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Maria Barbedo Ramalho Figueiredo Tavares

Pesquisa de alvos mitocondriais da ligase de ubiquitina APC/C^{Cdh1}

Searching for mitochondrial targets of the ubiquitin ligase APC/C^{Cdh1}

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Clara Isabel Ferreira Pereira, Investigadora Auxiliar no Instituto de Investigação e Inovação em Saúde (i3S) da Universidade do Porto e coorientação da Doutora Maria Paula Polónia Gonçalves, Professora Associada do Departamento de Biologia da Universidade de Aveiro.

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presidente	Doutor Artur Jorge da Costa Peixoto Alves Professor Auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro
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resumo

O Complexo promotor de anáfase/ciclossoma (APC/C) é uma ubiquitina ligase E3 envolvida na progressão do ciclo celular, com um papel na degradação dos reguladores do ciclo celular. É conservado em vários organismos, incluindo a levedura e mamíferos, e depende de duas proteínas adaptadoras, Cdc20 e Cdh1, que identificam os seus substratos, devido à presença de motivos de reconhecimento. Em humanos, a Cdh1 reconhece um conjunto mais abrangente de substratos do que a Cdc20, incluindo Drp1, uma proteína envolvida na fissão mitocondrial. Estudos do nosso grupo revelaram que os níveis de uma outra proteína mitocondrial, Atp2, uma subunidade da ATPsintase, aumentam na ausência de Cdh1 (trabalho não publicado). Esses dados sugerem que APC/C^{Cdh1} pode desempenhar um papel mais abrangente do que o esperado na regulação das proteínas mitocondriais. Desta forma, os objetivos deste trabalho foram investigar o papel da APC/C^{Cdh1} no controlo da função mitocondrial através de uma análise de proteómica e identificar os fatores envolvidos nesta regulação.

Utilizamos espectrometria de massa de alta resolução para avaliar o impacto da Cdh1 no proteoma mitocondrial de levedura. Com uma cobertura de ~97%, identificamos 12% de proteínas com uma abundância significativamente aumentada na ausência de *CDH1*. Estas incluíram subunidades de todos os complexos respiratórios, enzimas do ciclo de Krebs e reguladores da morfologia mitocondrial (incluindo Dnm1/Drp1). Estas classes de proteínas são essenciais para a adaptação das mitocôndrias ao metabolismo respiratório. Também identificamos 19% de proteínas cuja abundância era significativamente reduzida, associadas a processos não respiratórios. Curiosamente, quase todas as proteínas alteradas no mutante *cdh1*Δ possuem sítios de ligação de 4 fatores de transcrição (TF), Rpn4, Pdr3, Yap1 e Gcn4, sugerindo que Cdh1p pode regular proteínas mitocondriais, através da regulação de TFs. Concordante com esta hipótese, os níveis mais elevados de Atp2 e a elevada respiração mitocondrial na estirpe *cdh1*Δ, diminuíram após a deleção de *RPN4* e *YAP1*, indicando que estes TFs de resposta ao stresse atuam a jusante de Cdh1. Adicionalmente, o TF Yap1 é transcripcionalmente mais ativo nas células deficientes em Cdh1 e a sua ausência reduz o crescimento lento da estirpe *cdh1*Δ. Além disso, os níveis proteicos de Yap1 encontram-se significativamente reduzidos na fase G1 do ciclo celular, fase na qual a atividade de APC/C^{Cdh1} é máxima, sugerindo que Cdh1 regula a degradação do Yap1.

Em conclusão, estes resultados revelam uma nova função para o Cdh1 na regulação metabólica da mitocôndria, através da regulação do TF Yap1. A regulação do Yap1 durante o ciclo celular pode permitir acoplar o aumento da respiração mitocondrial, importante para assegurar a energia necessária para o ciclo celular, com a expressão das defesas antioxidantes, essenciais para eliminar as espécies reativas de oxigénio, subprodutos da respiração.

keywords *S. cerevisiae*, proteomics, mitochondria, Anaphase promoting Complex/Cyclosome, cell cycle, transcription factor

abstract

The Anaphase promoting Complex/Cyclosome (APC/C) is an E3 ubiquitin ligase involved in cell cycle progression by targeting cell-cycle regulators for degradation. It is conserved from yeast to humans and relies on two adaptor proteins, Cdc20 and Cdh1, which identify its substrates due to the presence of recognition motifs. In humans, Cdh1 recognizes a broader set of substrates than Cdc20, including Drp1, a protein involved in mitochondrial fission. Unpublished studies from our group revealed that Atp2, an ATPsynthase subunit, is also increased in the absence of Cdh1 suggesting that APC/C^{Cdh1} may play a broader role in mitochondrial function than expected. As such, the objectives of this work were to investigate the role of APC/C^{Cdh1} in mitochondrial function using a proteomic approach, and identify the players involved.
We used high-resolution mass spectrometry to obtain a global view of the impact of Cdh1 on the yeast mitochondrial proteome. With a coverage of ~97%, we found 12% of proteins have a significantly increased abundance in the absence of *CDH1*. These included subunits from all the respiratory complexes, enzymes from the Krebs cycle and regulators of mitochondria morphology (including Dnm1/Drp1). These protein classes are known to be essential for the adaptation of mitochondria to respiratory metabolism. We also found that 19% of the proteins were downregulated, being associated to non-respiratory processes. Interestingly, nearly all the *cdh1*Δ-altered proteins have the signature of 4 transcription factors (TFs), Rpn4, Pdr3, Yap1, and Gcn4, suggesting Cdh1 may be regulating mitochondrial proteins by targeting TFs. Supporting this hypothesis, the higher Atp2 levels and mitochondrial respiration in *cdh1*Δ strain decreased upon *RPN4* and *YAP1* deletion indicating these stress response TFs act downstream of Cdh1. In agreement, Yap1 is transcriptionally more active in the absence of Cdh1 and its absence decreases the slow growth of *cdh1*Δ cells. In addition, Yap1 protein levels are strongly decreased in the G1 phase of the cell cycle, in which APC/C^{Cdh1} activity peaks, suggesting Cdh1 targets Yap1 for degradation.
Overall, these results indicate a novel role for Cdh1 in the regulation of mitochondrial metabolic remodeling through Yap1 regulation. Regulation of Yap1 during cell cycle may allow coupling the known transient increase in mitochondrial respiration, important to meet cell cycle energetic demands, with the expression of antioxidant defenses, vital to mitigate the reactive oxygen species byproducts of respiration.

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Abbreviations

APC/C	Anaphase-promoting complex/cyclosome
CDKs	Cyclin-dependent protein kinases
Gal	Galactose
Glc	Glucose
o.n.	Overnight
OD_{600}	Optical Density at 600nm
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SCF	Skp1/Cul1/F-box
Ser	Serine
TBS	Tris-Buffered Saline
TF	Transcription Factor
Thr	Threonine
Ts	Temperature sensitive
TTBS	TBS with 0.05% (vol/vol) Tween-20
Wt	Wild-type
YPD	Yeast Peptone Dextrose
YPGal	Yeast Peptone Galactose

1. Introduction

1.1 Cell cycle

The cell division cycle is a necessary process in all organisms. It is highly conserved and characterized by a series of events that end in mitosis and the formation of two daughter cells. (Hartwell et al., 1974). The cell cycle has four phases: Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M). In *S. cerevisiae*, when cells achieve a threshold size in the late G1 phase in the presence of sufficient nutrients, they enter a period known as "Start," during which the bud emerges, DNA replication begins, and cells replicate their spindle pole body in preparation for mitosis and cytokinesis (Mendenhall & Hodge, 1998) (Figure 1). To guarantee that cell division does not occur under adverse conditions, cell cycle start, and advancement are strictly regulated, through cell cycle checkpoints (Shackelford et al., 1999; McGowan, 2002).

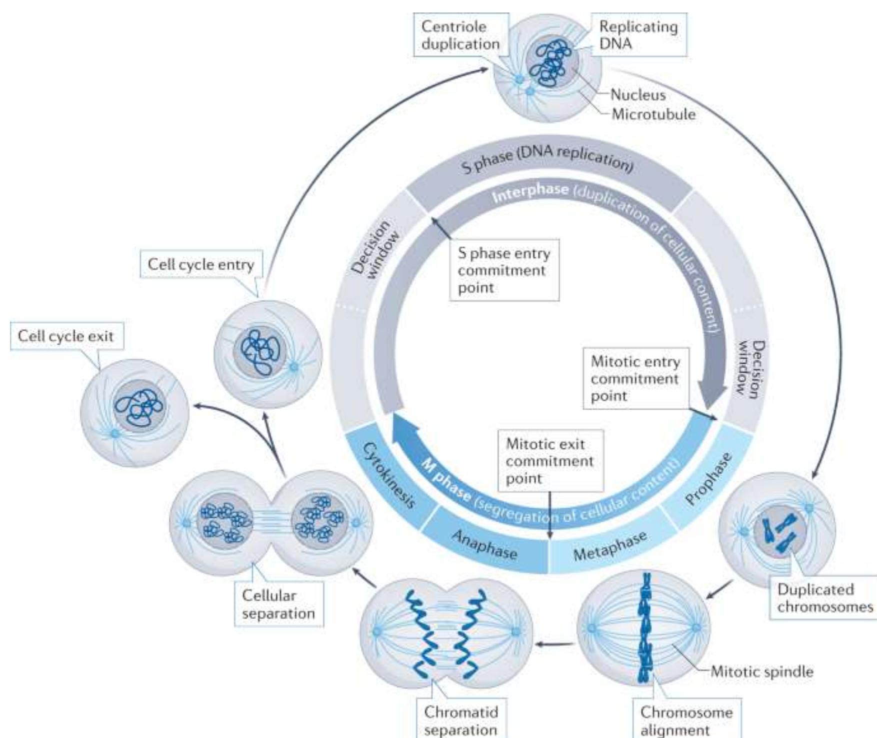

Figure 1. Cell division cycle. Retrieved from (Matthews et al., 2021).

1.2 Cell cycle regulation

Eukaryotic cell cycle events are regulated by an oscillating regulatory system, which is generated by variations in protein phosphorylation and ubiquitin-dependent protein degradation (Hartwell et al., 1974). The cyclin-dependent protein kinases (CDKs) and E3 ubiquitin ligase complexes such as SCF (Skp1/Cul1/F-box) and APC/C (anaphase-promoting complex/cyclosome) are at the core of this system. (Vodermaier, 2004). CDKs are serine/threonine kinases that regulate important processes, like DNA replication, through protein phosphorylation and are activated during the cell cycle in a sequential manner by binding to distinct cyclin molecules, which oversee kinase activity and substrate specificity. (Murray, 2004). Cyclins are a varied group of proteins that are distinguished by the presence of a cyclin box, which mediates binding to CDK. Variations in the sequence outside the cyclin box enable a differential regulation and expression levels fluctuations dependent of the cell cycle (Figure 2) (Lim & Kaldis, 2013). The cyclin–CDK appear to be linked to transcriptional regulation in some circumstances (Morgan, 1997). Budding yeast have six conserved CDKs, but only one, Cdc28, is required for mitotic and meiotic cell cycle control (Enserink & Kolodner, 2010), while the others CDKs are required for transcriptional regulation (Meinhart et al., 2005). In yeast, CDKs do not control all cyclic waves of gene expression (Orlando et al., 2008). Polo kinases have been demonstrated to affect cell cycle gene expression directly at late stage of the cell cycle by binding to and phosphorylating the transcription factors that regulate them (Fu et al., 2008).

Ubiquitin-dependent protein degradation occurs through a ubiquitin–proteasome system, which tags target proteins with ubiquitin for degradation by the 26S proteasome. Protein ubiquitination mediated by three enzymes: ubiquitin-activating enzymes (E1s), which are involved in ubiquitin activation and transfer, ubiquitin-carrying enzymes (E2s) and ubiquitin-protein ligases (E3s), which bring substrates and E2 together by transfer of ubiquitin to a specific substrate (Pickart, 2001). This connection of substrates and E2 enzymes determines the specificity of ubiquitin dependent proteolysis, underlying the importance of ubiquitin-protein ligases in the regulation of various processes, especially cell cycle. SCF and APC/C are the E3 ubiquitin-protein ligases most involved in the process of cell cycle regulation (Vodermaier, 2004). These E3 ubiquitin-protein ligases have different functions through the cell cycle. From late G1 to early M phase, SCF ubiquitylates substrates, whilst APC/C is active from anaphase

to the end of G1. Interactions between the SCF and the APC/C regulate the boundaries between their activities (Nakayama & Nakayama, 2006). SCF recognizes the phosphorylated APC/C inhibitors EMI1 and EMI2, causing their degradation and an increase in APC/C activity in the early M phase (Guardavaccaro, 2003; Margottin-Goguet et al., 2003). In G1 phase, APC/C recognizes SFC's subunit SKP2 and degrades it, decreasing SFC activity and accumulating cyclin dependent kinase inhibitors (CKIs). In G1/S transition, SFC's subunit SKP2 degrades CKIs while activating S-cyclin/cyclin dependent kinase 2 (CDK2). CDK2 dissociates APC/C co-activator Cdh1 from APC through phosphorylation, decreasing APC/C activity and enabling cyclin accumulation (Bashir et al., 2004; Wei et al., 2004).

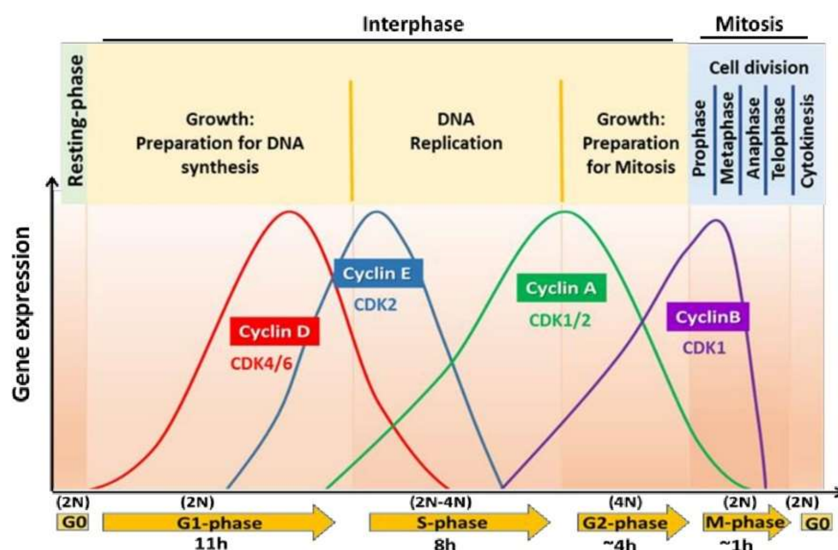

Figure 2.Oscillations of cyclin levels throughout the eukaryotic cell cycle. Adapted from (Shah, 2016)

1.3 Control of cell-cycle transcription

During the cell cycle, a transcription factor (TF) network is involved in generating the cell-cycle transcriptional program (Kelliher et al., 2018). TFs control and mediate cellular responses to environmental stimuli through sequence specific interactions with cis regulatory DNA elements within the promoters and enhancers of their target genes, stimulating or repressing their expression (Gordân et al., 2011). TFs can also bind to the promoters of other TFs that regulate periodic gene expression (Harbison et al., 2004). Additionally, TFs that are

expressed in the late stage of cell cycle can bind to other TFs involved in expression in early stages of the cell cycle (Di Talia et al., 2009). A high percentage of genes (>10%), in the budding yeast, *S. cerevisiae*, are transcribed with cell cycle periodicity (Wittenberg & Reed, 2005) and can be classified into clusters based on the timing of their expression and the TFs that regulate them (Haase & Wittenberg, 2014). Different groups of genes are transcribed at different cell cycle phases, including critical regulatory elements such as cyclins, CDKs, the protein degradation machinery and even proteins that apparently have no direct role in cell cycle activity (Wittenberg & Reed, 2005). The regulation of the abundance of these cell-cycle regulators by activating its phase-specific gene expression contributes to the cell cycle oscillatory mechanism. The TF network is linked to CDKs and APC/C, which provide a regulation system to control the cell cycle-dependent transcription, through direct phosphorylation of TFs in the case of CDKs (Cho et al., 2017). In fact, it has been reported that G1 cyclin-CDKs promote TF protein activation and accumulation in S/G2/M phases by impairing G1 transcriptional corepressors and APC^{Cdh1}, promoting the activity of TF network (Cho et al., 2019).

There are different models that explain the reason behind the cell cycle regulated transcription. One model proposes that cell cycle-regulated transcription is a mechanism for efficient use of energy resources, as transcription and translation are both energy-intensive processes, where specific genes are only expressed when required. Another model focuses on the construction of a needed element once per cycle, as in the construction of the complex required to initiate DNA replication, which is only built once, serving as control for cell cycle errors (Futcher, 2000; Simmons Kovacs et al., 2008). Yet another model focuses on unchanged protein renewal, in which post-translationally modified gene products are no longer active (Breeden, 2003). Independently of the model, which may not be exclusive, the cell cycle regulated transcription seems conserved (Orlando et al., 2007).

1.4 Anaphase-promoting complex

The anaphase-promoting complex is an E3 ubiquitin ligase required for the cell cycle in all eukaryotes. APC/C serves as a binding platform that brings together a specific substrate and an E2 co-enzyme, resulting in polyubiquitination and degradation of the substrate by the 26S

proteasome (Pines, 2011; Primorac & Musacchio, 2013). Without APC/C, cells cannot initiate the steps that are necessary for DNA replication later in S phase, separate their sister chromatids in anaphase, and, they cannot exit from mitosis and divide into two daughter cells, (Peters, 2002; Harper et al., 2002). APC/C is composed of many different subunits, 12 in humans and 13 in budding yeast (Figure 3), but it can only ubiquitylate substrates with the help of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating (E2) and an enzyme co-activator protein (Buschhorn et al., 2011).

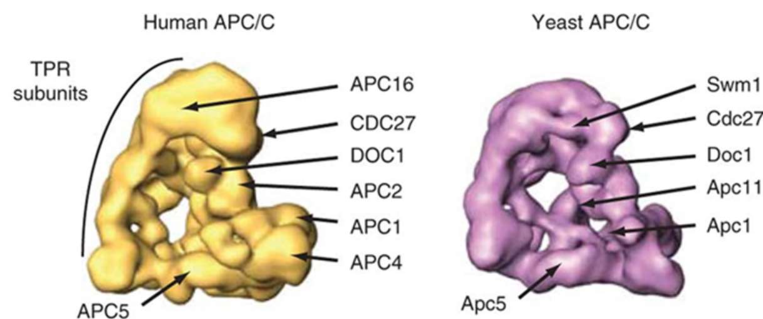

Figure 3. Three-dimensional structure of human APC/C vs yeast APC/C. Adapted from (Buschhorn et al., 2011).

The recruitment of substrates to the APC/C and the positioning of its catalytic domains for ubiquitin transfer occur simultaneously through the binding to one of two APC/C-specific coactivators, CDC20 or CDH1, which are encoded by all known eukaryotic genomes (Visintin et al., 1997; Buschhorn et al., 2011). Most APC/C substrates are recruited by interaction with the seven-blade β-propeller WD40 repeat domain in the C-terminal half of the APC/C activator subunit (Kraft et al., 2005). This domain contains binding sites for recruited substrates by recognizing short linear sequence motifs, called degrons, such as Destruction-box (D-box) (RXXLXXXXN/D/E) and KEN-box (XXKENXXXX), frequently found in APC/C's substrates (Figure 4) (Davey & Morgan, 2016). KEN-boxes are preferentially, but not exclusively, recognized by APC/C^{Cdh1} (Pfleger & Kirschner, 2000). The consensus sequence of destruction box can be found in many proteins, but not all these proteins are APC/C's substrates. Furthermore, some substrates only have an RxxL motif and are still recognized by APC/C,

indicating the last amino acid in the consensus is not stringently conserved (Li & Zhang, 2009). Thus, there must exist other sequence limitations that characterize real APC substrates that are not yet known (Qin et al., 2017). Phosphorylation can disrupt the interaction between the APC/C and a D-box motif, concealing the degradation motif. The interaction of phosphorylation and ubiquitination mechanism allows signal integration and rigorous control of cell cycle transitions (Holt, 2012).

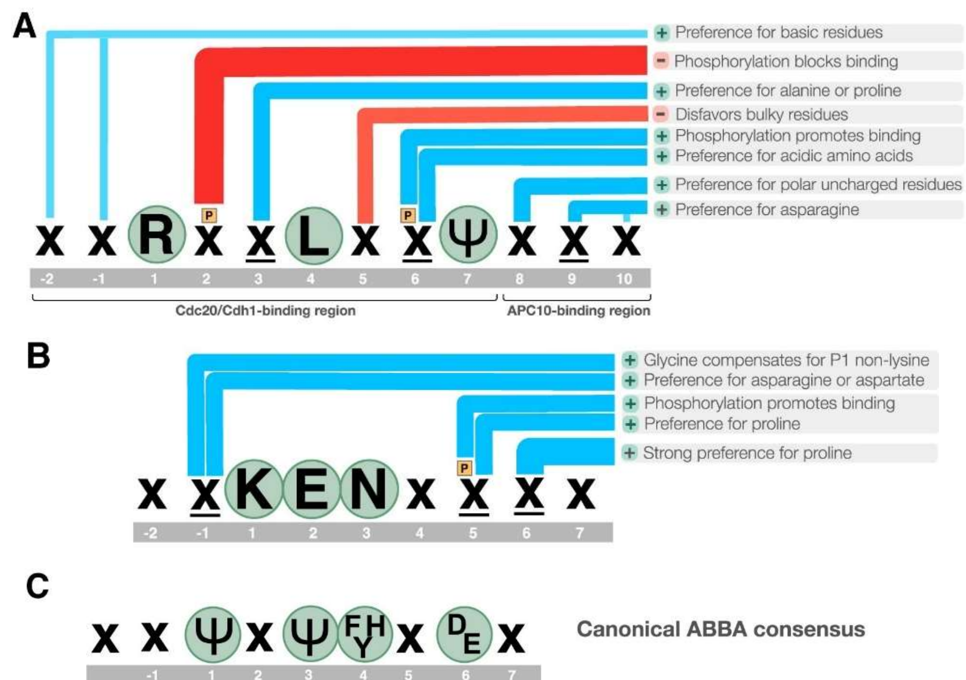

Figure 4. Sequence preferences of common of APC/C recognition motifs. (A) Sequence preference of the D box. ‘‘ψ’’ indicates a leucine, isoleucine, or valine residue; ‘‘x’’ indicates any residue; underlined ‘‘x’’ indicates any residue but with strong preferences for particular residues based on information from characterized degrons; orange ‘‘P’’ indicates phosphorylation of the position. (B) Sequence preference of KEN-box (C) Sequence preference of ABBA motif-binding pocket. Adapted from (Davey & Morgan, 2016)

Cdc20 is necessary for metaphase to anaphase transition (Li et al., 2007) while Cdh1 is required for G1/S transition, mitotic exit, degradation of regulators of cytokinesis and centrosome replication (Li et al., 2008; Garcí-Higuera et al., 2008). At the end of mitosis, various mitotic regulators, such as Cdc20, are degraded by APC/C^{Cdh1} (Prinz et al., 1998; Pfleger & Kirschner, 2000). In line with this, previous studies revealed that in the absence of Cdh1 cells accumulate mitotic errors and have difficulty in completing cytokinesis (Li et al., 2008; Garcí-Higuera et al., 2008). Also, APC/C may be necessary for DNA endoreplication since it was previously reported that in the absence of Cdh1, placental giant cells failed to form (Li et al., 2008) and that cyclin B1, essential for mitotic exit, is an APC^{Cdh1} substrate (King et al., 1995). Furthermore, APC/C^{Cdh1} may have a role in mitotic entry prevention in cells with damaged DNA, as targeting and destruction of polo like kinase 1 (Plk1) by APC/C^{Cdh1} was reported to induce mitotic exit in these cells (Bassermann et al., 2009).

APC/C coactivator binding is cell-cycle dependent and mediated by phosphorylation. In mitosis, Cdc20 associates with APC/C only if phosphorylation of APC/C by mitotic kinases, such as Cdk1, has occurred. On the other hand, before the beginning of metaphase, Cdh1 is inactivated through phosphorylation by different CDKs (Kramer et al., 2000). This Cdh1 inhibition by phosphorylation occurs in 11 Ser/Thr sites at the following positions: 12, 16, 42, 157, 169, 173, 176, 227, 239, 418, 436 (Zachariae et al., 1998). During mitotic exit, APC/C^{Cdc20} is inactivated by APC/C dephosphorylation and by Cdc20 targeting by APC/C^{Cdh1} (Huang et al., 2001). At the G1/S transition, APC/C^{Cdh1} is inactivated enabling the accumulation of APC/C targets and consequently DNA replication and mitosis (Figure 5) (Kramer et al., 2000).

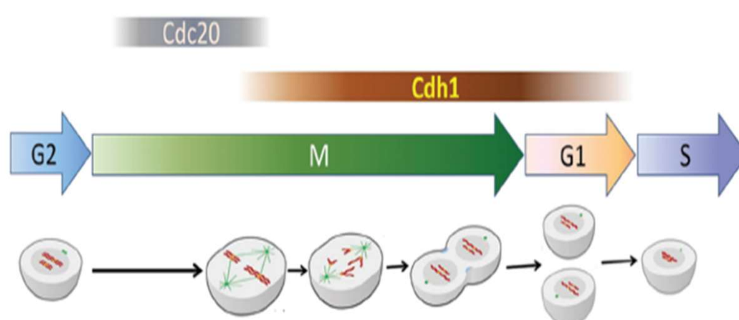

Figure 5. APC activation by Cdc20 and Cdh1 during the cell cycle. Adapted from (Qiao et al., 2010).

Studies across diverse organisms suggest that both molecular and dynamic functional aspects of Cdk–APC/C regulation may be conserved (Cross et al., 2011; Bertoli et al., 2013). In fact, D-box and KEN-box motifs are recognized by all known APC/C activators. However, there are other activators that do not bind to specific degrons. For instance, none of the known ABBA motifs can bind Cdh1 and the Cdc20 orthologue (FZY-1) in *C. elegans* (Figure 6) (Davey & Morgan, 2021).

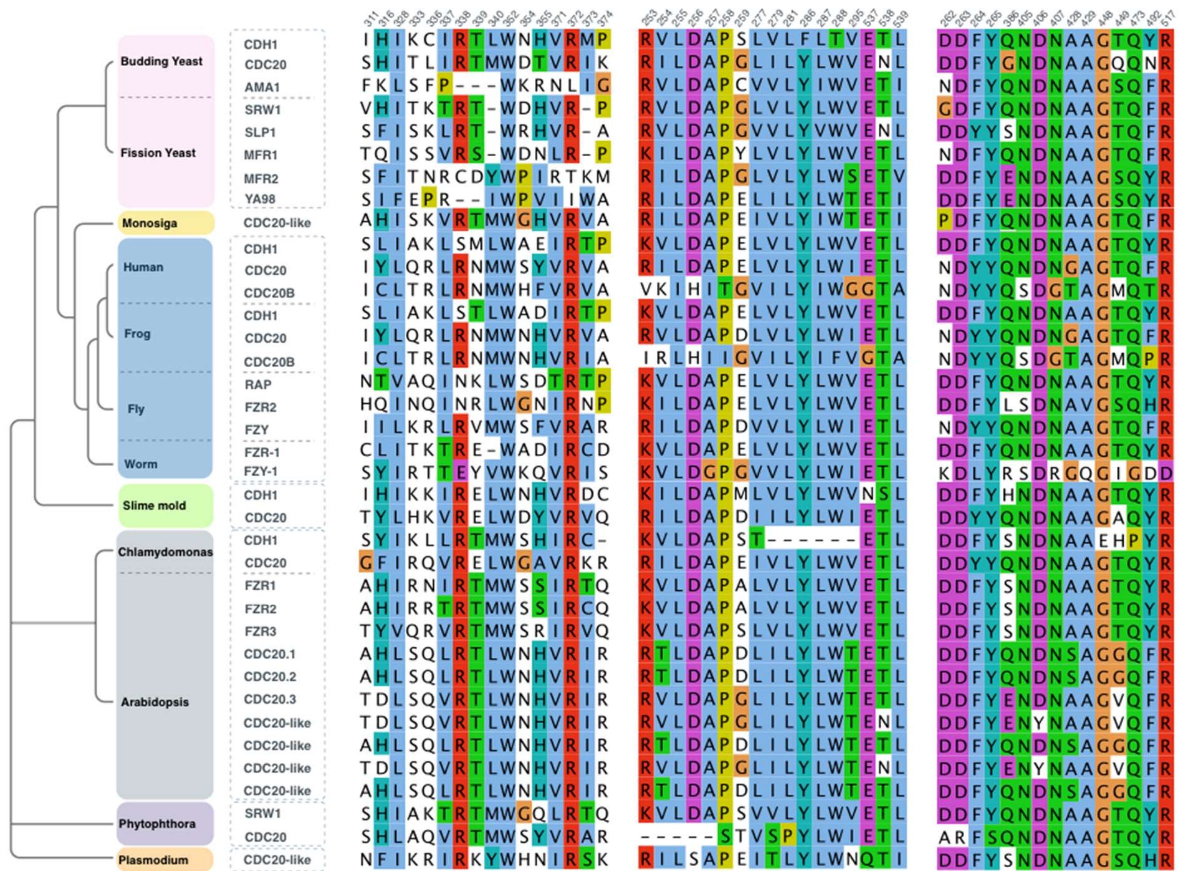

Figure 6. Conservation of the three APC/C degrons across several eukaryotic proteomes. Adapted from (APC/C Degron Repository).

1.5 Coordination of cell cycle with cellular activity

The cell cycle control network regulates cellular metabolism in order to meet the needs of cell division. APC/C^{Cdh1} is known as a regulator of cell cycle progression but it also as plays a role in cell metabolism regulation (Duan & Pagano, 2011). It was shown that APC/C^{Cdh1}

regulates the stability of metabolic enzymes, particularly enzymes involved in glycolysis and gluconeogenesis, in HeLa cells (Colombo et al., 2011). Furthermore, at G1/S transition, Cdk1 is responsible for various metabolic changes such as promotion of lipid metabolism in yeast (Kurat et al., 2009; Blank et al., 2019), trehalose catabolism (Ewald et al., 2016) and cell growth (McCusker et al., 2007).

Reactive oxygen species (ROS), oxidative stress, and the redox state in general, may all have a role in cell cycle regulation at certain stages. However, this link is still not completely understood (Patterson et al., 2019). In line with this, yeast cells show metabolic cycles in which most genes are expressed in a cyclic manner (Klevecz et al., 2004; Tu et al., 2005). Three phases constitute the metabolic cycle in yeast: Ox (oxidative, respiratory), R/B (reductive, building) and R/C (reductive, charging). In the Ox phase cells are preparing for cell division, with increase in ATP production through oxidative phosphorylation of previously accumulated acetyl-CoA and increased expression of genes involved in amino acid synthesis and ribosomes. In the R/B phase, the expression of genes involved in cell division and mitochondrial biogenesis increases, as oxygen consumption decreases, and mitochondria are rebuilt. The R/C cluster is enriched for genes involving fatty acid oxidation, glycolysis, stress associated response and protein degradation (Tu et al., 2005; Tu & McKnight, 2007).

Cell cycle impacts on mitochondrial functions in several aspects, including mitochondrial biogenesis, bioenergetics, and morphology (fission and fusion) (Lopez-Mejia & Fajas, 2015). Mitochondrial bioenergetics appears to be coordinated with cell cycle, with increased respiratory activity and increased mitochondrial membrane potential mainly occurring at S transition or G2/M in mammals (Martínez-Diez et al., 2006). In line with this, in yeast it was established a link between cell cycle and mitochondria that suggests a specific role for the import of proteins for mitochondrial activity and efficient progression through the cell division cycle (Figure 7) (Harbauer et al., 2014). This model suggests that in M phase, Cdk1 enhances translocase of outer membrane (Tom) activity through phosphorylation, promoting mitochondrial protein import. This import increases the biogenesis of mitochondria and mitochondrial respiration, enhancing energy production, to fuel the cell cycle, an energy demanding process (Shiota et al., 2015).

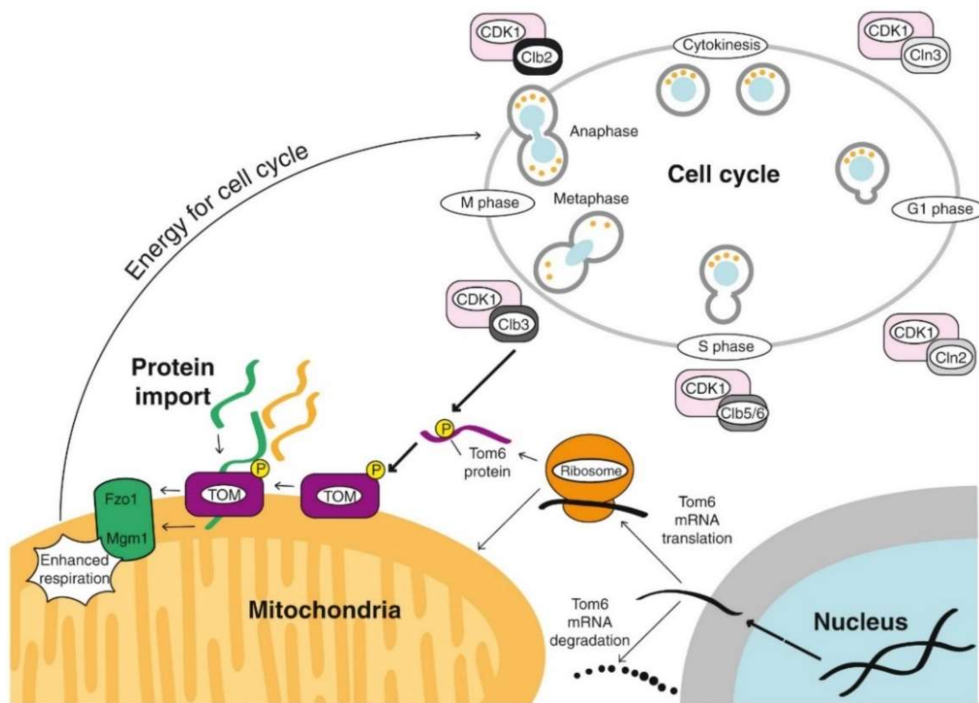

Figure 7. Cell cycle-dependent regulation of mitochondrial protein import through TOM complex regulation and energy production in yeast (Shiota et al., 2015).

This regulation of mitochondrial respiration by CDK in a cell cycle-dependent manner was also observed in mammalian cells (Wang et al., 2014). Mitochondrial fission and fusion influence mitochondrial architecture and thereby mitochondrial activity and inheritance. APC/C^{Cdh1} is known to target the pro-fission dynamin related protein (DRP)1, which is ubiquitinated and degraded by proteasomes, allowing mitochondrial fusion. In fact, it was previously demonstrated that in the presence of APC/C^{Cdh1}, Drp1 levels were lowest at G1 phase in HeLa cells (Horn et al., 2011). In G1/S transition, mitochondria shift from a tubular network to a fragmented state, believed to be of relevance for the appropriate segregation of the organelles and mtDNA into daughter cells during mitosis. In this transition, APC/C^{Cdh1} activity is inhibited by CDK through phosphorylation, enhancing levels of Drp1, leading to

mitochondrial fission. Later, in M/G1 and G1, APC/C^{Cdh1} targets Drp1 for degradation, promoting mitochondrial fusion (Horn et al., 2011; Taguchi et al., 2007). Recently, APC/C^{Cdh1} has also been reported to regulate mitochondrial function by targeting of Idh2 (isocitrate dehydrogenase), a protein responsible to produce NADPH in mitochondria, increasing the mitochondrial production of ROS (Lambhate et al., 2021).

1.6 Cdh1 targets beyond cell cycle

Though APC/C main function is ubiquitination of cell cycle substrates, in recent years a growing body of evidence indicates APC/C^{Cdh1} function is not restricted to controlling cell-cycle progression. Several studies implicate APC/C^{Cdh1} activation and subsequently ubiquitylation of substrates (many unknown) in the regulation of processes such as: genomic integrity (Lafranchi et al., 2014), apoptosis (Huang et al., 2012), senescence (Johmura et al., 2014), metabolism (García-Nogales et al., 2003), and stem cell regulation (Yang et al., 2011). Unlike the APC/C coactivator Cdc20, whose expression is restricted to proliferating cells, Cdh1 is also expressed in several mammalian tissues that predominantly contain differentiated cells, such as adult brain (Gieffers et al., 1999). In the brain, APC/C^{Cdh1} seems to control several processes such as axonal growth (Konishi et al., 2004), synaptic plasticity (Li et al., 2008), dendritic integrity, and memory (Pick et al., 2013). In addition to cell cycle defects, knockout of Cdh1 in neural progenitor cells causes a reduced number of cortical neurons (Delgado-Esteban et al., 2013). APC/C^{Cdh1} is also involved in establishing neuronal quiescence since it contributes to the inhibition of axon growth by targeting Id2 (inhibitor of differentiation 2) in the developing nervous system (Lasorella et al., 2006). APC/C^{Cdh1} has also been reported to control neurogenesis in the cerebellum via ubiquitination and degradation of the casein kinase 1δ (Penas et al., 2015). It has been reported that APC/C^{Cdh1} activity increases with Cdh1 dephosphorylation by the protein phosphatase 2A (PP2A), suggesting that dephosphorylation of Cdh1 by PP2A promotes APC/C^{Cdh1} function in neuronal differentiation (Delgado-Esteban et al., 2013). Furthermore, APC/C^{Cdh1} may control neuronal survival, as APC/C^{Cdh1} degradation of Pfkfb3 (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3) leads to glycolysis inhibition and maintains the antioxidant status of neurons (Herrero-Mendez et al., 2009). In light of this, it is

not surprising that Cdh1 has been associated to disorder such as microcephaly (Rodríguez et al., 2019), Alzheimer´s (Aulia & Tang, 2006) and Parkinson´s disease (Stieg & Cooper, 2016).

2. Aims

In humans, Cdh1 recognizes a broader set of substrates than Cdc20, including Drp1, a protein involved in mitochondrial fission (Horn et al., 2011). Recent work from our group revealed another protein, Atp2, the catalytic subunit of mitochondrial ATPsynthase, is increased in the absence of Cdh1 (unpublished work). This evidence raised the hypothesis that APC/C^{Cdh1} may play a broader role in the regulation of mitochondrial proteins. As such, the aims of this work were to investigate the role of APC/C^{Cdh1} in mitochondrial function using a proteomic approach and to explore the mechanisms and players involved.

3. Materials and Methods

3.1 Strains and growth conditions

All *Saccharomyces cerevisiae* cells used in this study are in a BY4741 yeast strain background (S288c derived) (Table 1).

Table 1. List of *S. cerevisiae* strains and plasmids used in this study

Strain	Genotype	Source
	Yeast Strains	
BY4741 (wild type)	MATα; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	EUROSCARF
*cdh1*Δ	BY4741*; cdh1*Δ*::kanMX4*	EUROSCARF
*cdh1*ΔHis3	*cdh1*Δ::*HIS3MX6*	This study
*yap1*Δ	BY4741; *yap1*Δ::*kanMX4*	EUROSCARF
*rpn4*Δ	BY4741; *rpn4*Δ::*kanMX4*	EUROSCARF
*gcn4*Δ	BY4741; *gcn4*Δ::*kanMX4*	EUROSCARF
*pdr3*Δ	BY4741; *pdr3*Δ::*kan*MX4	EUROSCARF
*gcn4*Δ*cdh1*Δ	BY4741; *gcn4*Δ::*kanMX4 cdh1*Δ::*HIS3MX6*	This study
*pdr3*Δ*cdh1*Δ	BY4741; *pdr3*Δ::*kanMX4 cdh1*Δ::*HIS3MX6*	This study
*yap1*Δ*cdh1*Δ	BY4741; *yap1*Δ::*kanMX4 cdh1*Δ::*HIS3MX6*	This study
*rpn4*Δ*cdh1*Δ	BY4741; *rpn4* Δ::*kanMX4 cdh1*Δ::*HIS3MX6*	This study
*bar1*Δ	BY4741; *bar1*Δ::*kanMX4*	EUROSCARF
*bar1*ΔHis3	BY4741; *bar1*Δ::*HIS3MX6*	This study
cdc28-1	BY4741; *cdc28-1*:*kan*MX4	EUROSCARF
*cdc28-1cdh1*Δ	BY4741; *cdc28-1*:*kan*MX4 *cdh1*Δ::*HIS3MX6*	This study
	Plasmids	
pAP1-LacZ	pRS415-AP-1-CYC1-LacZ	(Maeta et al., 2004)
pYap1-9Myc	pRS315-Yap1-9Myc	(Maeta et al., 2004)
pCdh1-m11	p416-GALL-HA3-HCT1-m11	(Zachariae et al., 1998)

Cells were grown in a rich medium YPD [(1% (w/v) yeast extract (Liofilchem), 2% (w/v) bactopeptone (Liofilchem), 2% (w/v) glucose (Liofilchem)] or YPGal [same as YPD except that glucose was replaced by 2% (w/v) galactose (Fisher Scientific)] or synthetic complete (SC) drop-out medium [2% (w/v) glucose (Liofilchem), 0.67% (w/v) yeast nitrogen base without amino acids (BD BioSciences) and appropriate amino acids or nucleotides (Sigma Aldrich) [0.008% (w/v) histidine, 0.008% (w/v) tryptophan, 0.04% (w/v) leucine and 0.008% (w/v) uracil]. For solid medium, 2% (w/v) agar (Liofilchem) was added.

Yeast cells were routinely grown at 26ºC, in an orbital shaker, at 140 rpm, with a 5:1 flask/culture volume ratio. Optical density at 600nm was measured using a Shimadzu UV-2401 PC spectrophotometer.

3.2 Construction of yeast mutant strains

3.2.1 Polymerase chain reaction

Mutants *gcn4*Δ, *pdr3*Δ, *rpn4*Δ, *yap1*Δ were obtained from EUROSCARF, and the correct ORF deletion confirmed by polymerase chain reaction (PCR) using an upstream ORF-specific primer and a primer specific to the deletion cassette (p83 R; Table 2).

To generate *cdh1*Δ::*HIS3* and *bar1*Δ::*HIS3* strains, the selection marker in the *cdh1*Δ::*kanMX4* and *bar1*Δ::*kanMX4* strains was replaced by *HIS3MX* amplified from pFA6a-hisMx6 plasmid (Longtine et al., 1998). To construct *gcn4*Δ*cdh1*Δ, *pdr3*Δ*cdh1*Δ, *yap1*Δ*cdh1*Δ, *rpn4*Δ*cdh1*Δ and *cdc28-1cdh1*Δ strains, *gcn4*Δ, *pdr3*Δ, *rpn4*Δ, *yap1*Δ and *cdc28-1* strains were transformed with a deletion cassette amplified by colony PCR from the *cdh1*Δ::*HIS3* strain, containing the selection marker *HIS3MX* and the flanking regions of *CDH1*.

Confirmation of gene deletion was performed by colony PCR using one primer with homology to the selection marker and the other homologous to the adjacent region of the gene mutated. Cell lysis was accomplished by heating the cell suspension in a microwave oven for approximately 30 seconds, before adding the reaction mix.

PCRs were performed in a T100™ Thermal Cycler (Bio-Rad) with a heated lid, in reaction mixtures using Supreme NZYTaq II 2x Green Master Mix (NZYtech). PCR programs used in this study are described in Table 2.

PCR products were examined by nucleic acid electrophoresis at 120V for 30 minutes, using 0.8-1% agarose gels containing GreenSafe Premium 0.04 µL/mL (NZYtech) and TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). DNA bands were detected upon UV fluorescence. PCR products were purified using NZYGelpure kit (NZYtech).

Table 2. Primers and respective PCR programs used in this study.

Gene	Primer	Nucleotide sequence	PCR program Annealing Temperature (ºC)	 Extension Time (s)	 Number of Cycles
BAR1HISMX6 **amplification**	Bar1_F Bar1_R	GAAATTACATGGCGAGTGTC CCGTGATAGTTTAATGGTCAG	47	60	35
bar1Δ::HIS3MX6 **confirmation**	Bar1_Conf_F p83_R	AGATAACGGCTCTTGCCG GTATAGCGACCAGCATTC	47	45	35
CDH1HISMX6 **amplification**	Cdh1_F Cdh1_R	CAACCTCACCAATAAAGTC GTGCAAGAAAAAGGTTCC	45	60	35
cdh1Δ::HIS3MX6 **confirmation**	Cdh1_Conf_F p83_R	CGCGGAAACTCTGAATGG GTATAGCGACCAGCATTC	47	45	35
rpn4Δ::kanMX4 **confirmation**	Rpn4_Conf_F p83_R	GTTCAAGGAGGAGGATAC GTATAGCGACCAGCATTC	46	45	35
yap1Δ::kanMX4 **confirmation**	Yap1_Conf_F p83_R	TGCATGAACACGAGCCAT GTATAGCGACCAGCATTC	47	45	35
pdr3Δ::kanMX4 **confirmation**	Pdr3_Conf_F p83_R	GCAACCATAGGTCTTAGG GTATAGCGACCAGCATTC	46	45	35
gcn4Δ::kanMX4 **confirmation**	Gcn4_Conf_F p83_R	ATCCAGGTTTACTCGCCA GTATAGCGACCAGCATTC	47	45	35

3.2.2 Yeast transformation

Lithium acetate/single-stranded carrier DNA/ polyethylene glycol method was used to transform yeast cells, as described by Gietz (Gietz & Schiestl, 2007). Briefly, yeast cells were grown overnight (o.n.) in YPD medium, diluted to OD_{600}=0.2, and grown to OD_{600}=0.6. Cells were collected by centrifugation, washed, and incubated with a transformation mix containing 240 µL 50% polyethylene glycol 3350 (PEG 3350) (Sigma Aldrich), 36 µL of 1.0 M lithium acetate (Sigma Aldrich), 25 µL boiled single-stranded carrier DNA (5.0 mg/mL), the appropriate amount of DNA and sterile water to a final volume of 360 µL assuring thorough homogenization. An aliquot with water instead of DNA was used as a negative control for every

transformation. Cells were incubated for 26ºC for 30 min followed by 30 min at 42ºC. After incubation, cells were gently transferred to selective media and allowed to recover at 26 °C for 4-5 hours on an orbital shaker. Cells were harvested by centrifugation, resuspended in sterile water, and plated in SC selection medium. For transformations using plasmid DNA the incubation period of 30 minutes at 26 °C was omitted as well as the recovery period. For the transformation of the temperature-sensitive (ts) *cdc28-1* strain cells were incubate at room temperature o.n.

Plates were incubated at 26 °C for 2-4 days to obtain transformants. Obtained colonies were restreaked on selective media plates.

3.3 Isolation of Mitochondria

Wild-type and *cdh1*Δ strains were grown o.n. in YPGal. Samples were collected at mid-log phase (OD_{600}~1.4), in triplicates. Cells were harvested at 7000 rpm for 5 min. Washed cells were suspended in digestion buffer [2M sorbitol; 1M phosphate, pH 7.5; 0.5M EDTA, 1% (vol/vol) β-mercaptoethanol] at a concentration of 10 grams cells (wet weight) to 30 mL. The cell wall was digested enzymatically with Zymolyase at 5mg/g of cells at 37ºC for 30 min. The spheroplasts were harvested by centrifugation at 7000 rpm and resuspended in ice-cold 0.5M homogenization buffer (0.5M sorbitol; 20mM Tris, pH 7.5; 1mM EDTA) and lysed using the Douce homogeneizer. Suspensions were transferred to small centrifuge flasks and subjected to 3 cycles of 15 min low speed/high-speed centrifugation (2500 rpm/13000 rpm) and resuspension cycles. When not analyzed immediately, the mitochondrial pellet was stored at -80 °C.

3.4 HPLC-MS/MS analysis of mitochondrial protein content

HPLC-MS/MS analysis of wt and *cdh1*Δ isolated mitochondrial extracts was performed at the i3S Proteomics Platform.

Briefly, triplicates from wt and *cdh1*Δ strains were solubilized with 100 mM Tris pH 8.5, 1% sodium deoxycholate, 10 mM tris(2-carboxyethyl) phosphine (TCEP) and 40 mM chloroacetamide for 10 minutes at 95ºC at 1000 rpm. Each sample was processed for proteomics analysis following the solid-phase-enhanced sample-preparation (SP3) protocol as described in

(Hughes et al., 2019). Enzymatic digestion was performed with Trypsin/LysC (2 micrograms) o.n. Protein identification and quantitation was performed by nanoLC-MS/MS composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific), as previously described (Osório et al., 2021). This equipment is composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific).

The raw data was processed using Proteome Discoverer 2.5.0.400 software (Thermo Scientific) and searched against the UniProt database for the *S. cerevisiae* Proteome 2020_03 together with a common contaminant database from MaxQuant (version 1.6.2.6, Max Planck Institute of Biochemistry). The Sequest HT search engine was used to identify tryptic peptides. Peptide confidence was set to high. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target FDR 1%, validation based on q-value. Protein label free quantitation was performed with the Minora feature detector node at the processing step. Precursor ions quantification was performing at the processing step with the following parameters: Peptides to use unique plus razor, precursor abundance based on intensity and normalization based on total peptide amount.

3.5 Western Blotting Analysis

For protein extraction, cells were harvested by centrifugation, washed and the pellets were suspended in phosphate buffer [50mM KH_2PO_4, 0.1mM EDTA, 1% (vol/vol) Triton, pH7.0] in the presence of protease inhibitors. Cells were lysed by vigorous shaking of the cell suspension in the presence of zirconia beads (short pulses of 1 min with 1 min intervals on ice), following centrifugation at 13400 rpm for 10 min, at 4ºC. Supernatants were collected and total protein concentration was determined using the Lowry method using bovine serum albumin as standard.

For loading, Laemmli sample buffer 4x (4% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue and 0.125M Tris HCl, pH 6.8 and 20% (v/v) β-mercaptoethanol) was added to 40 μg of protein samples and boiled for 5 min at 95ºC. Samples were loaded into a 12% SDS-PAGE gel, run at 70 V and electroblotted into a nitrocellulose membrane (Hybond-

ECL, GE Healthcare) at 50 mA for 1 hour. Pre-stained molecular weight marker proteins were used as standards for SDS-PAGE. Ponceau staining was performed to verify the quality of the transfer.

Membranes were blocked with 5% (w/v) dry milk in TBS (Tris-buffered saline) with 0.05% (v/v) Tween-20 (TTBS) [20 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween-20 (Merck), pH 7.6] for 1 hour at room temperature. Membranes were incubated o.n. at 4ºC with primary antibodies: anti-Atp2 (1:8000; Abcam), anti-Pgk1 (1:15000; ThermoFisher Scientific), anti-c-Myc (1:100, ThermoFisher Scientific) or anti-Cdc28 (1:50; Santa Cruz Biotechnology). After removing the primary antibodies, membranes were washed with TTBS and probed with appropriate secondary antibodies, anti-rabbit IgG (1:7000, Sigma) and anti-mouse IgG (1:6000, Molecular probes), for 1 hour at room temperature. After washing the membranes with TTBS, immunodetection was achieved by chemiluminescence using WesternBright™ ECL detection kit (Advansta), followed by exposure of membranes to LucentBlue X-ray films (Advansta).

A densitometry analysis was performed using Quantity One 1-D Analysis Software (Bio-Rad).

3.6 Oxygen consumption rate measurements

The oxygen consumption was measured polarographically in whole cells (1×10^8 in PBS buffer) from cultures grown in YPD media to late log phase, using a Clark-type oxygen electrode coupled to an Oxygraph plus system (Hansatech). Data were analyzed using the OxyTrace+ software. The respiratory rate was obtained by dividing the cyanide-sensitive oxygen consumption per min by the number of cells. The respiratory rate in the presence and absence of the inhibitor potassium cyanide (700 µM) was obtained by dividing the oxygen consumed per min by the number of cells used in the experiment.

3.7 Growth curve

In other to monitor growth for wt, *rpn4*Δ, *pdr3*Δ, *gcn4*Δ, *yap1*Δ, *cdh1*Δ, *rpn4*Δ*cdh1*Δ, *pdr3*Δ*cdh1*Δ, *gcn4*Δ*cdh1*Δ and *yap1*Δ*cdh1*Δ strains were pre-grown in YPD and used to inoculate a culture at OD_{600}=0.004 in YPGal. Cultures were incubated o.n and growth was then monitored for 24 hours by OD_{600} measurements, with initial OD_{600}=0.004.

3.8 H_2O_2 resistance

Wt, *cdh1*Δ, *yap1*Δ, *yap1*Δ*cdh1*Δ strains were grown o.n at 26ºC in YPD until OD_{600}=0.5. Each culture was then diluted to OD_{600}=0.1 and ten-fold dilutions were performed using PBS buffer. 7 µL of cells were spotted in YPD plates with 0, 0.75,1 and 2 mM of H_2O_2. Plates were incubated for 2 days at 26ºC.

3.9 β-Galactosidase assay

To evaluate Yap1 activity, wt and *cdh1*Δ cells were transformed with the vector pRS415-AP-1-CYC1-LacZ and 4 different colonies were evaluated for β-galactosidase activity. For that, cells were grown o.n in SC-glucose medium lacking leucine to exponential phase, and the β-galactosidase activity was measured in a liquid assay using o-nitrophenyl-β-D-galactoside (ONPG), as previously described (Ausubel et al., 1998), with the following modifications: a cellular extract was prepared in 100 mM Tris-HCl, 1 mM DTT, 10% (v/v) glycerol, and 40 µg of total protein was used in the assay.

3.10 Cell cycle arrest and Cdh1-m11 overexpression

To obtain synchronized cells, *bar1*Δ mutant strains were grown in YPD to OD_{600}=0.3 and arrested in G1 phase with 100 ng/ml α-factor for 3 hours at 30ºC, in S phase with 200 mM hydroxyurea for 4 hours at 30 ºC or in G2/M phase with 10 µg/mL nocodazole for 4 hours at 30ºC. Then, cycling, and arrested cells were collected by centrifugation at 4200 rpm for 2 min.

To obtain G1-synchronized cells using conditions other than alpha factor treatment, Gl arrest was achieved using temperature-sensitive mutants *cdc28-1* and *cdc28-1cdh1*Δ. These ts mutants were grown o.n. in YPD at 25 ºC to OD_{600} =0.3. Cells were incubated at restrictive temperature, 37°C, during 3 hours. After that time, cycloheximide (100 µg/mL) was added to arrest translation, cells were harvested at 0, 15, 30 and 60 min. by centrifugation at 4200 rpm for 2 min.

To induce Cdh1-m11 overexpression, cells carrying p416-*GALL-HA-CDH1-m11* or empty vector were grown o.n. in SC medium lacking leucine and uracil with 2% raffinose to

OD_{600}=0.5 and expression induced by adding 4% Galactose. Cells were harvested 0, 2, 3 and 4 hours after galactose addition, by centrifugation at 4200 rpm for 2 min.

3.11 Software used

STRING v11.0 (https://string-db.org/, accessed: 7 April 2021), a database of known and predicted protein-protein interactions, was used to build interaction maps and to establish a gene-ontology analysis between altered proteins obtained from the proteomics analysis of mitochondrial extracts (Szklarczyk et al., 2021). On the interaction maps, proteins are shown as nodes and the existence of interactions between them are represented by edges (connection between nodes). Edge thickness indicates the strength of the different interactions. Nodes with the same color represent specific clusters. This analysis was made using a medium confidence (0.400) and a PPI enrichment p-value $< 1.0\times 10^{-16}$.

In order to identify transcription factors for these upregulated and downregulated proteins identified by the proteomics approach, a database of yeast transcription regulatory associations, YEASTRACT+, was used (http://www.yeastract.com/index.php, accessed: 15 March 2021) (Monteiro et al., 2020).

Search on canonical recognition motifs was conducted using the APC/C recognition motif prediction software GPS-ARM v1.0, using a high threshold for D-box and low threshold for KEN-box (accessed: 1 March 2021) (Xue et al., 2005).

Statistical analysis was performed using GraphPad Prism v9.00.

4. Results and Discussion

4.1. Proteomic characterization of mitochondria from *cdh1*Δ cells

To evaluate the impact of Cdh1p on yeast mitochondria, *CDH1*-deleted and wt (control) mitochondrial proteome were analyzed by mass spectrometry. For that, cultures were grown under semi-respiratory conditions, using galactose as a carbon source, to obtain a higher mitochondrial mass. Since we already knew Atp2 is increased in *cdh1*Δ cells, we used the levels of this protein to establish the maximal growth phase in which the increase in Atp2 levels is observed. Since APC/C^{Cdh1} is involved in cell division, it is expected to be more active in exponential actively dividing cells. To select the growth phase for mitochondrial extraction, Atp2 levels in total protein extracts from wt and *cdh1*Δ were analyzed by Western blotting at different OD_{600}. Atp2 levels were higher in *cdh1*Δ cells compared to wt strain until mid-log phase (OD_{600}~2) (Figure 8). Unexpectedly, after OD_{600}~3 the tendency is inverted, with Atp2 levels decreasing in *cdh1*Δ cells. Thus, for this work, samples were collected at OD_{600}=1.4, in which differences were maximal, in triplicates.

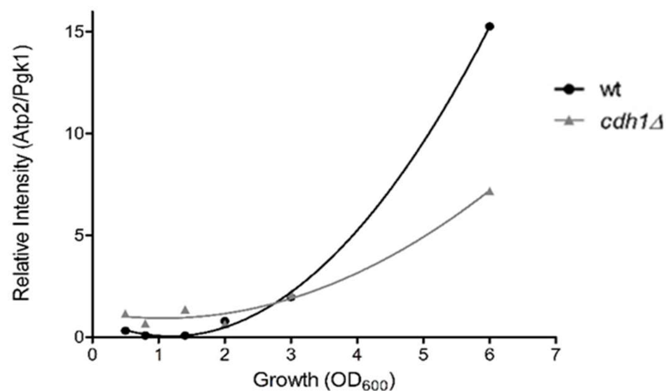

Figure 8. Atp2 levels in wt and *cdh1*Δ analyzed along growth by Western blotting. OD_{600}=1.4 is the growth phase in which Atp2 levels are most augmented in *cdh1*Δ cells compared to wt cells.

Although we observed higher Atp2p levels for *cdh1*Δ cells compared to wt strain in log phase cells, it appears to reverse the effect on Atp2p at higher growth phases. Preliminary data

on oxygen consumption (not shown) seems to confirm this trend, suggesting that in low-proliferating cells, Cdh1 may have an opposite effect on mitochondrial function. Interestingly in yeast, Cdh1 has a role in replicative lifespan (Smith et al., 2008). This suggests that likewise in post-mitotic mammalian cells (Almeida et al., 2005; Gieffers et al., 1999), Cdh1 may play a role in non-dividing yeast cells.

For mitochondrial extraction, the cell wall was digested enzymatically with Zymolyase-20T, and mitochondria were then isolated from the spheroplasts by differential centrifugation. Mitochondrial samples were then analyzed by high-resolution mass spectrometry (HPLC-MS/MS). Quantification of mitochondrial proteins was performed with normalization based on total peptide amount. A total of 932 mitochondrial proteins were identified, representing a coverage of 90-100%, depending on the reference proteome used (Vögtle et al., 2017; Morgenstern et al., 2017). A Student t-test was used to identify differential protein expression between *cdh1*Δ and wt cells. To analyze the changes in protein abundance, a cutoff of p-value <0.05 was applied. A total of 279 proteins were significantly regulated in the absence of *CDH1*, with 110 up-regulated proteins (12%) and 169 down-regulated proteins (18%) (Figure 9A).

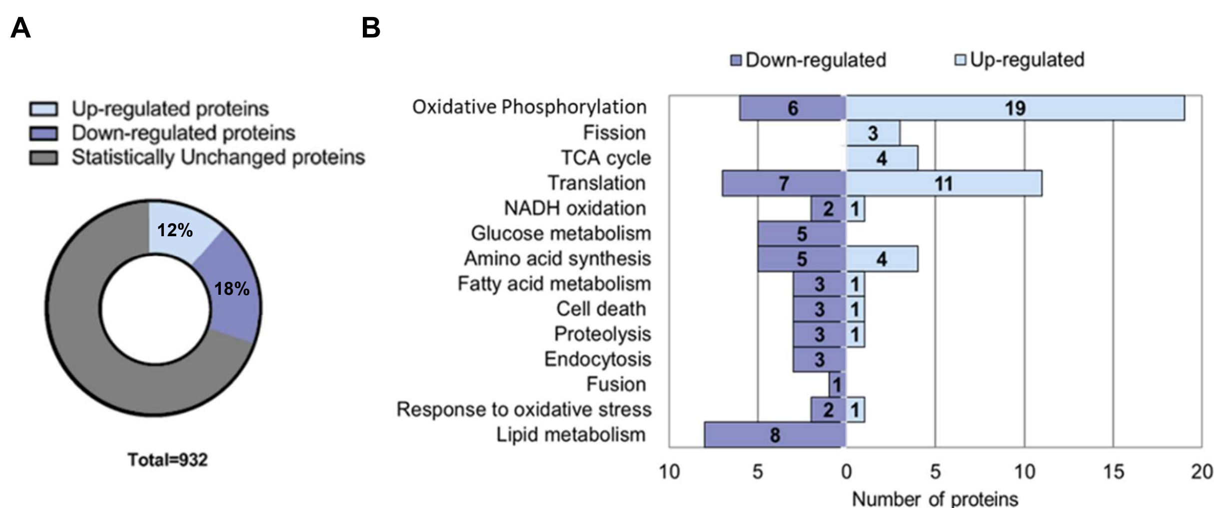

Figure 9. Proteome analysis of mitochondrial extracts in the absence of Cdh1. (A) Up-regulated proteins (p-value>0.05) and down-regulated proteins (p-value <0.05) in the absence of *CDH1*. (B) Gene ontology-term enrichment analysis on biological processes made for statistically changed proteins using STRING v11.0.

To make the analysis more stringent, log2 fold changes (log2FC) of the statistically significantly different proteins between *cdh1*Δ and control cells was calculated and a cutoff of log2FC>0.25 was set. This analysis resulted in 58 proteins being up-regulated (6%) and 105 down-regulated (11%).

To determine the possible trends in processes being regulated in the absence of Cdh1, a gene ontology-term enrichment analysis on biological processes was made using STRING v.11.0 database (Figure 9B). This analysis showed that among the 58 up-regulated proteins the most represented processes were: oxidative phosphorylation, including proteins from all the respiratory complexes, the Krebs cycle, and mitochondrial fission, including Dnm1 (Drp1 in mammals) and Fis1. On the other hand, the 105 down-regulated proteins include mostly unrelated proteins, with lipid metabolism, mitochondrial translation, amino acid synthesis and mitochondrial fusion being the most relevant down-regulated biological processes.

Additionally, two interaction maps (one for up-regulated proteins and one for down-regulated proteins) were built using the STRING v.11.0 database (Figure 10 and 11). For the up-regulated proteins (Figure 10) we can observe 3 major clusters: proteins associated with the respiratory chain (in red), proteins associated with translation (in yellow), proteins associated with fission (in blue). For the down-regulated proteins (Figure 11) we can observe various clusters: proteins associated with glucose metabolism (in red), proteins associated with complex assemble (in light green) and proteins associated with endocytosis (in light blue).

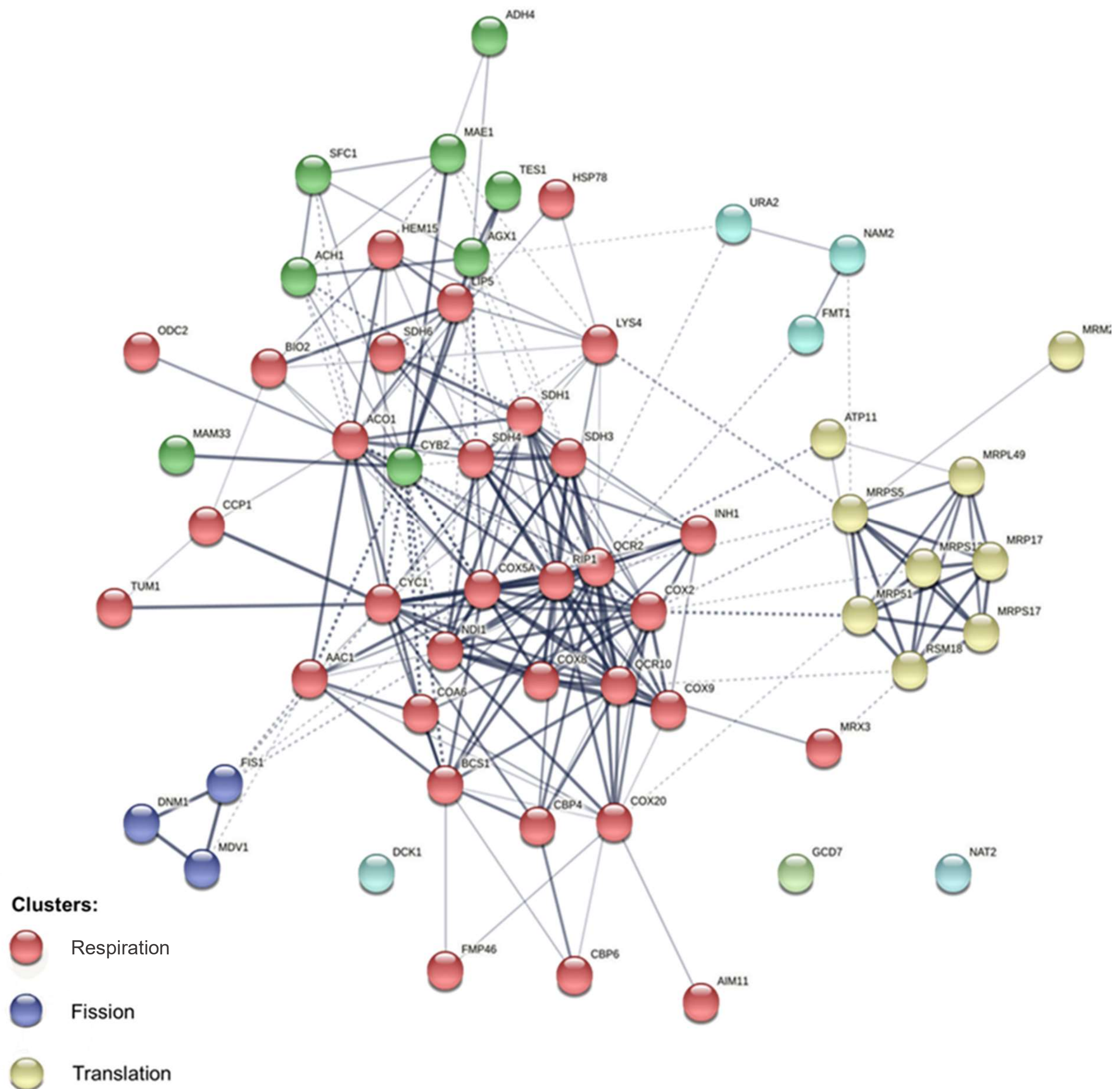

Figure 10. Proteins upregulated in the absence of Cdh1p are involved in the respiratory chain, the Krebs cycle and mitochondrial fission. Interaction network map built using STRING v11.0 based on the 58 up-regulated proteins detected in *S. cerevisiae cdh1*Δ strain. Proteins are shown as nodes and the existence of interactions between them are represented by edges (connection between nodes). Edge thickness indicates the strength of the different interactions. Nodes with the same color represent specific clusters. PPI enrichment p-value < 1.0×10^{-16}.

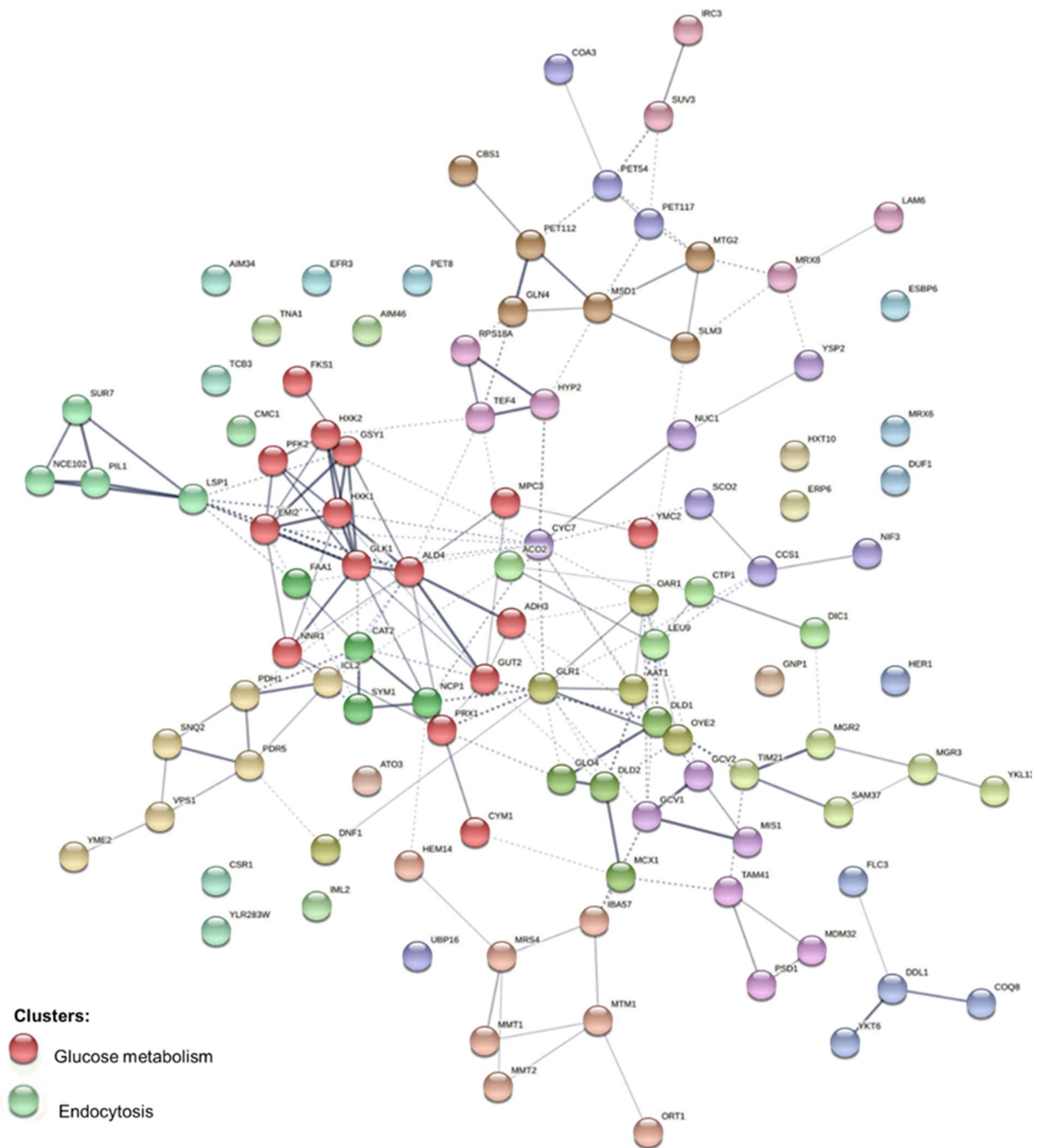

Figure 11. Proteins downregulated in the absence of Cdh1p are involved in various processes. Interaction network map built using STRING v11.0 based on the 105 down-regulated proteins in detected in *S. cerevisiae cdh1*Δ strain. Proteins are shown as nodes and the existence of interactions between them are represented by edges (connection between nodes). Edge thickness indicates the strength of the different interactions. Nodes with the same color represent specific clusters. PPI enrichment p-value < 1.0×10^{-16}.

These results demonstrate up-regulation of proteins involved in energy generation, such as the respiratory chain and Krebs cycle, as previously reported (Di Bartolomeo et al., 2020) for respiratory conditions. Consistent with our results it was also reported that proteins implicated in lipid metabolism and amino acid synthesis are downregulated in transitioning to respiratory phase (Casanovas et al., 2015; Di Bartolomeo et al., 2020). Thus, the gene-ontology analysis of altered proteins in the absence of Cdh1 points to a metabolic remodeling towards an increased respiratory metabolism. The only exception is that mitochondrial fusion is usually associated with respiratory metabolism and fission with fermentable conditions (Westermann, 2012) and we observe the opposite. This may be related with the fact that respiratory growth is usually studied at diauxic shift, in which cells are growth slowly and mitochondrial fusion usually occurs, and our studies focus on a potential effect in mitochondrial respiration associated to rapid cell division, in which mitochondrial fission usually predominates.

4.2 Identification of potential APC/C^{Cdh1} targets

Our initial hypothesis was that Cdh1 could target directly mitochondrial proteins, sending them for degradation. In line with this, in the absence of Cdh1, proteins with increased protein levels would potentially be Cdh1 targets. However, considering our results, in which we observed a high number of altered proteins, many downregulated, hence not direct targets, we postulated that Cdh1 may target mitochondrial proteins indirectly, through the regulation of transcription factors (TFs). To address this hypothesis, we searched for the signature of TFs that could be regulating the up and downregulated proteins in the *cdh1*Δ strain. For that, we used YEASTRACT+, a data base of yeast transcription regulatory associations (http://www.yeastract.com/index.php, accessed: 15 March 2021) (Monteiro et al., 2020). Interestingly, the isolated top 4 potential regulatory transcription factors identified for the *cdh1*Δ altered proteins were the same for the up and downregulated proteins (Table 3). This indicates that it was possible to regulate the levels of all the identified altered proteins in *cdh1*Δ cells by regulating a single TF.

Table 3. TFs Rpn4, Yap1, Pdr3 and Gcn4 regulate all proteins altered in *cdh1*Δ cells. Transcription factors identified for *cdh1*Δ up- and down- regulated proteins using Yeastract+ repository, using a > 94% coverage.

Transcription Factor	% in upregulated proteins	p-value	% in downregulated proteins	p-value
Rpn4	96.6	0.0001	94.3	0.00004
Yap1	96.2	0.0006	96.2	3.96×10^{-10}
Pdr3	94.5	0.0029	94.5	0.0018
Gcn4	94.8	0.0045	100.0	0.0000282

These TFs include: Rpn4, that stimulates expression of proteasome genes in response to stress (Xie & Varshavsky, 2001); Yap1, major factor involved in oxidative stress tolerance (Ikner & Shiozaki, 2005); Gcn4; transcriptional activator of amino acid biosynthetic genes (Hinnebusch & Natarajan, 2002) and Pdr3, transcriptional activator of the pleiotropic drug resistance network. (Delaveau et al., 1994). Remarkably, the TF Pdr3 was previously proposed as a Cdh1p interactor (Ostapenko et al., 2012). Therefore, we investigated the hypothesis that the targeting of transcription factors by APC/C^{Cdh1} may account for most of the mitochondrial alterations induced by Cdh1.

4.2.1 Identification of TFs with a role in *cdh1*Δ-induced mitochondrial respiration

The altered mitochondrial proteins identified in the proteomic analysis suggest an increase in respiratory metabolism in *cdh1*Δ cells. This result was confirmed by measuring oxygen consumption in whole cells of wt and *cdh1*Δ, using a Clark-type oxygen electrode. In cells grown to log phase, we observed an 20% increase in mitochondrial respiration in *cdh1Δ* cells (Figure 12A). In line with our hypothesis, that TFs mediate the role of Cdh1 in mitochondrial regulation, we predicted that its downregulation should suppress the *cdh1*Δ increase in mitochondrial respiration. For that, strains deleted both in *CDH1* and individual TFs were constructed. In addition to *cdh1*Δ cells, we found the absence of Yap1p alone also lead to a significant increase in oxygen consumption.

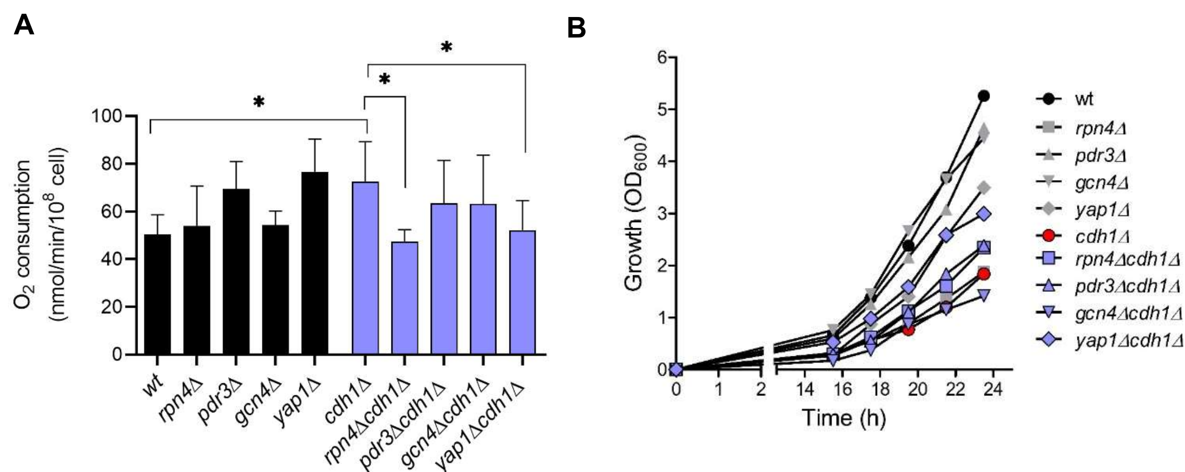

Figure 12. Transcription factors Rpn4p and Yap1p are involved in the *cdh1*Δ-increase in mitochondrial respiration. (A) The oxygen consumption was measured polarographically in whole cells from cultures grown to late log phase. The respiratory rate was obtained by dividing the cyanide-sensitive oxygen consumption per min by the number of cells. Values are the mean ± SEM (*n*=4); *, $p < 0.05$; one-way ANOVA (B) The growth of the indicated strains was monitored by measuring OD_{600} over time. Values are the mean (*n*=3); * p<0.05: wt vs *cdh1*Δ; wt vs *rpn4*Δ *cdh1*Δ; wt vs *pdr3*Δ *cdh1*Δ and wt vs *gcn4*Δ*cdh1*Δ, n.s.: wt vs *yap1*Δ*cdh1*Δ; Welch´s t test of the area under the curve.

Notably, the high mitochondrial oxygen consumption in cells lacking *CDH1* decreased upon *RPN4* and *YAP1* deletion but not upon *PDR3* and *GCN4* deletion (Figure 12A). These results suggest that the transcription factors Rpn4p and Yap1p are involved in the *cdh1*Δ-increase in mitochondrial respiration. Interestingly, Rpn4p is a downstream target of Yap1p (Yokoyama et al., 2006), suggesting Yap1p pathway is important for Cdh1p-mediated effects in mitochondria. In addition, the growth curves of single deleted TF and TF *CDH1* double mutants were analyzed. Deletion of *CDH1* resulted in a significant slow growth (Figure 12B), which was previously attributed to the accumulation of substrates important for cell cycle progression such as Clb2p and Ase1p (Visintin et al., 1997). The slow growth of *cdh1*Δ cells was not significantly affected by *GCN4, PDR3* and *RPN4* deletion but is decreased by *YAP1*

deletion (Figure 12B). This result indicate *CDH1* interacts genetically with *YAP1*, suggesting a functional relation.

Since our results point to Yap1p and Rpn4p as Cdh1p downstream effectors, their role was further evaluated. For that, we evaluated Atp2p levels (which increased in *cdh1Δ* cells) in total protein extracts from *cdh1*Δ, *yap1*Δ and *rpn*4 single and double mutants, were analyzed Western blotting. Our results demonstrate that the deletion of both *RPN4* and *YAP1* lead to a decrease in Atp2 levels in *cdh1*Δ cells, though only statistically significant for *RPN4* (Figure 13). Together with the results from mitochondrial respiration, these results confirm that *YAP1* and *RPN4* act downstream Cdh1 in the control of mitochondrial protein levels.

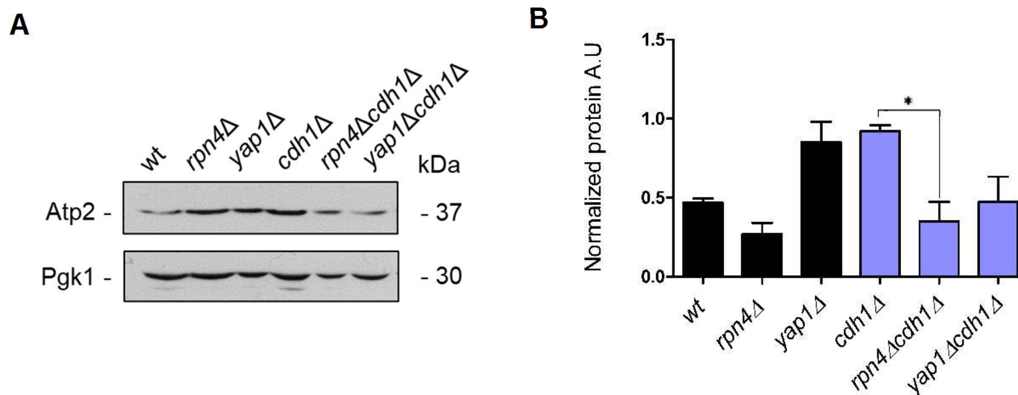

Figure 13. Deletion of both *RPN4* and *YAP1* in *cdh1*Δ cells lead to a decrease in Atp2p levels. Immunodetection of Atp2p on total proteins extracts of *rpn4*Δ, *yap1* Δ, *rpn4*Δ*cdh1*Δ and *yap1*Δ*cdh1*Δ cells. Pgk1p is shown as loading control. Quantification of data is shown in (B).Values are the mean ± SEM (*n*=4);*, p<0.05; one way ANOVA; Tukey's test.

4.2.2 Searching of APC/C recognition motifs in TFs

To assess if Rpn4 and Yap1 are targets of Cdh1, we searched for the presence of APC/C^{Cdh1} recognition motifs using the APC/C recognition motif prediction software GPS-ARM 1.0. This software only predicts two canonical degrons: destruction box (D-box, RXXLXXXXN/D/E, where X indicates any amino acid) and the KEN-box (XXKENXXXX). This search revealed that Rpn4 contains one KEN-box and one D-box and Yap1p has one D-

box (Figure 14), suggesting that Rpn4 and Yap1 may be APC/C^{Cdh1} targets. However, the APC/C motifs are very common in the proteome (Pfleger & Kirschner, 2000) and, thus, need to be experimentally validated.

Figure 14. Rpn4 and Yap1 contain common recognition motifs of APC/C^{Cdh1}. (A) Sequence preference of Rpn4 degrons. (B) Sequence preference of Yap1 degron.

Despite both Rpn4 and Yap1 being promising APC/C^{Cdh1} targets, Yap1 was selected to be further evaluated as a potential Cdh1 target. This was based on the fact that the deletion of *YAP1* had a stronger impact in *cdh1*Δ growth and *YAP1* overexpression was already reported to cause cell cycle arrest (Niu et al., 2008) showing this TF has a role in cell cycle. In addition, Rpn4 has a very short half-life ($t_{1/2} \approx 2$ min) (Xie & Varshavsky, 2001), making it more unlikely to be regulated during cell cycle progression.

4.3 Evaluating Yap1 as a potential APC/C^{Cdh1} target

4.3.1 Yap1 activity in *CDH1*-deleted cells

Yap1 is a major oxidative stress regulator in yeast (Rodrigues-Pousada et al., 2008). If APC/C^{Cdh1} targets Yap1 for degradation, Yap1 should be more abundant and active in the absence of Cdh1, leading to an increased oxidative stress resistance. To test this hypothesis, we evaluated *cdh1*Δ cell growth in the presence of an oxidative stress inducer, H_2O_2. In *yap1*Δ, we found a strong decrease in H_2O_2 resistance, as expected due to its vital role in the transcriptional response to oxidative stress. In the absence of Cdh1p, cells presented a higher oxidative stress

resistance, compared with wild-type cells, particularly evident at the higher H_2O_2 concentrations. This increased resistance of *cdh1*Δ cells is dependent on Yap1 since *YAP1* deletion restored H_2O_2 sensitivity (Figure 15A).

To assess changes in Yap1 activity, wt and *cdh1*Δ cells were transformed with a *YAP1*-lacZ reporter plasmid that contains the bacterial LacZ coding region under the control of three Yap1 binding sites (Maeta et al., 2004). The reporter gene LacZ, encodes the enzyme β-galactosidase, that will be quantitatively produced upon Yap1p activation and give origin to a colorimetric product. To measure Yap1-responsive β-galactosidase activity, cells were grown to exponential phase and collected in the absence of any stressor. The *cdh1*Δ strain showed an increase in β-galactosidase activity (1.5-fold) compared to control cells (Figure 15B). These results indicate that Yap1 transcriptional activity increases in cells lacking Cdh1p, which is in accordance with our hypothesis that Yap1 is a target of APC/C^{Cdh1}.

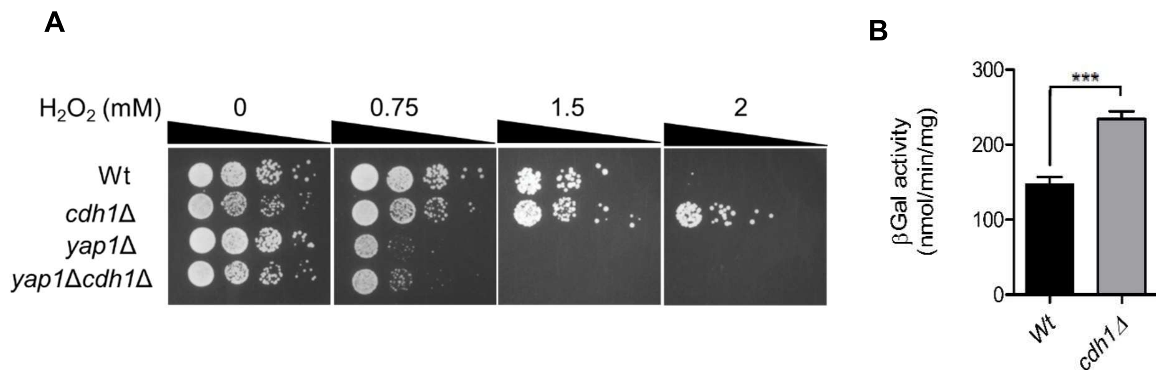

Figure 15. Oxidative stress resistance and Yap1 activity increases in Cdh1 deficient cells. (A) Tenfold serial dilutions of wt, *cdh1*Δ, *yap1*Δ and *yap1*Δ*cdh1*Δ strains were plated in rich solid media (YPD) in the absence or presence of 0.75, 1.5 and 2 mM H2O2. (B) wt and *cdh1*Δ cells expressing a *YAP1*-LacZ reporter were grown to exponential phase and β-galactosidase activity was determined. Values are the mean ± SEM (*n*=4); ***, $p < 0.001$, Student's t-test.

4.3.2 Yap1p levels in cell cycle phases

Since APC^{Cdh1} only becomes active from late mitosis to G1, proteolysis of its targets is expected to occur in the G1 phase, leading to a decrease in its levels. To further evaluate Yap1p

as potential APC^{Cdh1} target, Yap1 protein levels in different cell cycle phases or in normal growing exponential cells (cycling) was analyzed by Western blotting. For that, we used *bar1*Δ mutants (Bar1 cleaves and inactivates α-factor), transformed with pRS315-Yap1-9Myc. To obtain synchronized cells, early exponential phase cells were treated for 3h with α-factor, 4h with hydroxyurea, or 4h with nocodazole, which arrest cells in G1, S and G2/M phase, respectively. Hydroxyurea induces a DNA replication checkpoint arrest in S phase by deoxyribonucleotide synthesis inhibition (Weinert et al., 1994), whereas nocodazole induces checkpoint arrest at G2/M phase by microtubule polymerization inhibition (Jacobs et al., 1988). The results show that Yap1 levels significantly decreased in the G1 phase (Figure 16), supporting Yap1 as a putative substrate of APC/C$^{Cdh1.}$

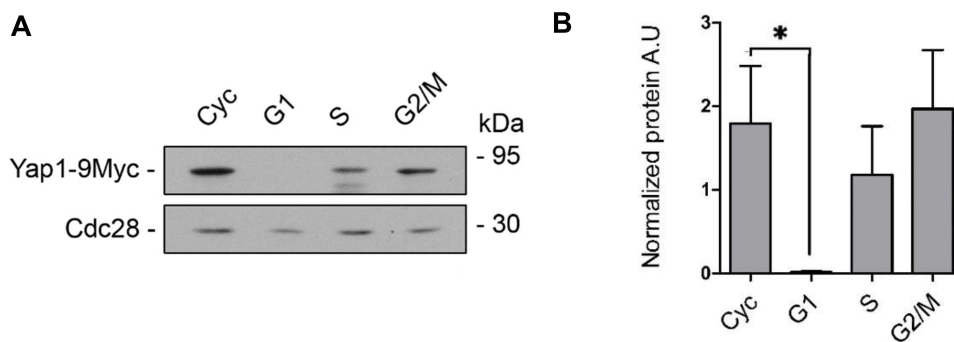

Figure 16. Yap1p levels significantly decreases in G1 phase, supporting Yap1p as a putative APC/C^{Cdh1} substrate. (A). Immunodetection of Yap1p on total proteins extracts of cycling cells (cyc) and cells treated with α-factor (G1), hydroxyurea (S) or nocodazole (G2/M). Cdc28, a protein constant during cell cycle, is shown as loading control. Quantification of data is shown in (B). Values are the mean ± SEM (*n*=4);**, $p<0.01$; one way ANOVA; Tukey's test.

4.3.3 Establishing methods to evaluate Yap1 levels upon *CDH1* deletion/overexpression

If Yap1 was degraded in G1-arrested cells at G1 due to APC/C^{Cdh1} activity, *CDH1* deletion should increase Yap1 protein levels and Cdh1 overexpression decrease Yap1 protein levels. Since *cdh1*Δ cells cannot be efficiently synchronized with α-factor (Amon et al., 1994;Irniger & Nasmyth, 1997), we generated a *cdc28-1cdh1*Δ strain by deleting *CDH1* in a

cdc28-1 ts mutant, that arrests at G1 upon shifting to 37 °C. Wt cells were first synchronized with *cdc28-1* at 37 ºC, cycloheximide was added to prevent translation, and Yap1 levels throughout 60 min or 5h in wt non-synchronized cells and analyzed by Western blotting. Yap1 half-life was strongly decreased in *cdc28-1* strain (Figure 17B), compared to non-synchronized cells (Figure 17A), confirming the Yap1 instability at G1 phase achieved by synchronization with α-factor (Figure 16). Further work is required to optimize time points in *cdc28-1* cells at which Yap1 half-life is obtained, to test in these conditions if Yap1 is more stable in the *cdc28-1cdh1*Δ mutant.

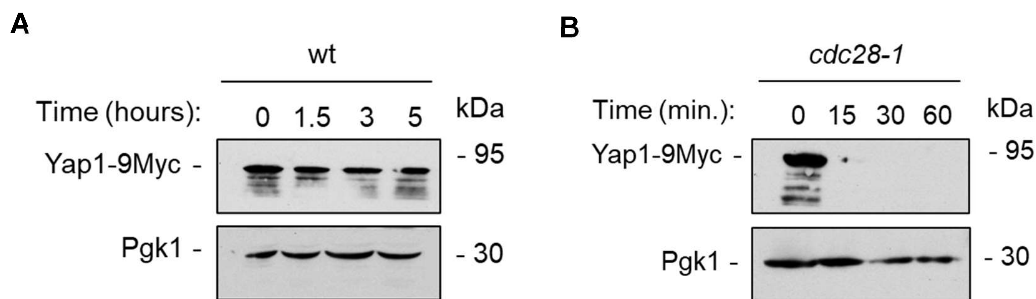

Figure 17. Yap1p half-life decreased in cells synchronized in G1, compared to non-synchronized cells. Preliminary data (n=1) of immunodetection of Yap1 on total proteins extracts of: (A) wt cells harvested at 0, 1.5, 3 and 6 h after cycloheximide addition. Pgk1 is shown as loading control. (B) *cdc28-1* cells synchronized in G1, harvested at 0, 15, 30 and 60 min. after cycloheximide addition.

To evaluate the effect of Cdh1 overactivation in Yap1 levels, simple *CDH1* overexpression is not efficient, since Cdh1 activity is inhibited by phosphorylation by cyclin-dependent kinase (CDK) in 11 residues (Robbins & Cross, 2010). As such, to achieve Cdh1 overactivation, we used an allele, Cdh1-m11, that lacks all 11 CDK phosphorylation sites (Ser/Thr sites were changed to Ala at the following positions: 12, 16, 42, 157, 169, 173, 176, 227, 239, 418, 436) (Zachariae et al., 1998). To assess if Cdh1 overactivation decreases Yap1 levels, cells were transformed with p416-*GALL-HA-CDH1-m11* or empty vector and grown in galactose to induce Cdh1-m11 expression. Yap1 levels analyzed measured by Western blotting. For shorter time points (until 90 min) we found no differences in Yap1 levels (not shown). When

increasing the overexpression for longer times, preliminary results suggest overexpressing CDH1-m11 leads to a decrease in Yap1 levels over time compared to empty vector control cells (Figure 18). Further work will be needed to confirm this result.

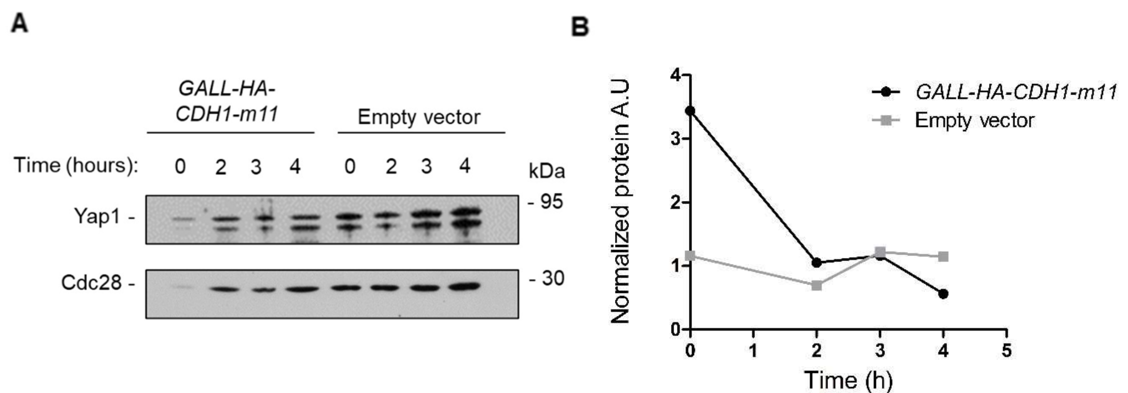

Figure 18. Overexpressing Cdh1-m11 leads to a decrease in Yap1 levels. Preliminary data (n=1) of immunodetection of Yap1p on total proteins extracts of CDH1-m11 overexpressing and empty vector cells over time after induction with 4% galactose for indicated times. Cdc28 is shown as loading control. Quantification is shown in (B).

5. Conclusions and Future Perspectives

In this work, we investigated the role of APC/C^{Cdh1} in the control of mitochondrial function using a proteomic approach. APC/C^{Cdh1} has a well-known role in ubiquitination of cell cycle substrates, regulating cell cycle processes such as G1/S transition and mitotic exit. Besides its function in cell cycle regulation, Cdh1 has been reported to have a role in different processes such as the regulation of mitochondrial morphology (Horn et al., 2011).

We investigated the role of Cdh1 in mitochondrial proteome and found both up- and down-regulated proteins in the absence of Cdh1, suggesting Cdh1 has a broader impact on mitochondrial protein content than currently known. The fact that we observed a high percentage of down-regulated proteins also indicates Cdh1 is not targeting the mitochondrial proteins directly. We found 4 TFs, Rpn4, Pdr3, Yap1 and Gcn4 that could be regulating the expression of proteins altered in *cdh1*Δ cells. Supporting our analysis, the TF Pdr3 was

previously reported as a Cdh1 interactor and potential target (Ostapenko et al., 2012).

The mitochondrial remodeling towards a respiratory metabolism in *cdh1*Δ cells as suggested by the proteomic analysis, was confirmed by measuring oxygen consumption in whole cells of wt and *cdh1*Δ. Since the proteomic analysis was performed using the same amount of mitochondria, further evaluation of mitochondrial size and mass in whole cells is required to assess if Cdh1 may also play a role in mitochondrial biogenesis. Previous studies have shown that mitochondrial respiration is synchronized with cell cycle progression (Harbauer et al., 2014; Shiota et al., 2015). Our results suggest that APC/C^{Cdh1} regulation of Yap1 may contribute to this coordination of mitochondrial function with the cell cycle progression. Indeed, the high mitochondrial oxygen consumption in *cdh1*Δ cells decreases upon deletion of *YAP1* or *RPN4*, a downstream target of Yap1 (Yokoyama et al., 2006), suggesting that these transcription factors are involved in the induction of mitochondrial respiration in *cdh1*Δ cells.

Our data is consistent with Yap1 being a direct target of APC^{Cdh1}, since Yap1 activity increases in cells lacking Cdh1 and it is more unstable at G1 phase, a cell cycle phase in which Cdh1 is more active. Moreover, *YAP1* expression is not cell cycle regulated (Spellman et al., 1998), confirming that the decrease of Yap1 protein levels at G1 are due to an increase in Yap1 degradation. Our hypothesis that Yap1 is a target of Cdh1 and may play a role during the cell cycle, is supported by previous studies showing that *YAP1* deletion (Yi et al., 2016) or overexpression (Niu et al., 2008) cause cell cycle arrest in S phase and G2/M phase, respectively. To confirm Yap1 as Cdh1p substrate, Yap1 levels upon Cdh1 deletion/overexpression will be evaluated. Notably, we found that Yap1 contains one D-box, a recognition motif for Cdh1. As such, Yap1 should be further confirmed as APC/C^{Cdh1} target by mutating the conserved residues of the D-box by site-direct mutagenesis and evaluating its impact on Yap1 protein stability. In addition, co-immunoprecipitation experiments should be conducted.

Yap1 is a transcription factor activated under oxidative stress conditions, that triggers the transcription of genes encoding antioxidant defenses during the diauxic shift, by delocalization from the cytoplasm to the nucleus (Ikner & Shiozaki, 2005). Oxidative stress is caused by ROS, and in most types of cells the main source of ROS is the mitochondria (Gray, 2013). High concentrations of ROS cause oxidative damage to nucleic acids, proteins, and lipids

(Cooke et al., 2003; Dickinson & Chang, 2011). In fact, ROS induce the accumulation of double-strand breaks (DSBs) that becomes more accentuated in the absence of Yap1, indicating that Yap1 can have a rule in the cell cycle as a DNA damage response molecule (Yi et al., 2016). Furthermore, it has been reported that oxidative stress, both in yeast and humans, leads to an arrest in G1, allowing for cellular damage removal and/or repair (Brien & Dawes, 1998; Alic et al., 2001). It has also been noted that in synchronized cells the production of ROS increases during the cell cycle, with a peak occurring in mitosis (Havens et al., 2006; Patterson et al., 2019). Considering the role of Yap1 in oxidative stress tolerance and the increase in ROS production with mitochondrial respiration, we suggest that Yap1 regulates mitochondrial respiration and transcription of genes involved in oxidative stress tolerance simultaneously to mitigate the oxidative damage to the cell. Our results regarding Yap1 degradation support this hypothesis, since mitochondrial respiration increases in G2/M transition (Harbauer et al., 2014; Wang et al., 2014), where we observed Yap1 is more stable (Wang et al., 2014). Additionally, Yap1 degradation has been indicated as a mechanism that assures a G2 delay in response to a stress, supporting the Yap1 role in the regulation of G2 phase (Yokoyama et al., 2006). Taken all together, Cdh1 may regulate Yap1 during cell cycle, ensuring that higher respiration is accompanied by increased antioxidant enzyme expression. This is supported by our observation that cells are more resistant to oxidative stress in the absence of Cdh1 in a Yap1-dependent manner. This hypothesis should be confirmed by measuring the expression of Yap1-regulated genes (e.g., SOD1, TRX2, GSH1, GLR1) and/or the activity or levels of antioxidant defences (e.g., glutathione, superoxide dismutase).

In summary, our study shows a novel role for Cdh1 in the regulation of mitochondrial respiration, revealing a novel mechanism on how mitochondrial ATP production is coordinated with the energy-demanding cell cycle progression. It also lead to the uncovering of a potential novel Cdh1 substrate, the TF Yap1. The regulation of Yap1 by APC/C^{Cdh1}, may enable to connect the known transient increase in mitochondrial respiration, that fuels the cell cycle, with the expression of antioxidant defenses, crucial in destruction of ROS, byproducts of respiration.

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