showed such a major effect on CDG toxicity, we wanted to investigate the effects of deletions of downstream targets of Sch9. In detail, we looked for gene deletions, which caused a diminished rescue effect of rapamycin treatment. One downstream target is the protein kinase Rim15, which controls different developmental processes. When TORC1 is inhibited through rapamycin treatment,

Rim15 is phosphorylated and then translocates to the nucleus, where it leads to transcriptional activation. Sch9 negatively regulates Rim15 in the absence of rapamycin (Pedruzzi et al. 2003b). Deletion of *RIM15* caused increased cell death both in the *tet*$_{off}$-WT and *tet*$_{off}$-*WBP1* background (Figure 13 B), in accordance with Rim15's role in stress response (Bridges et al. 2012a). Rapamycin treatment was able to restore survival in the Δ*rim15* mutant as well as in the wildtype cells (Figure 13 B). Moreover, we observed no difference in the underglycosylation status of CPY in the Δ*rim15* deletion strain (Figure 13 D).

Rim15 directly interacts with another major downstream target of Sch9, Msn2/Msn4. Msn2/Msn4 is a transcription factor that activates genes involved in the heat shock and stress response. They are both required for autophagy induction and lifespan extension, upon Sch9 inactivation (Yorimitsu et al. 2007a) (Pedruzzi et al. 2000) (Lin et al. 1998). In addition, PP2A can control the transcriptional activator Msn2/Msn4 through TORC1 signaling (Bohovych et al. 2016). Flow cytometry of PI-stained cells revealed that the Δ*msn2/msn4* deletion strain had a similar phenotype as the Δ*rim15* deletion strain (Figure 13 A), arguing that these stress response factors are not part of the rapamycin-mediated rescuing cascade. CPY glycosylation was only partially affected in Δ*msn2/msn4* cells (Figure 13 C).

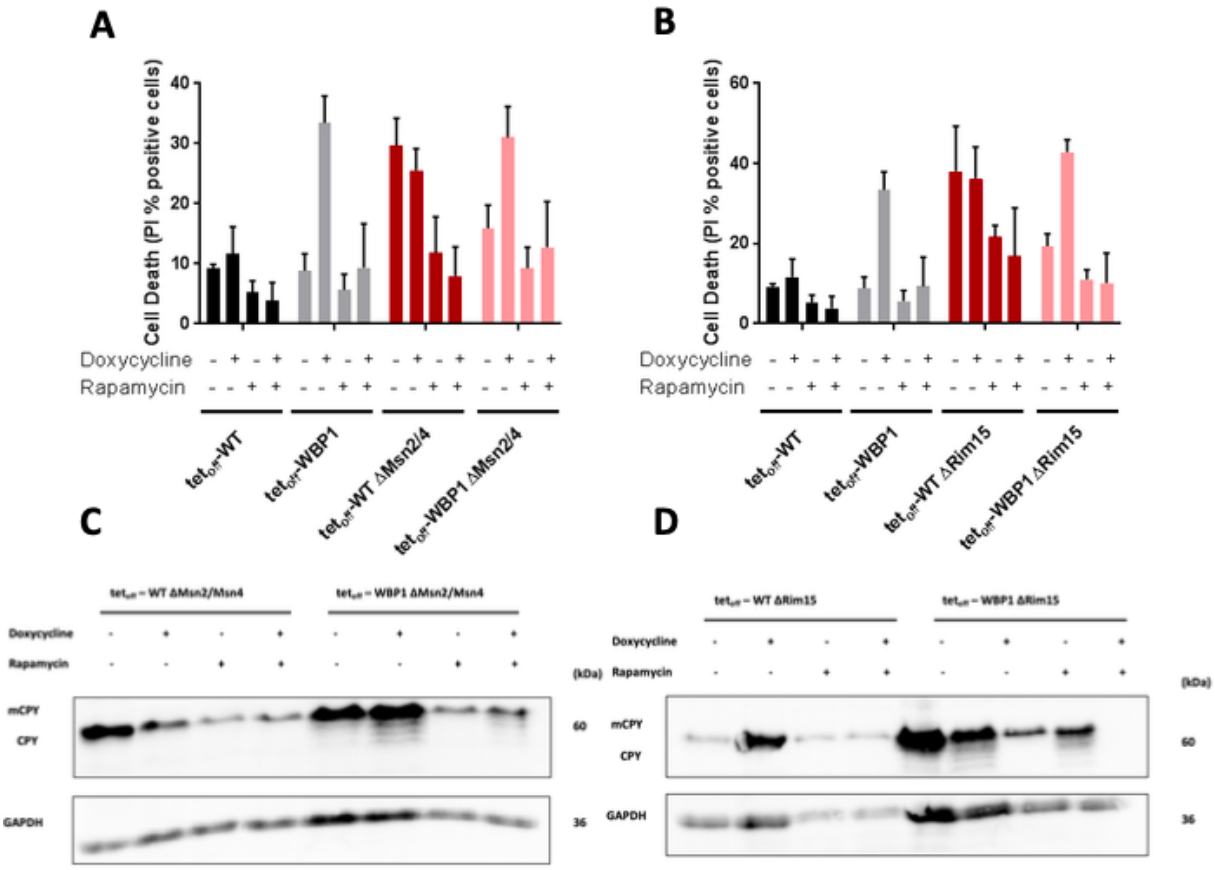


Figure 13: Deletion of Msn2/Msn4 and Rim15 have no influence on CDG toxicity and decrease survival in the wildtype *per se*. (A-B) Flow cytometry of PI stained tet$_{off}$-*WBP1* mutant cells with or without deletion of *MSN2/4* (A) or *RIM15* (B) and respective controls. Cells were incubated with 100 ng/ml doxycycline and 40 nM rapamycin for 72h respectively (+ = treated, - = untreated). **(C-D)** immunoblot of the *MSN2/MSN4* (C) and *RIM15* (D) deletion strain in the glycosylation defective background and wildtype background using CPY antibody and GAPDH as loading control. Data represents means ± s.e.m. *n* = 3.

Because Rim15 and Msn2/Msn4 deletion showed no evidence to be involved in rapamycin-mediated rescue, downstream of Sch9, we decided to explore other genes involved in Sch9 signaling. Two important transcription factors regulated by Sch9 are the forkhead transcription factor Hcm1 and the transcriptional repressor Maf1. Under nutrient-rich conditions, Sch9 phosphorylates Hcm1, which inhibits its nuclear import. Hcm1 gets activated when glucose levels are low, but not completely consumed, which indicates that this transcription factor might mediate an early response to changing environmental conditions There is also possible crosstalk between Hcm1 and Msn2/Msn4 via Rim15 (e.g. overexpression of Hcm1 leads to upregulation *RIM15* and *MSN2/MSN4*) (Rodríguez-Colman et al. 2013). In a Δ*hcm1* deletion strain, knockdown of *WBP1* still showed cell death rates comparable to *WBP1*-depleted control cells. In

addition, rapamycin was still able to completely abolish the toxic effects in Δ*hcm1* cells as assessed by PI staining and flow cytometry (Figure 14 A). Furthermore, deletion of HCM1 did not affect CPY glycosylation patterns (Figure 14 C).

Maf1 is a transcriptional regulator, which is activated upon nutrient deprivation or stress conditions by dephosphorylation and enters the nucleolus to repress polymerase III, which drives tRNA synthesis. When nutrients become available, TORC1 signaling leads to Maf1 phosphorylation and thus inactivation, and polymerase III can form the initiation complex (Huber et al. 2009) (Roberts et al. 2006). Deletion of *MAF1* showed similar effects as deletion of *HCM1*, namely toxicity upon *WBP1* knockdown, but no influence on the rescue effect of rapamycin (Figure 14 B). Again, CPY glycosylation remained unaffected (Figure 14 D).

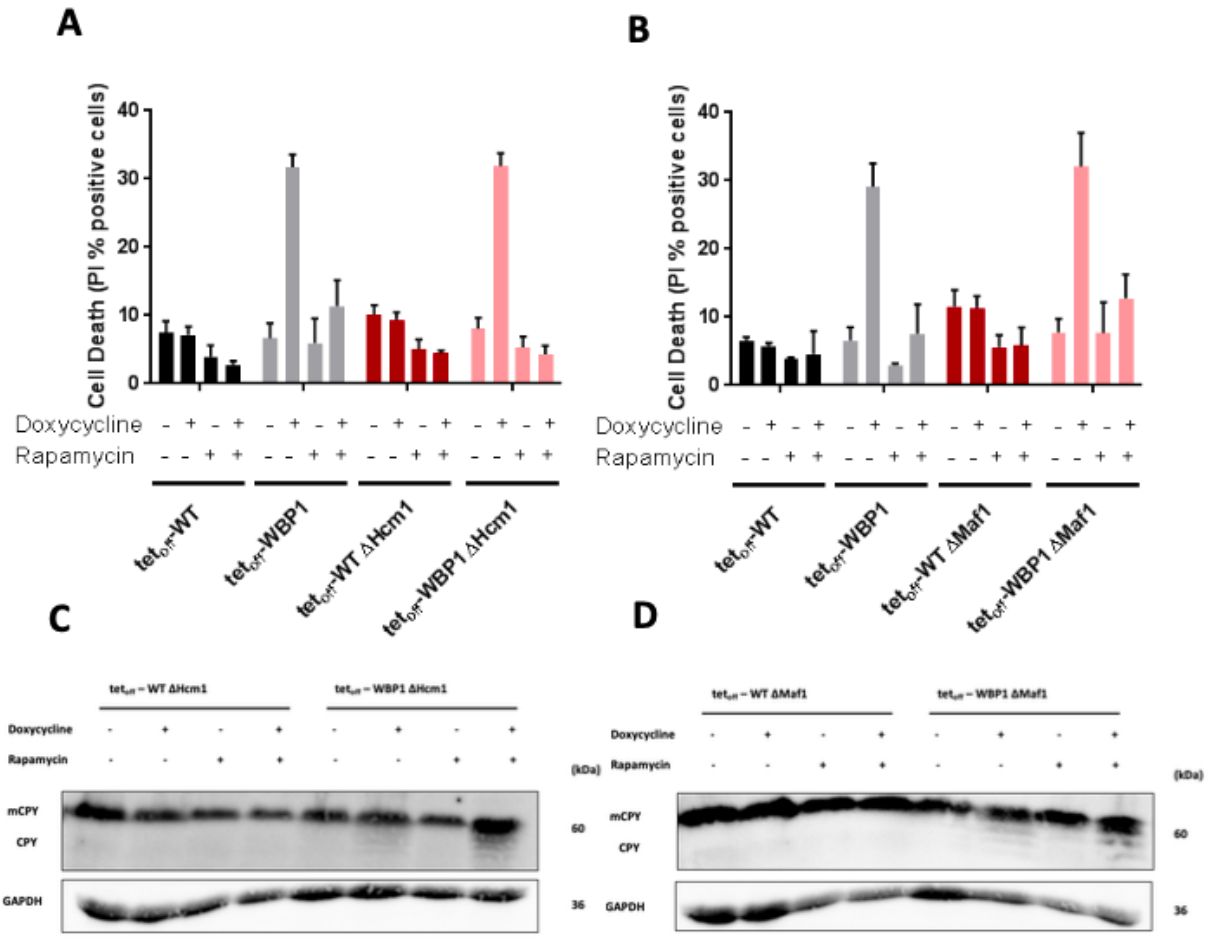


Figure 14: Deletion of *HCM1* and *MAF1* have no influence on CDG toxicity. (A-B) Cell death rate measured by PI-staining of tet_{off}-*WBP1* mutant cells with or without deletion of *HCM1* (A) or *MAF1* (B) and respective controls via flow cytometry. Cells were incubated 3 days with 100 ng/ml doxycycline and 40 nM rapamycin respectively (+ = treated, - = untreated). **(C-D)** Immunoblot of *HCM1* (C) and *MAF1* (D) deletion strains in the glycosylation defective and wildtype strain using CPY antibody and GAPDH as loading control. Data represents means ± s.e.m. *n* = 3.

Dot6 another downstream target of Sch9 and is one of the regulators of ribosome biogenesis. Upon Sch9 inhibition, Dot6 is hypophosphorylated and together with Tod6 and Stb3, Dot6 recruits a histone deacetylase to promoters for ribosomal biogenesis, leading to reduced transcription (Oh et al. 2018). We generated a *DOT6* deletion in the N-glycosylation defective background but did not observe a difference between Δ*dot6* and wildtype cells, neither upon *WBP1* knockdown, nor upon rapamycin treatment (Figure 15 A). Cells devoid of DOT6 did not exhibit altered CPY glycosylation pattern as determined by immunoblotting (Figure 15 B).

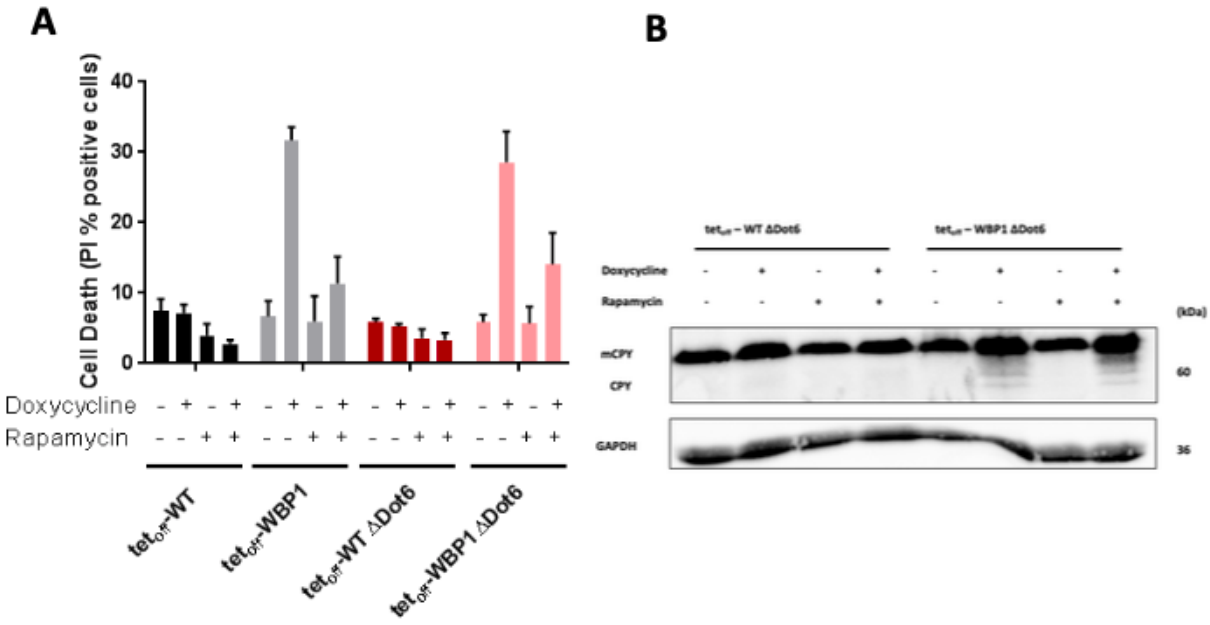


Figure 15: **Deletion of *DOT6* has no influence on CDG toxicity. (A)** Flow cytometry of PI-stained tet_{off}-*WBP1* mutant cells with or without deletion of *DOT6* and respective controls. Cells were treated with 100 ng/ml doxycycline and 40 nM rapamycin for 72h respectively (+ = treated, - = untreated). **(B)** Immunoblot of the *DOT6* deletion strain using CPY antibody and GAPDH as loading control. Data represents means ± s.e.m. *n* = 3.

5.6 Deletion of TORC1 targets ATG1 and RTG2 has differential effects on CDG toxicity

Since deletion of Sch9 downstream targets showed no effect on CDG toxicity, we decided to track down other proteins involved in TORC1 signaling. One major target is the autophagy-related protein Atg1, which is regulated by TORC1 directly, but also by Sch9 (Bridges et al. 2012a). Atg1 is a serine/threonine protein kinase, which activates autophagy initiation when TORC1 is inhibited (e.g. nutrient deprivation or rapamycin treatment). Atg1 activity depends on the phosphorylation status of its interaction

partner Atg13, which itself is a target of TORC1. Upon nutrient deprivation, Atg13 is no longer hyperphosphorylated and can bind to Atg1 which further can develop its full activity together with Atg13 (Oh et al. 2018). In glycosylation-defective cells, deletion of ATG1 did not exacerbate toxicity (Figure 16 A). However, rapamycin treatment was less effective in restoring viability in ∆atg1 cells as compared to cells with intact ATG1 (Figure 16 A), indicating that at least some of the positive effects of rapamycin treatment might require intact autophagy signaling. CPY glycosylation was not affected by deletion of ATG1 (Figure 16 C).

Another target of TORC1 is Rtg2, which is required for mitochondrial to nucleus ("retrograde response") signaling. Rtg2 promotes metabolic remodeling (Liao and Butow 1993). Similar to PP2A inactivation, RTG2 deletion caused increased cell death in wildtype cells as well as in N-glycosylation-defective cells per se (Figure 16 B). This observation is in line with the known role of Rtg2 as a longevity mediator (Liu et al. 2003). While rapamycin treatment recovered the cell death rate to normal levels both in N-glycosylation-competent and –deficient cells, knockdown of WBP1 alone did not cause further toxicity in ∆rtg2 cells (Figure 16 B). Deletion of RTG2 did not affect CPY glycosylation patterns (Figure 16 D).

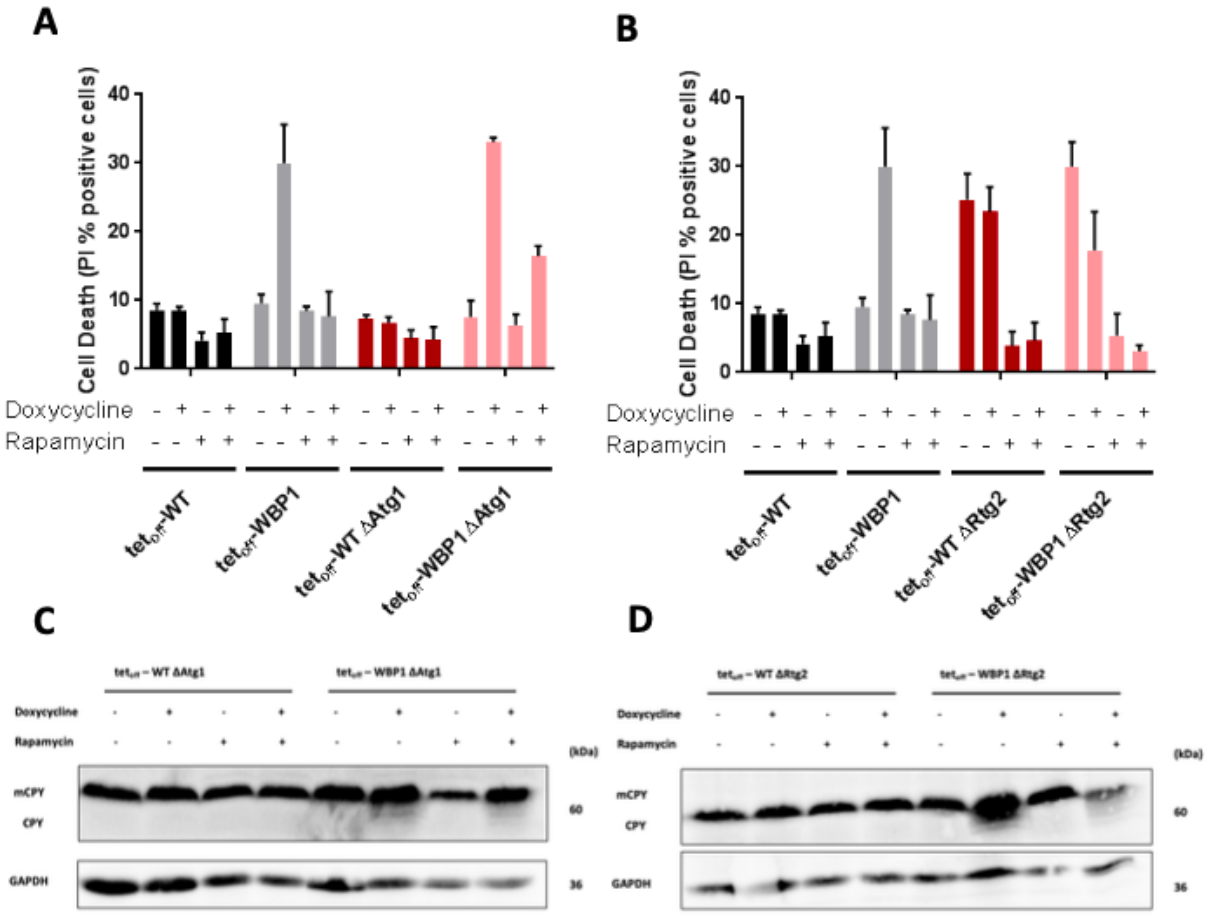


Figure 16: Deletion of ATG1 and RTG2 affect CDG toxicity differentially. (A-B) Flow cytometry of PI-stained tet$_{off}$-*WBP1* mutant cells with or without deletion of *ATG1* (A) or *RTG2* (B) and respective controls. Cells were treated with 100 ng/ml doxycycline and 40 nM rapamycin for 72h respectively (+ = treated, - = untreated). **(C-D)** Immunoblots of the *ATG1* (C) or *RTG2* (D) deletion strains using CPY antibody and GAPDH as loading control. Data represents means ± s.e.m. *n* = 3.

5.7 Rapamycin has toxic effects in an *ATG6* deletion strain

As Atg1 seemed to play a role in rapamycin-mediated rescue, we decided to test a knockout of *ATG6/VPS30*, which is the yeast homolog of the mammalian phosphatidylinositol-3 kinase, Beclin-1. Previous findings implicated ATG6 in rapamycin-mediated rescue of N-glycosylation defects (Moitzi 2012). Of note, Atg6 It is both involved in the autophagy process and vacuolar protein sorting. Mutations or deletions of the *ATG6* gene lead to sensitivity to agents that induce UPR and defects in autophagy, vacuolar protein sorting, ERAD and retrograde transport from the endosome to the Golgi (Ohsumi 1999) (Patricie Burda et al. 2002). In our model, deletion of *ATG6* rendered cells sensitive to defective N-glycosylation, although to a lesser extent than wildtype cells (Figure 17 A). Intriguingly, rapamycin did not reduce

cell death in Δ*atg6* cells but even increased PI-positive cell populations both with and without knockdown of *WBP1* (Figure 17 A). CPY glycosylation remained unaffected by deletion of *ATG6* (Figure 17 B).

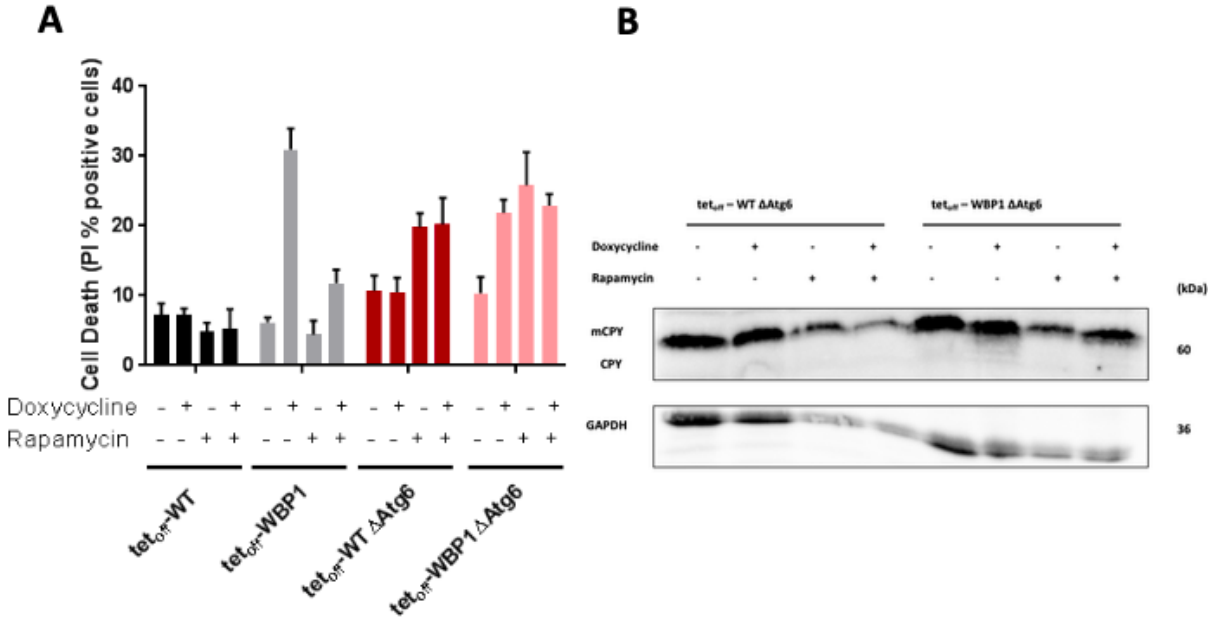


Figure 17: Cells devoid of Atg6 still display CDG toxicity, but are sensitive to rapamycin treatment. (A) Flow cytometry of PI-stained tet_{off}-*WBP1* mutant cells with or without deletion of *ATG6* and respective controls. Cells were treated with 100 ng/ml doxycycline and 40 nM rapamycin for 72h respectively (+ = treated, - = untreated). **(B)** Immunoblot of the *ATG6* deletion strain using CPY antibody and GAPDH as loading control. Data represents means ± s.e.m. *n* = 3.

5.8 Rapamycin reduces toxicity of Alg6 knockdown in *Drosophila melanogaster*, but not in Pmm2 and Cog7 knockdown flies

Finally, we decided to test rapamycin's beneficial effects in a multicellular organism, the fruit fly *Drosophila melanogaster*. Therefore, we treated glycosylation-defective *daughterless* (da)-Gal4 x Alg6RNAi, Cog7RNAi and Pmm2RNAi flies, which were isogenized to w^{1118} flies for 6 generations with 2 µM rapamycin (see Methods for details on the feeding regimen). Of note, the da-Gal4 driver line drives expression in early stages of embryonal development and therefore is well suited as a model for CDG effects in humans. As a readout, we counted the eggs, larvae that developed from those eggs and additionally the number of pupae. We compared those to control (w^{1118} flies) and to untreated glycosylation-defective flies. Indeed, in (da)-Gal4 x Alg6RNAi flies, rapamycin feeding led to a significant higher amount of eggs and larvae for treated flies compared to the untreated controls ($p<0.05$ for eggs and $p<0.01$ for larvae), and an almost significant higher number of pupae ($p=0.081$) (Figure 18 A-C).

In Cog7RNAi flies, we could not observe any difference between the treated and the untreated flies. The Pmm2 knockdown flies showed a tremendous retardation in development and were not able to eclose (Figure 18 A-B).

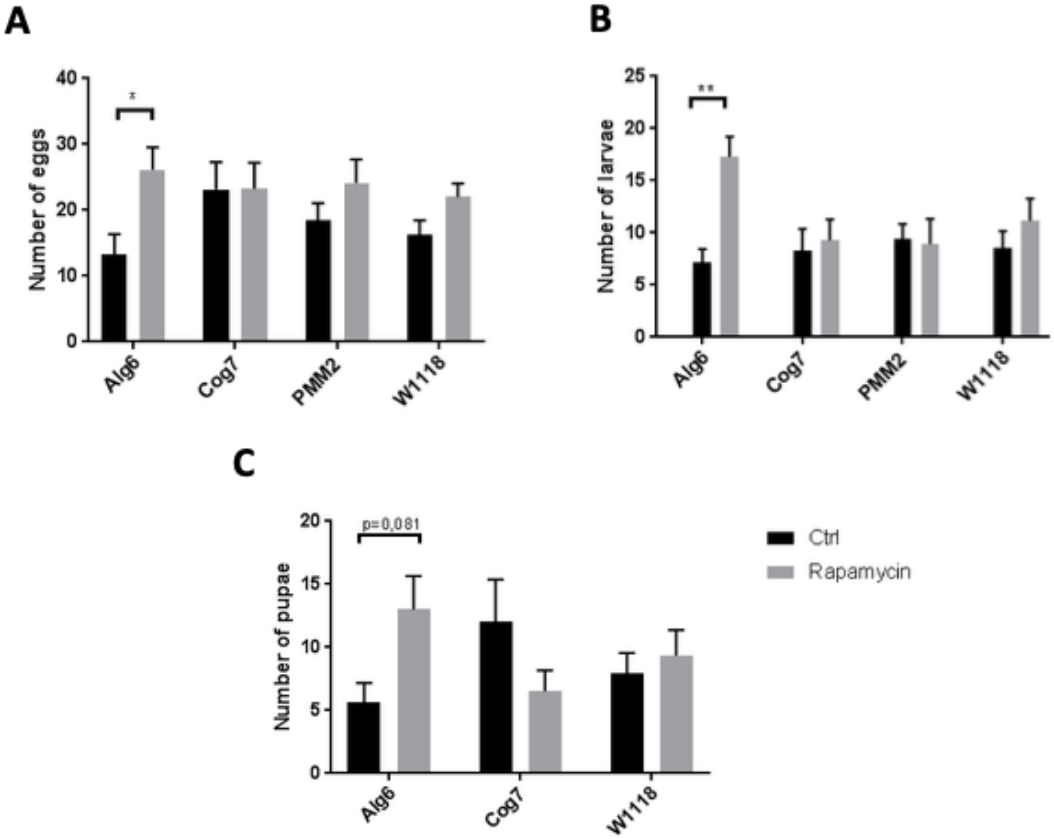


Figure 18: Rapamycin treatment rescues the toxic effects of Alg6 knockdown in *Drosophila melanogaster*. (A-C) Developmental assay of male RNAi and control lines crossed with female da-Gal4 driver on food with or without 2 µM rapamycin for 24h. Flies were kept under constant 25°C and 70% humidity. (A) number of eggs laid on the food after 24 hours incubation of 3 fertilized female flies on Bloomington standard food with or without 2 µM rapamycin (Ctrl). (B) Number of larvae developed out of the eggs before and (C) number of those larvae which eclosed to pupae. See Methods for details on the experimental procedure. Additional data is shown in Supplementary figure 1 and Supplementary figure 2.

6. Discussion

Congenital disorders of glycosylation are a fatal and rare disease. Since it was first discovered in the 1980 more than 100 different types of glycosylation defects in different glycosylation pathways have been discovered. As of today, only three of them can be treated. Depending on the CDG-type the symptoms can be very diverse, but typically include severe malfunction of different organ system that effect the entire body. Especially the peripheral and central nervous system are affected and often CDG end in early death.

Rapamycin, the autophagy inducer and inhibitor of TOR, has repeatedly been shown to extend lifespan in several experimental models, although those effects have not been explored in humans, yet. Rapamycin is already a licensed drug, used for immunosuppression after organ transplantations. There beneficial effects have to be set against the side effects. (Li, Kim, and Blenis 2014)

Given the diversity of the disease it is difficult to find the right model organism to work with. With *Saccharomyces cerevisiae*, an established model organism with an N-glycosylation machinery comparable to human glycosylation pathway and *Drosophila melanogaster*, the fruit fly where one can examine development, we wanted to validate previous findings on the beneficial effects of rapamycin on CDG toxicity and further identify cellular key players.

6.1 Sch9 seems to play a tremendous role in rescue of CDG toxicity

Our initial findings, using a yeast model with conditional knockdown of the OST subunit *WBP1*, challenged the involvement of autophagy in the pathology of CDG. First, autophagy was not reduced upon defective N-glycosylation but rather increased (Figure 8), which arguably reflects a cellular repair mechanism in response to misfolded proteins. Interestingly, others have suggested that tunicamycin treatment, which also interferes with N-glycosylation, induces autophagy. Moreover, transcriptome analyses revealed that *ATG* genes are upregulated in temperature-sensitive *wbp1-1* cells at 37°C (Moitzi, 2012). Our control experiments demonstrated that autophagic flux is inhibited at 37°C (Figure 6). Thus, it is possible that the upregulation of ATG genes reflects a cellular attempt to overcome a temperature-mediated blockage of autophagic flux.

Second, one of the suggested mediators of rapamycin-induced rescue, the autophagy-related protein Atg6/Vps30 (Moitzi, 2012), can be part of two protein complexes, (i)

complex I with Atg14, Atg38, Vps34, and Vps15, which is essential for autophagosome formation and (ii) complex II, with Vps38, Vps15 and Vps34, which regulated vacuolar protein sorting (Backer 2008). Deletion of the Atg6 interactor Vps34 has been shown to strongly inhibit TORC1, therefore deletion of Atg6 might interfere with TORC1 activation and thus recapitulate the effects of rapamycin, even if the autophagic machinery is compromised (Bridges et al. 2012a).

To understand the mechanistic network of rapamycin-mediated amelioration of CDG toxicity, we decided to investigate downstream targets of TORC1. Our data demonstrate that rapamycin treatment does not abolish the N-glycosylation defect, caused by *WBP1* knockdown (Figure 8). Therefore, we concluded that TORC1 inhibition must cause effects downstream of defective N-glycosylation, for example by inhibiting a death signal or by reinstating defective protein sorting.

Given the central role of Sch9 as a TORC1 effector kinase, we generated an *SCH9* deletion in the conditional N-glycosylation defective strains to investigate whether Sch9 was involved in CDG toxicity. Indeed, Sch9 deletion improved lifespan overall (Figure 11 A), similar to previous reports that demonstrated a longevity phenotype upon *SCH9* disruption (Yorimitsu et al. 2007b). It should be noted though, that it is still unclear whether Sch9 actually only acts downstream of TORC1 or might also regulate TORC1 activity. There have been several studies that have shown that Sch9 could be both: a downstream target of TORC1 and an individual target for rapamycin (Smets et al. 2008).

Given the range of targets of Sch9, we hypothesized, that if Sch9 is involved in CDG toxicity and rapamycin-mediated rescue, it was likely that another downstream target of Sch9 might be involved. Hence, we generated deletions of Maf1, Hcm1, Dot6, Rim15 and Msn2/Msn4 in the N-glycosylation defective mutant. *MAF1*, *HCM1* and *DOT6* knockout all displayed a similar phenotype, namely decreased survival upon *WBP1* knockdown, and improved survival upon concomitant rapamycin treatment.

Those three proteins regulate different downstream pathways: Maf1 is a major regulator of Polymerase III and represses its activation under a variety of conditions. It gets phosphorylated by Sch9 and its phosphorylation status also controls its subcellular localization (e.g. in the nucleus). *MAF1* deletion has influences on the lipid homeostasis and glucose metabolism (Pluta et al. 2001) (S. Zhang et al. 2018) (Mierzejewska and Chreptowicz 2016).

Dot6 is involved in ribosome biogenesis signaling. together with Tod6. Both are regulated through Sch9. Dot6 phosphorylation causes repression of RNA-Polymerases I and III. As a result, the transcription of ribosomal genes are blocked, which impacts cell size and protein synthesis (Huber et al. 2011) (Lippman and Broach 2009). Since neither Maf1, nor Dot6 seemed to play a role in rapamycin-mediated rescue of CDG toxicity, the involvement of ribosomal biogenesis can most likely be ruled out.

Hcm1 is part of the forkhead family of transcription factors in eukaryotes and involved in Rim15 and Msn2/Msn4-related stress signaling via Sch9 (Rodríguez-Colman et al. 2013). It regulates genes that are involved in chromosome organization, spindle dynamics and budding. Hcm1 is also involved in adaption to stress conditions in a nutrient-dependent manner. When Sch9 is active Hcm1 is localized in the cytosol, when Sch9 is blocked, translocation of Hcm1 into the nucleus increases and the transcription switches to stress response (Rodríguez-Colman et al. 2013) (Swinnen et al. 2014). Rim15 and Msn2/Msn4 regulate similar downstream targets. Like Hcm1, they activate the stress response in an Sch9-dependent fashion. Msn2/Msn4 reacts to environmental changes such as temperature shifts and can induce expression of heat shock genes. Our data showed that deletion of these transcription factors lead to decreased survival, both in the tet_{off}-WT and tet_{off}-*WBP1* strain background (Figure 13 A). The same phenotype we could observe for deletion of *RIM15* (Figure 13 B), which is involved in stress response as well (Yorimitsu et al. 2007b) (Kaeberlein et al. 2005). Msn2/Msn4 can also be regulated by the phosphatase PP2A. PP2A, which has two catalytical subunits (Pph21 and Pph22). can regulate stress response by Msn2/Msn4 activation (Reiter et al. 2013). PP2A is particularly important in nitrogen signaling and is activated during starvation. Deletions of both catalytical subunits reportedly lead to decreased survival (Georis et al. 2011), which we could observe in our deletion strains as well (Figure 12). Surprisingly, in PP2A-deficient cells, we observed a slight increase in survival upon *WBP1* knockdown, although this effect was only apparent on day one. As PP2A had severe effects both on cell growth and morphology, we did not pursue that in detail. Pharmacological inhibition or conditional knockdown of PP2A might be better suited to examine PP2A's role in rapamycin-mediated rescue of CDG toxicity.

6.2 UPR is not upregulated upon *WBP1* depletion

The UPR is activated during ER-stress and/or through mislocalized or unfolded proteins. Interestingly crosstalk to TORC1 signaling has been suggested, although there is a lack of precise mechanistic insight (Appenzeller-Herzog and Hall 2012). In addition, prolonged UPR has been suggested to induce PCD (Fribley, Zhang, and Kaufman 2009) (Senft and Ronai 2015). Upon *WBP1* knockdown, underglycosylated (and probably mislocalized) proteins accumulate, as apparent by underglycosylated CPY (Figure 8 A,B). Consequently, we wanted to address if *WBP1* knockdown led to increased UPR signaling. We performed a beta-galactosidase assay using a UPRE-lacZ reporter construct, but could not detect any activation in the doxycycline-treated *tet$_{off}$-WBP1* strain compared to *tet$_{off}$*-WT cells (Figure 9 B). There are three possible explanations: (i) UPR activation in our system might have more complex kinetics than expected, meaning that UPR might be activated transiently and then repressed again. Since we did not observe any increase in lacZ expression, it is unlikely though that *WBP1*-depleted cells suffer from prolonged activation of UPR. (ii) In contrast to tunicamycin-induced glycosylation defects, disruption of “late” N-glycosylation (Wbp1 mediates the final step of glycan transfer to proteins) does not activate UPR. (iii) Lack of *WBP1* might lead to ER-stress but might also interfere with UPR signaling, ultimately leading to cell death, because the cell fails to respond to the accumulation of misfolded proteins. During ER-stress Ire1, a transmembrane kinase, is activated and activates the (non-canonical) cytosolic splicing of *HAC1* mRNA. Hac1 is a transcriptional activator and binds directly to the UPRE. To test this hypothesis, we attempted to reinstate UPR by introducing Hac1p^{i}, the spliced form of Hac1, which activates UPRE-controlled genes without preceding Ire1 activation (J S Cox and Walter 1996). However, these experiments did not produce any conclusive results (data not shown) and we failed to validate expression of the constructs (courtesy of Peter Walter). Nevertheless, our results suggest that CDG toxicity may be connected to impaired UPR signaling, and this hypothesis is worth exploring in future studies.

6.3 Autophagy is only partially involved in rapamycin-mediated rescue

In a previous study it was shown that rapamycin improves lifespan in N-glycosylation defective yeast and is has been suggested that rapamycin treatment reinstalls the autophagic flux in an Atg6-dependent manner (Moitzi 2012). Atg6/Vps30, the yeast homolog to Beclin-1, is part of the phosphatidylinositol-3 kinase complex, which is

involved both in autophagic signaling and vacuolar protein sorting (Kihara et al. 2001) (Kametaka et al. 1998). In our hands, CDG toxicity was not accompanied by autophagy inhibition of autophagy (Figure 8). When testing the effect of *ATG6* deletion in the *tet$_{off}$-WBP1* strain, we did not observe a rescue effect by rapamycin, in accordance with the findings by Moitzi (Moitzi, 2012). However, when comparing all control conditions, rapamycin was actually toxic upon *ATG6* deletion (Figure 17 A) This increased sensitivity in Δ*atg6* cells to rapamycin has been observed before (Bridges et al. 2012b). Mutations in Atg6 do not only interfere with autophagy and vacuolar protein sorting, but also lead to lower phosphatidylinositol-3 phosphate levels and increase sensitivity to agents that induce UPR (Kihara et al. 2001) (Patricie Burda et al. 2002). In conclusion, it is unlikely, that Atg6 represents a specific mediator of rapamycin-mediated rescue of CDG toxicity. In fact, Moitzi showed that rapamycin was still able to rescue toxic effects of tunicamycin-induced N-glycosylation defects in other autophagy-deficient strains (Moitzi, 2012). Here, we explored the effects of *ATG1* deletion, which is another downstream target of TORC1 signaling. Although rapamycin improved survival in Δ*atg1* cells, the relative rescuing effect was lower than in wildtype cells (Figure 16 A). In sum, rapamycin-induced autophagy might partially account for the rescue effect, although our data suggest that other factors must be involved as well.

6.4 Rapamycin has beneficial effects in the multicellular organism *Drosophila*

In this work we could demonstrate that rapamycin improves survival in a conditional N-glycosylation-defective *Saccharomyces cerevisiae* strain to wildtype levels. Because of these promising results in yeast, we wanted transfer our findings to a multicellular organism. Moitzi showed that rapamycin improved development in different *Drosophila* strains with deficient N-glycosylation (Moitzi, 2012). In an attempt to validate these findings, we treated selected N-glycosylation defective *Drosophila* lines with rapamycin. A first attempt that strictly followed the suggested protocol (Nadege Minois, personal communication) failed, because the number of eggs was too low to achieve meaningful results (data not shown). When using more females per vial, we observed that rapamycin improved the number of eggs, larvae and pupae in da-Gal4 x Alg6RNAi flies (Figure 18 A-C; Supplementary figure 1 A-D; Supplementary figure 2 A-C). A disruption of Alg6 leads to a defect in the attachment of the first out of three glucose

residues to the LLO precursor. This leads to an accumulation of a nonglycosylated LLO precursor. Patients with an Alg6 mutation show moderate psychomotor retardation, muscular hypotonia, strabismus and seizure (Grünewald et al. 2000). The results were unexpected, since Alg6 was one of the lines, where no positive effect of rapamycin had been observed by Moitzi (Moitzi, 2012).

In Cog7RNAi flies there was no difference between the treated and the untreated ones. In Pmm2 knockdown flies, rapamycin could not reinstate survival since the defect was so bad that larvae did not further develop at all (Figure 18 A-B Supplementary figure 1 A,C Supplementary figure 2 A). The discrepancy to the findings by Moitzi might be explained by slight variations in food composition or differences in other environmental factors, which can influence the expression of the RNAi construct (Bosch, Sumabat, and Hariharan 2016).

6.5 Conclusions

CDGs are a very rare and devastating disease, which lead to severe health problems or early death. As the field is constantly growing, there is much need to investigate (i) mechanistic details and (ii) identify novel treatment options. Furthermore, there is also a lack of cellular model systems to test therapeutic drugs on (Thiesler et al. 2016).

In this study we could show that rapamycin improves lifespan of a conditional N-glycosylation-defective yeast model, in a Sch9-dependent manner. Other than previously suggested, autophagy was only partially involved in rapamycin-mediated rescue. So far, we did not discover any further downstream target of Sch9, affecting the survival in a positive way. There are two possible explanations: First, rapamycin-mediated rescue might be mediated by another target of Sch9, not tested here (some targets are essential for growth and can therefore not be tested in the same manner). Second, a combination of downstream effects that can complement each other to some degree might mediate the beneficial effects of rapamycin. This could be tested by employing combinations of gene deletions.

Surprisingly, we did not observe increased UPR signaling, although we cannot completely rule out that defects in UPR signaling contribute to CDG toxicity. Here, further investigation concerning a potential UPR blockage has to be done.

Rapamycin also revealed some promising results in the glycosylation-defective Alg6 knockdown Drosophila model. Nevertheless, these results should be strengthened by further experiments, including control experiments for knockdown efficiency.

However, these results also need to be confirmed in mammals. In fact, mouse models for CDG, e.g. Slc35-deficient mice, are available. Since rapamycin is already used in medicine to treat patients, for example after organ transplantations, there already exists valuable data on drug tolerance, side effects and other parameters relevant for therapeutic use.

7. Abbreviations

ALG	Asparagine linked glycosylation
ANOVA	Analysis of variance
ApoC-III	Apolipoprotein C-III
Atg	Autophagy related gene
Asn	Asparagine acid
Asp	Asparagine
BSA	Bovine serum albumin
CDG	Congenital disorder of glycosylation
CFU	Colony forming units
COG	Conserved oligomeric Golgi complex
COP-I	Vesicular coat complex I
CPY	Carboxypeptidase Y
ddH_2O	Double-distilled water
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleosid-triphosphate
Doxy	Doxycycline
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FKBP12	FK506 binding protein
FUC	Fucose
Fruc-6-P	Fructose-6-phsophate
GAL	Galactose
GalNac	*N*-Acetylgalactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glc	Glucose
GlcA	Glucuronic acid
GlcNac	*N*-acetylglucosamine
GPI-Anchor	Glycosylphosphatidylinositol anchor

GPT	GlcNac phosphotransferase
GRP 78	Glucose regulated protein 78
HCl	Hydrogen chloride
HPLC	High pressure liquid chromatography
IdoA	Iduronic acid
IEF	Isoelectric focusing
kDa	Kilo Dalton
Leu	Leucine
LiAc	Lithium acetate
LLO	Lipid-linked oligosaccharide
Man	Mannose
Man-1-P	Mannose-1-Phosphate
Man-6-P	Mannose-6-Phosphate
MPI	Phosphomannose isomerase
MS	Mass spectrometry
mTORC	Mammalian target of rapamycin complex
NaCl	Sodium chloride
NaOH	Sodium hydroxid
NCR	Nitrogen catabolite repression
OD	Optical density
ONC	Over night culture
OST	Oligosacherlytransferase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase Chain Reaction
PEG	Polyethylen glycol
PI	Propidium iodide
PminCMV	Minimal promotor of cytomegalovirus
PMM2	Phosphomannomutase II
PP2A	Protein phosphatase 2A
Pol III	Polymerase III
Rapa	Rapamycin
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species

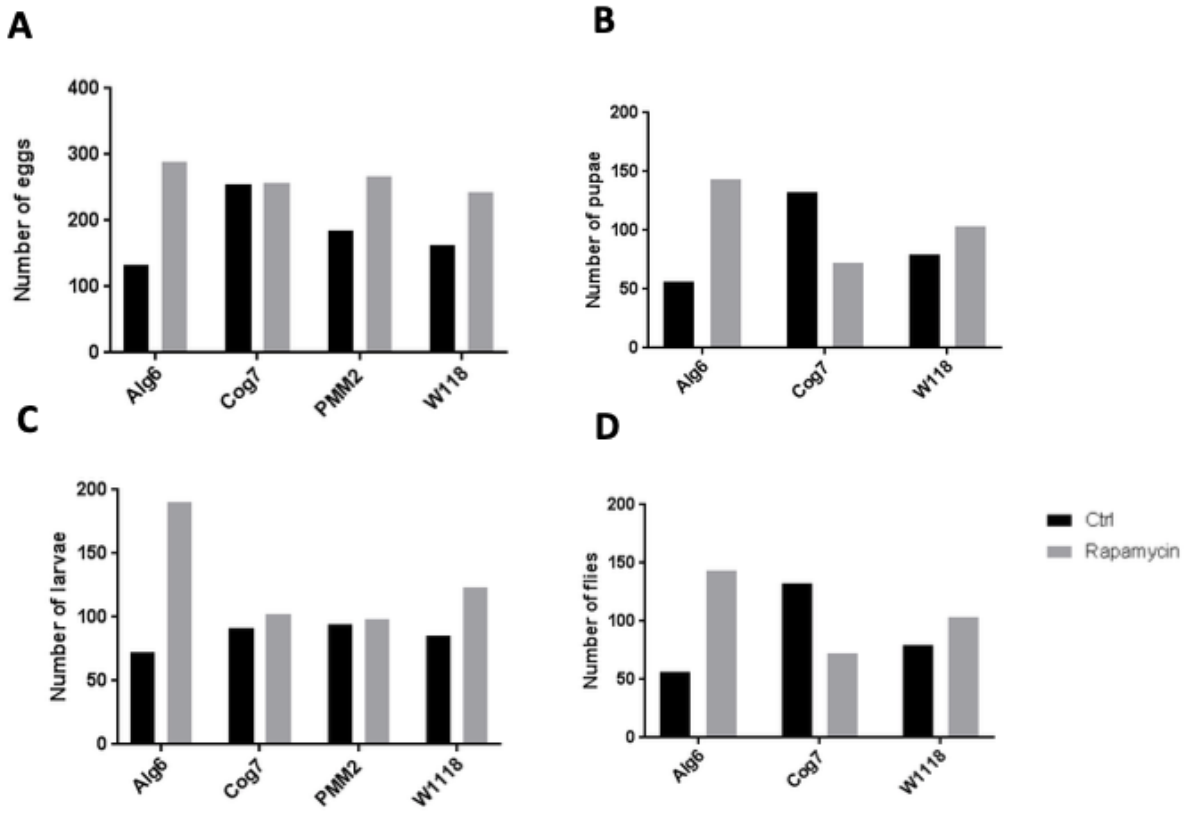
rpm	Rounds per minute
RT	Room temperature
SA	Sialic acid
S. Cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodiumdodecylsulfate
Ser	Serine
S-6-K	Serine-6-kinase
SMD	Synthetic minimal medium with glucose
SMG	Synthetic minimal medium with galactose
SNARE	SNAP (soluble NSF attachment protein) receptor
SYA	Sucrose yeast agar medium
TAE	Tris base-acetic acid-EDTA-buffer
TE	Tris EDTA buffer
tetR	Tet repressor protein
tf	Transferrin
Thr	Threonine
tm	Melting temperature
TRE	Tetracycline Response Element
Trp	Tryptophane
tTA	Tetracycline-controlled transactivator
Tyr	Tyrosine
TOR	Target of Rapamycin
Tris	Tris(hydroxymethyl)aminomethane
UAS	Upstream activating sequence
UPR	Unfolded Protein Response
UPRE	UPR element
untr	Untreated
VPS	Vacuolar protein sorting
Wbp1	Wheat germ binding protein 1
WT	Wild Type
YPD	Yeast peptone dextrose
Xyl	Xylose

8. Supplementary

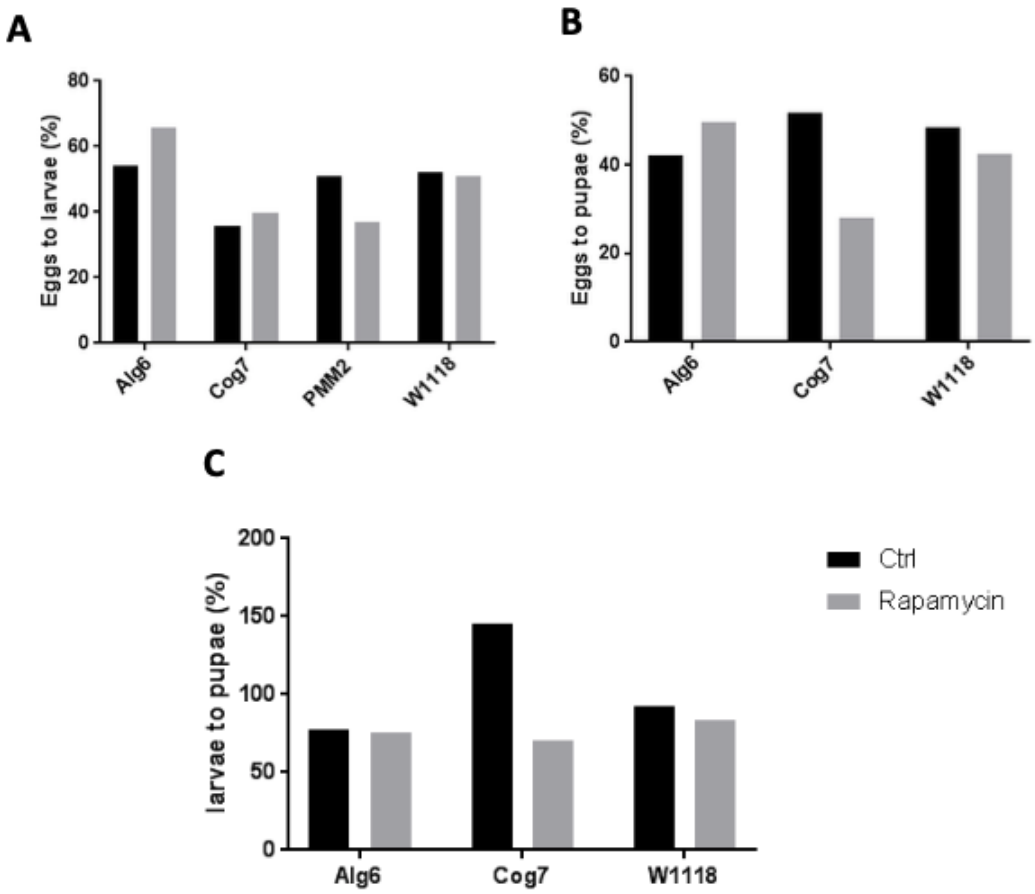
Supplementary table 1: Currently known CDG-types (adapted from (Scott et al. 2014)

Disorder	Genetic and biochemical defect
N-linked Glycosylation disorders	
ALG1–CDG	ALG1 β -1,4 Mannosyltransferase
ALG2-CDG	ALG2 α -1,3 Mannosyltransferase
ALG3-CDG	ALG3 α -1,3 Mannosyltransferase
ALG6-CDG	ALG6 α -1,3 Glucosyltransferase
ALG8-CDG	ALG8 α -1,3 Glucosyltransferase
ALG9-CDG	ALG69α -1,2 Glucosyltransferase
ALG11-CDG	ALG11 α -1,2 Mannosyltransferase
ALG13-CDG	ALG13 UDP-GlcNAc Transferase
ALG14-CDG	ALG14 UDP-GlcNAc Transferase
RFT1-CDG	RFT1 Man5GlcNAc2 Flippase
MPDU1-CDG	MPDU1 Mannose-P-Dolichol
TUSC3-CDG	TUSC3 OST complex subunit
MAGT1-CDG	MGAT1 OST complex subunit
DDOST-CDG	DDOST OST complex subunit
STT3A -CDG	STT3A OST complex subunit
STT3B-CDG	STT3B OST complex subunit STT3B
SSR4-CDG	SSR4 TARP complex associated
MGAT2-CDG	MGAT2 GlcNAc Transferase II
DPAGT1-CD	DPAGT1 GlcNAc-1-P Transferase
GCS1-CDG	MOGS α -1,2 Glucosidase
Multiple Pathways involving N-glycans	
PMM2-CDG	PMM2 Conversion of Man-6-phosphate to Man-1-phosphate
MPI-CDG	MPI Conversion of Fruct-6-phosphate and Man-6-phosphate
DHDDS-CDG	DHDDS Dehydrodolichol Diphosphate Synthase
DOLK-CDG	DOLK (DK1) Dolichol Kinase
SRD5A3-CDG	SRD5A3 Polyprenol Reductase
DPM1-CDG	DPM1 Dol-P-Man Synthase Complex
DPM2-CDG	DPM2 Dol-P-Man Synthase Complex
DPM3-CDG	DPM3 Dol-P-Man Synthase Complex
SLC35C1-CDG	FUCT1 GDP-Fucose Transporter
B4GALT1-CDG	B4GALT1 β -1,4 Galactosyltransferase
SLC35A1-CDG	SLC35A1 CMP-Sialic Acid Transporter
COG1-CDG	COG1 Golgi-ER Retrograde Transport
COG4-CDG	COG4 Golgi-ER Retrograde Transport
COG5-CDG	COG5 Golgi-ER Retrograde Transport
COG6-CDG	COG6 Golgi-ER Retrograde Transport

COG7-CDG	COG7 Golgi-ER Retrograde Transport
COG8-CDG	COG8 Golgi-ER Retrograde Transport
ATP6V0A2-CDG	ATP6V0A2 Golgi vesicular pH Regulator
TMEM165-CDG	TMEM165 Golgi pH Regulator
PGM1-CDG	PGM1 Conversion of Glc-1 Phosphate and Glc-6 Phosphate
PGM3-CDG	PGM3 Conversion of GlcNAc6 P and GlcNAc-1-P
SLC35A2 –CDG	SLC35A2 UDP-Galactose Transporter
MAN1B1-CDG	MAN1B1 α -1,2 Mannosidase



Supplementary figure 1: Total number of eggs, larvae, pupae and flies. (A-D) Developmental assay of male RNAi and control lines crossed with female da-Gal4 driver on food with or without 2 µM rapamycin for 24h. Flies were kept under constant 25°C and 70% humidity. (A) number of eggs laid on the food after 24 hours incubation of 3 fertilized female flies on Bloomington standard food with or without 2 µM rapamycin (Ctrl). (C) Number of larvae developed out of the eggs before and (B) number of those larvae which eclosed to pupae and (D) number of hatched flies out of the pupae.



Supplementary figure 2: Percentage of eggs developed to larvae and pupae; and larvae which developed to pupae. (A-C) Developmental assay of male RNAi and control lines crossed with female da-Gal4 driver on food with or without 2 µM rapamycin for 24h. Flies were kept under constant 25°C and 70% humidity. (A) Larvae developed out of the eggs after 24 hours incubation of 3 fertilized female flies on Bloomington standard food with or without 2 µM rapamycin (Ctrl). (B) Pupae developed out of the eggs before and (C) Percentage of those larvae which eclosed to pupae. See Methods for details on the experimental procedure.

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