UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Programa de Pós-Graduação em Tecnologia Bioquímico-Farmacêutica

CLAUDIA AZNAR ALESSO

“Estudo da função biológica e molecular de *YJL077C* – ORF de função desconhecida – envolvimento na resposta antioxidante e na sensibilidade ao cobre em *Saccharomyces cerevisiae*”.

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Souza.

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“Estudo da função biológica e molecular de *YJL077C* – ORF de função desconhecida – envolvimento na resposta antioxidante e na sensibilidade ao cobre em *Saccharomyces cerevisiae*”.

Comissão Julgadora de Defesa de Mestrado

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Professora Dra. Gisele Monteiro de Souza

1º examinador

2º examinador

São Paulo, de de 2014

Dedico esse trabalho aos
meus pais que me ensinaram
a perseguir meu ideal com
dedicação e coragem.

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"Pesquisar é ver o que outros viram, e
pensar o que nenhum outro pensou."

(Albert Szent-Gyorgyi)

RESUMO

Saccharomyces cerevisiae teve seu seqüenciamento genômico finalizado em 1996, sendo composto de cerca de 6600 ORF's ("open reading frames", quadros abertos de leitura) das quais aproximadamente 20% estão anotadas como não caracterizadas e de função molecular desconhecida. Com o objetivo de caracterizar algumas dessas ORF's relacionadas aos processos antioxidantes, escolhemos a proteína Yjl077cp. Através de nossos experimentos foi possível relacionar a proteína Yjl077cp com a resposta antioxidante, pois a linhagem mutante para o gene *YJL077C* apresentou letalidade quando exposta por 24 horas a uma concentração de 3mM de peróxido de hidrogênio (H₂O₂). Testes de tolerância ao cobre, demonstraram que esse metal na concentração de 10mM, adicionado diretamente ao meio de cultura YPD, após um período de 4 horas de incubação afeta a cinética de crescimento, e após 24 horas de incubação, essa concentração é letal para a linhagem mutante. Observamos que a adição de CuSO₄, causa modificação de pH do meio de cultura YPD, foram realizados experimentos para que verificássemos o efeito da acidificação do meio de cultura na viabilidade de Δ *ics3*, através de experimentos de tolerância em meio YPD ácido (pH 4.0) e com pH corrigido com adição de CuSO₄. De acordo com os resultados obtidos, o efeito da letalidade de 10 mM de CuSO₄ observado sem correção de pH, não ocorre quando em meio de cultura com o pH corrigido (pH6.0), demonstrando que, a letalidade da linhagem Δ *ics3* é causada pelo acréscimo do metal e a mudança de pH (ácido, pH4.0). Testes de ICP-AES detectaram uma maior absorção intracelular de cobre na linhagem mutante Δ *ics3* em condições de pH 4.0, quando comparada a absorção desse mesmo metal em condições de pH 6.0, demonstrando que grandes quantidades de cobre, estão sendo incorporadas para o meio intracelular, devido a acidificação do meio, sugerindo o envolvimento de Δ *ics3* às ações das VATP-ases. Em conjunto esses resultados demonstram que o gene *ICS3* é importante para a manutenção da viabilidade celular de *S. cerevisiae* crescida em meio de cultura com pH ácido e em condições de altas concentrações de CuSO₄.

O objetivo do presente trabalho foi entender a relação de *ICS3* na resposta antioxidante e sensibilidade ao cobre através de experimentos de tolerância e em meio YPD ácido (pH 4.5) e com pH corrigido após adição de CuSO₄.

Palavras chave: *Saccharomyces cerevisiae*; cobre; espécies reativas de oxigênio (ROS); estresse oxidativo; YJL077C (ICS3); V-ATPase.

ABSTRACT

Saccharomyces cerevisiae had its genome sequencing completed in 1996, consisting of about 6600 ORF's ("open reading frames" open reading frames) of which approximately 20% are annotated as uncharacterized and unknown molecular function. In order to characterize some of these ORF's related to antioxidants processes, we chose Yjl077cp protein. Through our experiments it was possible to relate the Yjl077cp protein with the antioxidant response, because the mutant strain to YJL077C gene showed lethality when exposed for 24 hours at a concentration of 3 mM hydrogen peroxide (H₂O₂). Copper tolerance tests demonstrated that this metal at concentration 10mM added directly to the medium YPD culture after a 4-hour period of incubation affects the growth kinetics, and after 24 hours of incubation, these concentration is lethal to mutant strain. We found that the addition of CuSO₄, causes pH modification in the YPD culture medium, experiments were conducted to we check the effect of acidification of the culture medium on the viability of Δ ics3 through experiments of acid tolerance in YPD medium (pH 4.0) and pH adjusted with addition of CuSO₄. According to the results, the effect of mortality of 10 mM CuSO₄ observed without pH correction, does not occur when the culture medium with the pH adjusted (pH6.0), demonstrating that the lethality of the strain Δ ics3 is caused by adding the metal and the change of pH (acid, pH4.0). ICP-OES tests detected greater intracellular uptake of copper in the mutant strain Δ ics3 under conditions of pH 4.0 when compared to absorption of the same metal under conditions of pH 6.0, demonstrating that large amounts of copper, are being incorporated into the intracellular medium due to acidification of the medium, suggesting the involvement of Δ ics3 to the actions of the VATP-ases. Together, these results demonstrate that *ICS3* gene is important for maintaining cell viability of *S. cerevisiae* grown in culture medium at acidic pH, and under conditions of high concentrations of CuSO₄.

The objective of this study was to understand the relationship *ICS3* in antioxidant response and sensitivity to copper through experiments tolerance and YPD medium acid (pH 4.5) and fixed pH after addition of CuSO₄.

Keywords: *Saccharomyces cerevisiae*, copper, reactive oxygen species (ROS), oxidative stress; *YJL077C* (*ICS3*); VATPase.

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LISTA DE SÍMBOLOS

α 1-antitrisina: Glicoproteína de 52KDa

α -sinucleína: Fosfoproteína Pré-Sináptica

a3: Isoforma da Subunidade a da VATP-ase

a4: Isoforma da Subunidade a da VATP-ase

Ace1: Fator de Transcrição Cupro-Dependente

Ace1p: Proteína que Atua como Fator de Transcrição, possui um Domínio Obrigatório de Cobre

AD: Doença de Alzheimer

ALS: Esclerose Amiotrófica Lateral

Amt1p: Proteína que atua como Fator de Transcrição, possui um Domínio Obrigatório de Cobre

ATP: Adenosina Trifosfato

ATx1p: Chaperona em *Saccharomyces cerevisiae*

°C: Graus Celsius

C: Símbolo Químico do Carbono

Ca: Símbolo Químico do Cálcio

Ccc2p: Cupro ATP-ase em *Saccharomyces cerevisiae*

Ccs1: Chaperona em *Saccharomyces cerevisiae*

Cd: Símbolo Químico do Cádmio

Células β : Células Endócrinas nas Ilhotas de Langerhans do Pâncreas responsáveis por Sintetizar o Hormônio Insulina

Citocromo C-oxidase: Cupro Enzima de Membrana Mitocondrial Interna

Co: Símbolo Químico do Cobalto

Coll: Cobalto Divalente

Cox17: Chaperona de Mitocôndria em *Saccharomyces cerevisiae*

CPY: Carboxipeptidase Y

CTNS: Gene Humano que Codifica a Proteína Cistinosina

CTR1: Transportador de Alta Afinidade pelo Cobre em *Saccharomyces cerevisiae*

CTR3: Transportador de Alta Afinidade pelo Cobre em *Saccharomyces cerevisiae*

Cu: Símbolo Químico do Cobre

CuSO₄: Símbolo Químico do Sulfato De Cobre

CuI: Símbolo Químico do Iodeto de Cobre

CuZnSOD: Cobre Zinco Superóxido Dismutase

CysS-SCys: Cistina

DNA: Ácido Desoxirribonucleico

EDTA : Ácido Etilenodiamino tetra-acético

ERS1: Gene de Função Conhecida (transportador de cistina vacuolar) em Leveduras *Saccharomyces cerevisiae*

Fe: Símbolo Químico do Ferro

Fet3: Cupro Enzima de Membrana Plasmática

FeSO₄: Símbolo Químico do Sulfato de Ferro

Fre1p: Redutase de Superfície Celular em *Saccharomyces cerevisiae*

Fre2p: Redutase de Superfície Celular em *Saccharomyces cerevisiae*

g: Gramas

G: Gravitacional

GC: Complexo de Golgi

HCl: Símbolo Químico do Ácido Clorídrico

HD: Doença de Huntington

His: Aminoácido Histidina

H₂O : Símbolo Químico da Água

H₂O₂ : Símbolo Químico do Peróxido de Hidrogênio

H₂SO₄ : Símbolo Químico do Ácido sulfúrico

HO₂[•] : Símbolo Químico do Radical Hidroperoxila

HOCl : Símbolo Químico do Ácido Hipocloroso

Hsp70: Proteína de Choque Térmico

ICP-AES: Espectrometria de Emissão Atômica (Método de Medição Quantitativa de Níveis dos Elementos de Pequenas Amostras)

ICS1/YIR004W: Gene da Levedura *Saccharomyces cerevisiae* de Função Molecular e Biológica conhecidas, envolvido no metabolismo do cobre

ICS1: Gene de *Saccharomyces cerevisiae* de Função Molecular e Biológica conhecidas, envolvido no metabolismo do cobre

ICS3/YJL077C: Gene de *Saccharomyces cerevisiae* de Função Molecular e Biológica desconhecidas. Sensibilidade aumentada ao cobre

K: Símbolo Químico do Potássio

KCl: Símbolo Químico do cloreto de Potássio

kDa: kilo Daltons

l: Litros

leu: Aminoácido Leucina

m: Massa

M: Molar

Mac1p: Proteína que Atua como Fator de Transcrição, possui um Domínio Obrigatório de Cobre

MATa: Mating Type a em *Saccharomyces cerevisiae*

met: Aminoácido Metionina

Mg: Símbolo Químico do Magnésio

Mg: Miligrama

Mn: Símbolo Químico do Manganês

Mo: símbolo químico do molibdênio

MP: Membrana Plasmática

ml: Mililitros

mM: Milimolar

NaOH: Símbolo Químico do Hidróxido de Sódio

NRAMP: Proteína Natural de Macrófago Associado à Resistência

nM: Nanomolar

nm: Nanômetro

N: Símbolo Químico do Nitrogênio

Ni: Símbolo químico do Níquel

NO: Símbolo Químico do Óxido Nítrico

O: Símbolo Químico do Oxigênio

$^1\text{O}_{2\text{G}}$: Oxigênio *singlet*

$\text{O}_2^{\cdot -}$: Símbolo Químico do Ânion Superóxido

O_2 : Símbolo Químico do Oxigênio Molecular

OD: Densidade Óptica

$\text{OH}^{\cdot}$: Símbolo Químico do Radical Hidroxila

$\text{ONOO}^{\cdot}$: Símbolo Químico do Peroxinitrito

ORF's: Open Reading Frame (Quadro de Leitura Aberta)

P: Símbolo Químico do Fósforo

PD: Doença de Parkinson

Pkr1p: Fator Agregado da Unidade V_0 da VATP-ase em *Saccharomyces cerevisiae*

pH: Potencial Hidrogeniônico

PM: Peso Molar

PMSF: FenilmetilSulfonil Fedrila

RL: Radical livre

RNA: Ácido Ribonucleico

RNS: Espécies Reativas de Nitrogênio

ROS: Espécies Reativas de Oxigênio

RPM: Rotações por minuto (expressa agitação)

S: Símbolo Químico do Enxofre

S. cerevisiae: *Saccharomyces cerevisiae*

SD: Meio de cultura mínimo para levedura

Se: Símbolo Químico do Selênio

SNC: Sistema Nervoso Central

SOD: Superóxido Dismutase

SOD1: Cupro Enzima Citosólica, Superóxido Dismutase 1

Stv1p: complexo da V-ATPase da Levedura da Subunidade V₀, presente em V-ATPases localizadas possivelmente no Complexo de Golgi e Endossomos.

Stv1p: Gene Presente em V-ATPases possivelmente do Complexo de Golgi e Endossomos

U: Uracila, Base Nitrogenada do RNA

UFC: Unidade Formadora de Colônia

UV: Ultra Violeta

V: Volume

V-ATPase: H⁺ATPase Vacuolar

H⁺ ATPase: Bomba de Próton

V₀: Subdomínio Transmembranar da V-ATP-ase em *Saccharomyces cerevisiae*

V₁: Subdomínio Citossólico da V-ATP-ase em *Saccharomyces cerevisiae*

Vma: Fenótipo Parcial de V₀ em *Saccharomyces cerevisiae*

Vma12p: Fator Agregado da Unidade V₀ da V-ATP-ase em *Saccharomyces cerevisiae*

Vma21p: Fator Agregado da Unidade V₀ da V-ATP-ase em *Saccharomyces cerevisiae*

Vma22p: Fator Agregado da Unidade V₀ da V-ATP-ase em *Saccharomyces cerevisiae*

Voa1p: Fator Agregado da Unidade V₀ da V-ATP-ase em *Saccharomyces cerevisiae*

Vph1p: Complexo da VATPase da levedura da subunidade V0, presente em VATPases de Vacúolo.

Vph1p: Gene Presente nas VATPases de vacuolo

Vps: Mutantes Defeituosos para a Biogênese Vacuolar.

Vps15p: Gene *Saccharomyces cerevisiae* de Função Molecular e Biológica conhecidas, envolvida no metabolismo do cobre

Vps34p: Gene de *Saccharomyces cerevisiae* que possui atividade fosfatidilinositol-3-quinase

V₀: Domínio hidrofóbico da V-ATP-ase, de aproximadamente 260 kDa, constituído de cinco subunidades (a,d,c,c', c'') que juntas formam um canal protônico

V₁: Complexo periférico de 570 kDa da V-ATP-ase composto por oito subunidades (A-H), responsáveis pela hidrólise de ATP

VS: Vesículas Secretórias

WD: Doença de Wilson

WT BY4741: *Wild Type* - Linhagem Selvagem para a levedura *Saccharomyces cerevisiae*

YIR004W: Gene da Levedura *Saccharomyces Cerevisiae* de Função Biológica e Molecular desconhecidas

YJL077C: Gene da Levedura *Saccharomyces Cerevisiae* de Função Biológica e Molecular desconhecidas

YjL077cp: Proteína da Levedura *Saccharomyces cerevisiae* de Função Biológica e Molecular desconhecidas

YKO: Coleção de Mutantes de *Saccharomyces cerevisiae* -Knock-out

YPAD: Meio rico de Cultivo para Levedura com adição de Adenina

YPD: Meio rico de Cultivo para Levedura

Zn: Símbolo Químico do Zinco

µL: Microlitros

µm: Micrômetro

µM: Micromolar

$\Delta yjl077c$: Linhagem Mutante para o Gene *YJL077C* em Leveduras *Saccharomyces cerevisiae*

$\Delta ers1$: Linhagem Mutante para o Gene *ERS1* em Leveduras *Saccharomyces cerevisiae*

$\Delta ics3$: Linhagem Mutante para o Gene *ICS3* em Leveduras *Saccharomyces cerevisiae*

$\Delta ics1$: Linhagem Mutante para o Gene *ICS1* em Leveduras *Saccharomyces cerevisiae*

$\Delta vph1$: Linhagem Mutante para o Gene *vph1*

ΔM : Variação de Massa

ΔV : Variação de Volume

%: Por cento

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1. INTRODUÇÃO

1.1 ROS (espécies reativas de oxigênio) e estresse oxidativo

Segundo Halliwell e Gutteridge (Halliwell and Gutteridge 2007), radical livre é qualquer espécie capaz de existir independentemente, que possui um ou mais elétrons desemparelhados em sua última camada eletrônica. Essa configuração faz dos radicais livres espécies de alta reatividade química, de meia vida relativamente curta e altamente instáveis. O radical livre mais simples é o átomo de hidrogênio, pois o mesmo tem apenas um elétron que é, portanto, desemparelhado.

Existem compostos derivados de O_2 que são tão reativos quanto os radicais livres, mas que não possuem elétrons desemparelhados na última camada eletrônica como, por exemplo, o peróxido de hidrogênio (H_2O_2), o oxigênio *singlet* ($^1O_{2G}$) e o ácido hipocloroso (HOCl). Assim sendo, a expressão “espécies reativas de oxigênio” (ROS) é utilizada para incluir tanto radicais de O_2 como substâncias não radicalares, que são agentes oxidantes e/ou facilmente convertidos em radicais (Halliwell and Cross 1994). Além dessas, existem ainda as espécies reativas de nitrogênio (RNS), sendo o óxido nítrico (NO), importante sinalizador celular, e o peroxinitrito ($ONOO^-$) os principais representantes (Jamieson 1998; Halliwell 2007; Roberts, Smith et al. 2010; Yang, Ding et al. 2013).

As espécies reativas de oxigênio são moléculas produzidas continuamente durante processos metabólicos. Em determinados níveis são sinalizadores moleculares que regulam diferentes processos biológicos, dentre eles, a resposta inflamatória (Murphy, Holmgren et al. 2011; Reddi and Culotta 2011; Scherz-Shouval and Elazar 2011). Vários trabalhos têm explorado os mecanismos que ligam ROS e a inflamação, demonstrando que as ROS mitocondriais atuam como moléculas

transdutoras de sinais que provocam o aumento da expressão de subgrupos de citocinas inflamatórias por diferentes vias moleculares (Naik and Dixit 2011). O maior foco na teoria do envelhecimento está centrado para o acúmulo do estresse oxidativo que conduz a mutações mitocondriais, e danos oxidativos (Gandhi and Abramov 2012).

Já o oxigênio é essencial para as funções normais dos organismos eucariotos. O seu papel na sobrevivência está associado ao seu grande potencial redox, o que o faz um excelente agente oxidante, capaz de aceitar elétrons facilmente de substratos reduzidos. Em condições fisiológicas do metabolismo celular aeróbico, o O_2 , sofre redução tetravalente, com aceitação de quatro elétrons, resultando na formação de H_2O . A propriedade tóxica do O_2 decorre de sua propensão para a produção de ROS, originada por uma falha no transporte de elétrons da cadeia respiratória, e fatores externos como exposição a metais pesados, radiação UV, herbicidas, poluentes atmosféricos, xenobióticos dentre outros (Halliwell and Gutteridge 2007; Dos Santos, Teixeira et al. 2012; Yang, Dong et al. 2013). Durante este processo, ocorre a formação de intermediários reativos, como o radical ânion superóxido ($O_2^{\cdot-}$), o radical hidroperoxila ($HO_2^{\cdot}$), o radical hidroxila ($OH^{\cdot}$), e o peróxido de hidrogênio (H_2O_2). A produção excessiva de ROS pode conduzir a diversas formas de dano celular (HALLIWELL e GUTTERIDGE, 2007).

O acúmulo de espécies reativas de oxigênio, definido como estresse oxidativo, causa danos à estrutura das biomoléculas: DNA, lipídeos, carboidratos e proteínas, além de outros componentes celulares. No ácido nucleico causa danos mutacionais, incluindo quebra da fita simples ou dupla, modificação de bases, sítios abásicos, e ligação cruzada entre DNA-proteína, comprometendo sua funcionalidade

e integridade (Salmon, Evert et al. 2004; Dimitrov, Pesheva et al. 2013; Yang, Dong et al. 2013). As proteínas podem sofrer oxidação em diferentes vias, resultando na formação de carbonilas proteicas (Berlett and Stadtman 1997). O dano oxidativo em lipídios geralmente envolve a lipoperoxidação, em hidroperóxidos lipídicos lábeis, por radicais $\text{OH}^{\bullet}$. Nas mitocôndrias, o dano oxidativo pode resultar em depleção energética, acúmulo de mediadores citotóxicos e morte celular. O reconhecimento da célula frente à adaptação ao estresse ou à morte celular é importante para a compreensão e estudo dos mecanismos redox e as doenças (Cecconi and Levine 2008; Abeliovich 2011; Mizushima and Komatsu 2011; Scherz-Shouval and Elazar 2011; Lee, Giordano et al. 2012).

As mitocôndrias são organelas intracitoplasmáticas envoltas por duas membranas e estão presentes na quase totalidade das células eucariontes (Pulkes and Hanna 2001; Kanki, Klionsky et al. 2011; Lee, Giordano et al. 2012). A principal função atribuída à mitocôndria é a de prover energia à célula. Calcula-se que aproximadamente 90% do ATP necessário às atividades biológicas seja produzido por essa organela, além de estarem envolvidas com a biossíntese de pirimidinas e do grupo heme da hemoglobina. O espectro da disfunção mitocondrial é vasto e inclui disfunção na cadeia respiratória, estresse oxidativo, produção reduzida de ATP, desregulação do cálcio, transição da permeabilidade mitocondrial, com abertura de poro, perturbação na dinâmica mitocondrial e um ajuste (espaço) mitocondrial desregulado (Petti, Crutchfield et al. 2011; Gandhi and Abramov 2012). Aproximadamente 102 doenças hereditárias estão associadas a defeitos mitocondriais, como por exemplo, Parkinson (PD) (Schapira 1996), Huntington (HD) (Gu, Gash et al. 1996) e Alzheimer (AD) (Bonilla, Tanji et al. 1999).

Em mamíferos o estresse oxidativo pode estar relacionado com o envelhecimento, apoptose, câncer, diabetes mellitus (Castino, Davies et al. 2005),

arteriosclerose (Roberts, Smith et al. 2010), doenças neurodegenerativas (Rodriguez-Rocha, Garcia-Garcia et al. 2013) e ateroscleroses (Morikawa, Nojiri et al. 2013).

1.2 *Saccharomyces cerevisiae* como modelo experimental

Saccharomyces cerevisiae é um dos organismos eucarióticos mais simples para o estudo de fenômenos biológicos. O fato de ser um microrganismo unicelular bem estudado (possui informações funcionais disponíveis para maior parte dos seus genes), não patogênico, de rápido crescimento, fácil manipulação genética, manter alto nível de conservação funcional com o genoma humano e com outros eucariotos superiores, faz desse microrganismo, um sistema modelo muito útil para estudos genéticos (Dos Santos, Teixeira et al. 2012).

Seu sistema genético apresenta duas fases estáveis: haplóide, o que facilita o isolamento e a caracterização de mutantes definidos; e diplóide, na qual podem ser realizados estudos de interações alélicas ou efeitos de dose, dentre outros.

O fato de *S. cerevisiae* ter seu genoma totalmente sequenciado, permite a rápida identificação de genes envolvidos na resposta antioxidante com função específica, bem como de ortólogos em outros organismos. Muitos dos quais são homólogos funcionais de mamíferos, comprovados através da complementação da deleção do mutante da levedura, o que faz com que esse modelo seja uma ferramenta poderosa possibilitando a compreensão de mecanismos moleculares utilizados por outros organismos na regulação de diversos tipos de estresse, como o oxidativo, que pode estar associado a patologias neurodegenerativas, respostas imune deficientes, doenças hematológicas, câncer, entre outras (Steinmetz, Scharfe

et al. 2002; Batista-Nascimento, Pimentel et al. 2012; Dos Santos, Teixeira et al. 2012).

Essa levedura apresenta aproximadamente cerca de 6600 ORF's (*open reading frames*), sendo que 20% dos genes estão anotados como não caracterizados e de função molecular não definida (<http://www.yeastgenome.org/cache/genomeSnapshot.html>).

Segundo Penã-Catillo, estes 20% de genes constam como não caracterizados, pois são expressos somente sob condições específicas, não apresentando vantagens ou facilidades de estudos funcionais em larga escala. Além disso, muitos desses genes codificam para proteínas com funções redundantes na célula, o que dificulta sua caracterização a partir da observação do fenótipo de linhagens mutantes. Outro aspecto importante a se considerar, é que dentre estes genes ainda não caracterizados em *S. cerevisiae* podem constar genes com funções específicas para sobrevivência deste fungo em suas condições naturais sem ortólogos humanos (Pena-Castillo and Hughes 2007).

Em uma triagem realizada pelo nosso grupo no site "Saccharomyces Genome Database" (<http://www.yeastgenome.org/>), encontramos 217 mutantes viáveis de função desconhecida. Todos foram expostos a condições de crescimento na presença de agentes oxidantes e desses, 7 linhagens apresentaram aumento de sensibilidade ao peróxido de hidrogênio e ao *tert*-butil hidroperóxido, indicando que essas ORF's podem estar relacionadas com a defesa celular contra o estresse oxidativo. Dentre os sensíveis escolhemos a linhagem com ausência do gene *YJL077C*, de processo biológico e função molecular desconhecidos como alvo desse trabalho (Auesukaree, Damnernsawad et al. 2009).

1.3 O gene *YJL077C*

Através de estudos funcionais (em larga escala) da levedura foi observado que a linhagem mutante para o gene *YJL077C* apresenta maior sensibilidade ao cobre (Entian, Schuster et al. 1999) o que lhe rendeu o nome de *ICS3* ("increased copper sensitivity"). Uma ferramenta de bioinformática (www.genemania.org) desenvolvida por Costanzo e colaboradores (Costanzo, Baryshnikova et al., 2010) que permite o mapeamento de interações proteínas/proteínas, interações genéticas, físicas, dentre outras, foi utilizado para estabelecer interações entre o gene *ICS3* e outros genes/proteínas de funções conhecidas.

Figura 1: Mapa de interações gerado pela ferramenta de bioinformática *Genemania* (www.genemania.org) (Costanzo, Baryshnikova et al. 2010). As linhas e suas cores indicam

diferentes interações: azul=interação física (Yang, Siganos et al. 2006); rosa=co-expressão gênica (Costanzo, Baryshnikova et al. 2011). A espessura da linha representa a força dessas interações (estimadas pelo programa de acordo com o número de observações em diferentes trabalhos publicados, tipo de experimento, dentre outros). Em destaque dentro do quadrado, o gene de interesse nesse estudo (*ICS3*). Destaca-se a interação com o gene *ERS1*, que dentre as apresentadas é a mais significativa, segundo a ferramenta de bioinformática.

De acordo com esse mapa, *ICS3* interage genética e fisicamente com *ERS1*. *ERS1* possui um ortólogo em humanos, denominado *CTNS*, cuja função é o transporte de cistina dos lisossomos para o citoplasma. Em outro estudo em larga escala, foi observado que a interação genética entre os genes *ICS3* x *ERS1* gera uma menor capacidade proliferativa e formação de colônias pequenas no duplo mutante (Costanzo, Baryshnikova et al. 2010).

Outro trabalho demonstrou que $\Delta ics3$ é hipersensível a Sortin 2, um composto sintético que interfere no tráfego vacuolar de proteínas. Este trabalho foi realizado monitorando-se o tráfego de CPY (carboxipeptidase Y) (Rothman, Howald et al. 1989; Takeshige, Baba et al. 1992), uma hidrolase vacuolar solúvel, que migra do complexo de Golgi para o vacúolo via endossomo (Seaman, McCaffery et al. 1998; Reddy and Seaman 2001; Norambuena, Zouhar et al. 2008; van der Vaart, Griffith et al. 2010). Isso sugere que *ICS3* pode ter função relacionada ao metabolismo vacuolar.

1.4 Vacúolos

Nas leveduras, o vacúolo é a organela responsável pela degradação de proteínas, reciclagem de nutrientes, estoque de componentes biológicos e homeostase do pH citossólico (Rees, Lee et al. 2004). Além disso, possui papel importante na regulação da concentração de íons metálicos necessária para funções

metabólicas essenciais e para a detoxificação de íons metálicos potencialmente tóxicos (Ramsay and Gadd 1997; Singh, Kaur et al. 2007). O tráfego de proteínas em eucariotos é essencial para a composição de membranas, sendo o vacúolo, um dos compartimentos chave para este sistema (Alibhoy and Chiang 2011). Sortin 2 é um composto químico sintético de baixa massa molecular capaz de afetar o sistema de tráfego endomembranar de proteínas vacuolares em *S. cerevisiae* (Norambuena, Zouhar et al. 2008).

Diversos mutantes defeituosos para a biogênese vacuolar já foram identificados (Coonrod and Stevens 2010). Estes mutantes são conhecidos com *vps*, sendo distribuídos em seis classes de acordo com sua morfologia vacuolar. Mutantes da classe A possuem vacúolos aparentemente normais, no entanto, apresentam defeitos no tráfego de proteínas. Mutantes classe B possuem vacúolos fragmentados, enquanto que mutantes classe C não possuem qualquer estrutura vacuolar reconhecível. Já os mutantes classe D, possuem defeitos na herança vacuolar, produzindo células-filhas com a aparência de mutantes classe C, enquanto mutantes de classe E acumulam proteínas vacuolares no compartimento pré-vacuolar pois apresentam defeitos no tráfego de membranas desse compartimento para o vacúolo ou Golgi. O último grupo de mutantes, o classe F, possui vacúolos com aparência normal e vacúolos fragmentados (Eide, Clark et al. 2005; Obara, Sekito et al. 2006).

1.5 V-ATPases

Embora parte da energia necessária para a organização e manutenção da composição das organelas celulares envolvidas nas vias secretórias seja provida pelos processos metabólicos citoplasmáticos, a maior parte é provida por bombas de

íons. A H^+ -ATPase (V-ATPase) provê muita dessa energia na forma de uma força próton-motora. A ação de V-ATPases, além de organizar a composição de organelas, também as acidifica. Os níveis de acidificação de cada organela são dependentes dos sistemas de transporte secundários e das propriedades específicas de cada V-ATPase (Nelson, Perzov et al. 2000; Borrelly, Boyer et al. 2001; Marino, Fais et al. 2010; Meena, Thakur et al. 2011; Finnigan, Cronan et al. 2012; Rios, Cabedo et al. 2013).

O gradiente eletroquímico gerado pelas V-ATPases é requerido para um conjunto de processos biológicos como tráfego vesicular, exocitose, fusão de membrana, desenvolvimento, degradação macromolecular, estoque e mobilização de nutrientes, homeostase iônica e regulação do pH, além da acidificação das vesículas e de organelas, de forma que, a perda da função das V-ATPases é letal para a maioria dos organismos, com exceção de poucas espécies de fungos (Kawasaki-Nishi, Nishi et al. 2001; Perzov, Padler-Karavani et al. 2002; Oot and Wilkens 2010).

Várias linhas de pesquisa têm demonstrado que funções anormais das V-ATPases contribuem para patologias humanas tais como, virulência, diabetes, metástase, doença de Alzheimer e doença de Parkinson e resistência a quimioterápicos (Futai, Oka et al. 2000; Beas-Zarate, Rivera-Huizar et al. 2001; Grazioso, Moretti et al. 2005; Vitvitsky, Garg et al. 2012). Várias patologias genéticas têm sido associadas a mutações nas isoformas da subunidade a, como por exemplo, a acidose tubular renal associada à isoforma a4, enquanto osteoporose está associada a mutações na isoforma a3. No entanto, pouco se sabe sobre os mecanismos que regulam o tráfego de vários complexos de V-ATPases para compartimentos celulares específicos (Finnigan, Hanson-Smith et al. 2011; Finnigan, Ryan et al. 2011; Finnigan, Cronan et al. 2012).

S. cerevisiae é um excelente modelo experimental para estudar as V-ATPases, pois o complexo enzimático não é requerido para sua viabilidade. A deleção de qualquer componente proteico da V-ATPase resulta em um número específico de crescimento e fenótipos celulares, incluindo sensibilidade ao excesso de metais e uma vasta acidificação vacuolar, pois as leveduras utilizam o gradiente de prótons gerados por essas enzimas para manter os níveis intracelulares e regular a homeostase iônica (Finnigan, Ryan et al. 2011). Níveis tóxicos de metal são sequestrados para dentro da levedura através de bombas de troca de prótons antipórtter, de forma que a disfunção das V-ATPases resulta numa diminuição da acidificação vacuolar e tornam as células sensíveis ao excesso de níveis de cátions divalentes tais como cálcio e zinco (Finnigan, Cronan et al. 2012). As estruturas das V-ATPases em *S. cerevisiae* são compostas por 14 diferentes subunidades e dois subdomínios:

- Citossólico V1: que é um setor catalítico solúvel responsável pela hidrólise de ATP, composto pelas subunidades (A, B, C, D, E, F, G e H).
- Transmembranar V0: parte integral do poro da membrana, realiza a troca de prótons através da bicamada lipídica, é composto pelas subunidades (a, c, c', c'', d e e), requerendo a presença de 5 fatores proteicos agregados (Vma21p, Vma22p, Vma12p, PKr1p e Voa1p), que auxiliam como componentes chaperônicos de V0, sendo necessários para a função completa da enzima (Finnigan, Hanson-Smith et al. 2011; Finnigan, Cronan et al. 2012).

Figura 2 – Estrutura e mecanismo da V-ATPase. (A) Disposição das subunidades da V-ATPase, destacando os domínios V₁ (amarelo) e V₀ (verde). (B) Mecanismo de rotação da V-ATPase, as subunidades rotatórias são destacadas em azul, enquanto as estacionárias são mostradas em laranja. A hidrólise de ATP causa mudanças conformacionais na subunidade A, que dirige a rotação do rotor (seta vermelha). (C) Mecanismo do transporte de prótons através de V₀. Prótons (pontos vermelhos) entram no complexo V₀ através de um canal hemi-citoplasmático (Adaptado de Cipriano, 2008).

A regulação da função das V-ATPases pode ocorrer através da rápida dissociação dos dois subcomplexos por uma montagem diferencial ou diferenças na localização do complexo enzimático (Kawasaki-Nishi, Nishi et al. 2001; Oot and Wilkens 2010).

Leveduras contêm dois complexos diferentes de V-ATPases de acordo com a incorporação de uma das duas isoformas da subunidade V0, e diferem na montagem, abundância proteica, acoplamento, e dissociação reversível.

- Vph1p: está presente nas V-ATPases de vacúolo,

- Stv1p: está presente em V-ATPases localizadas em outros compartimentos intracelulares, possivelmente Complexo de Golgi (GC) e endossomos (Kawasaki-Nishi, Nishi et al. 2001; Tarsio, Zheng et al. 2011). A deleção dos genes *vph1p* e *stv1p* individualmente, causa um fenótipo parcial Vma. Segundo dados publicados por Maureen Tarsio e colaboradores (Tarsio, Zheng et al. 2011) a deleção de ambos os genes, mimetiza os efeitos da supressão de qualquer uma das isoformas da subunidade da V-ATPase. A ausência de *stv1p* proporciona a oportunidade de distinguir as contribuições das ATP-ases localizadas no vacúolo, de forma que $\Delta vph1$ parece perder toda ou maior parte de sua atividade de V-ATPase em vacúolos isolados, sendo que os mutantes para $\Delta vph1$ apresentam forte sensibilidade a metais como, por exemplo, o Zn^{+2} refletindo o papel crítico do vacúolo na detoxificação de íons metálicos (Nelson, Perzov et al. 2000; Perzov, Padler-Karavani et al. 2002). A superexpressão de *STV1* pode compensar parcialmente a perda de *VPH1*, mas, os conteúdos dos complexos *Stv1p* das V-ATPases apresentam importantes diferenças bioquímicas, a partir dos conteúdos do complexo *Vph-1*, sugerindo que as isoformas das subunidades não são completamente intercambiáveis (Perzov, Padler-Karavani et al. 2002; Tarsio, Zheng et al. 2011).

1.6 Cobre

Células vivas são compostas principalmente por carbono (C), nitrogênio (N) e oxigênio (O) (Petti, Crutchfield et al. 2011). Além disso, necessitam de outros elementos químicos para sua sobrevivência, tais como fósforo (P), selênio (Se), enxofre (S), que são importantes como componentes de macromoléculas, o zinco (Zn), importante como cofator requerido para a integridade estrutural de enzimas, o cobre (Cu) e o ferro (Fe) como cofatores da atividade enzimática e o cálcio (Ca) como segundo mensageiro na transdução de sinal intracelular. Pela importância que esses elementos químicos apresentam na bioquímica celular, mecanismos eficientes para obtenção, utilização, armazenamento e regulação desses nutrientes são necessários, a fim de se evitar um acúmulo excessivo e uma consequente toxicidade (Rees, Lee et al. 2004; Eide, Clark et al. 2005).

Elemento Químico	Fontes Comuns	Funções Celulares
Carbono	Açúcares	Maior elemento estrutural das células de leveduras em conjunto com hidrogênio, oxigênio, e nitrogênio. Catabolismo de compostos de carbono, também fornece energia.
Hidrogênio	Prótons de ambientes ácidos	Motivo-próton transmembrana vital para o metabolismo da levedura.
Oxigênio	Ar	Substrato respiratório e outras funções oxidativas mistas
Nitrogênio	Sais de NH ₄ , uréia, aminoácidos	Estrutural e funcionalmente como amina orgânica em proteínas e enzimas
Fósforo	Fosfatos	Transdução de energia, ácido nucleico, estrutura de membrana, balanço iônico, atividade enzimática
Potássio	Sais de potássio	Balanço iônico, atividade enzimática
Magnésio	Sais de Magnésio	Atividade enzimática, estrutura celular e enzimática
Enxofre	Sulfatos e metionina	Aminoácidos sulfidrílicos e vitaminas
Cálcio	Sais de cálcio	Possível segundo mensageiro na transdução de sinal
Cobre	Sais cúpricos	Pigmentos redox
Ferro	Sais de ferro	Heme-proteínas, citocromo
Manganês	Sais de manganês	Atividade enzimática
Zinco	Sais de zinco	Atividade enzimática
Níquel	Sais de níquel	Atividade urease
Molibdênio	Na ₂ MoO ₄	Metabolismo nítrico, vitamina B12

Tabela 1: Nutrientes necessários para a célula e suas respectivas funções.
(Aptado de: Walk, M Graemer - "Yeast physiology and Biotechnology")

Vários estudos em leveduras têm chamado a atenção para os mecanismos de utilização de nutrientes, entretanto, a maioria desses estudos tem focado sobre o metabolismo de nutrientes específicos, sem considerar os efeitos desses sobre outros elementos. Desta forma, apesar do crescente conhecimento dos mecanismos que controlam a homeostase de diferentes nutrientes, pouco se sabe sobre como as células eucarióticas lidam com os múltiplos elementos químicos obtidos a partir do meio ambiente. Um estudo realizado em 2005 abordou essa questão combinando tecnologias genômicas e de espectrometria de emissão atômica (ICP-AES), um método preciso de medição quantitativa de níveis dos elementos a partir de pequenas amostras (Komaromy-Hiller 1999; Schuller, Auffermann et al. 2013). Utilizando a coleção de mutantes YKO provenientes do projeto *Saccharomyces* Genome Deletion (aproximadamente 5000 linhagens) o trabalho detectou mutantes com padrões de acúmulo de múltiplos íons. Assim, eles observaram que mutações de apenas um único gene podem alterar a obtenção e/ou o acúmulo de diferentes grupos de nutrientes (Eide, Clark et al. 2005).

O metabolismo do cobre e os mecanismos que controlam a concentração de seus níveis intracelulares são alvos de intensos estudos, uma vez que as deficiências em seus níveis, transporte e localização têm sido associados a várias doenças humanas (Adamo, Brocca et al. 2012; Arguello, Raimunda et al. 2012).

O cobre é um traço nutricional essencial para as células (Jo, Loguinov et al. 2008), pois é necessário como cofator catalítico para processos biológicos, tais como respiração, transporte de ferro, proteção contra o estresse oxidativo, produção de hormônios, pigmentação, coagulação sanguínea, crescimento e desenvolvimento normal das células (Puig and Thiele 2002; Leary, Cobine et al. 2007; Leary and Winge 2007; Yasokawa, Murata et al. 2008). Esse metal é cofator de

aproximadamente 20 enzimas em células eucarióticas superiores como, citocromo-c oxidase, lisil oxidase, ceruloplasmina, (Castillo-Duran and Uauy 1988; Fernandez-Calvino, Bermudez-Couso et al. 2012), Superóxido dismutase (SOD) etc (Babior, Kipnes et al. 1973; Cobine, Ojeda et al. 2004; Leary, Cobine et al. 2007). Assim como outros metais, o cobre tem um papel importante na expressão gênica. Proteínas como Ace1p, Mac1p e Amt1p, que atuam como fatores de transcrição, possuem um domínio obrigatório de cobre, necessário para a ligação ao DNA (Uauy, Olivares et al. 1998; Cabiscol, Piulats et al. 2000; Cohen, Nelson et al. 2000; Kommuguri, Bodiga et al. 2012). Mac1p e Ace1p são proteínas que têm sua expressão aumentada em resposta as concentrações intracelulares de cobre. Enquanto Mac1p é transcrita em situações de baixas concentrações de cobre, Ace1p têm sua transcrição ativada em altas concentrações. Esse perfil de expressão demonstra que essas proteínas interagem com repertórios completamente diferentes de genes, o que irá gerar respostas fisiológicas diferentes (De Freitas, Wintz et al. 2003).

Figura 3: Esquema de mecanismo da regulação da expressão gênica por proteínas cupro dependentes: Íons de cobre entram no núcleo celular e ligam-se às proteínas reguladoras (Ace1, Mac1 e Amt1). Esta ligação ativa-as para interagirem com a sequência específica dos elementos que

respondem aos metais na 5' dos genes regulados por estes elementos. (Adaptado de De Freitas, Wintz et al. 2003).

O cobre em altas concentrações é tóxico, podendo participar de reações de oxidação metal-catalisadas gerando ROS como, por exemplo, o radical hidroxila, o peróxido de hidrogênio e o ânion superóxido que estão intimamente ligados ao estresse oxidativo, provocando dano celular, oxidação proteica e danos ao DNA (Berlett and Stadtman 1997; De Freitas, Wintz et al. 2003; Farrugia and Balzan 2012; Rios, Cabedo et al. 2013). Por outro lado, a deficiência de cobre provoca perturbações no sistema imunológico, além de causar disfunções neurológicas e anormalidades hematológicas, mais comumente neutropenia e anemia (Stafford, Bokil et al. 2013), bem como o aparecimento de mitocôndrias aumentadas em células eritropoiéticas (Bustos, Jensen et al. 2013).

Em *S. cerevisiae* íons de cobre são necessários para pelo menos três enzimas principais; essas cupro-enzimas incluem: a citosólica SOD1, a de membrana plasmática Fet3 e a da membrana mitocondrial interna citocromo c-oxidase (Cobine, Ojeda et al. 2004). Mecanismos homeostáticos existem nas células para regular a concentração intracelular de íons de cobre, mantendo um equilíbrio desse metal e minimizando seus efeitos deletérios (Pena, Lee et al. 1999; Cohen, Nelson et al. 2000; Cobine, Ojeda et al. 2004).

O cobre extracelular é reduzido pelas redutases de superfície celular Fre1p e Fre2p e, é transportado pela membrana plasmática pelos transportadores de alta afinidade pelo cobre Ctr1 e Ctr3. Dentro das células a chaperona Cox17, localizada exclusivamente nas mitocôndrias, facilita a entrega de cobre para o complexo citocromo-c oxidase, necessário para a respiração aeróbica. Ccs1, uma outra chaperona, provê cobre para a SOD1, uma proteína envolvida na proteção celular contra o estresse oxidativo. A Atx1p que entrega cobre a vesículas relacionadas ao

complexo de Golgi, enquanto Ccc2p, uma cupro- ATPase, bombeia cobre para dentro desta organela (Pena, Lee et al. 1999; Pagani, Villarreal et al. 2007).

Figura 4: Desenho esquemático de enzimas que participam da regulação homeostática de metais em *S.cerevisiae*. As redutases Fre1 e Fre2, de superfície de membrana reduzem o cobre que é transportado por Ctr1 e Ctr3, proteínas de alta afinidade ao cobre, e dentro das células Cox17 localizada nas mitocôndrias facilita o delivery do cobre para a Sod1 enquanto, Atx1p faz o delivery desse metal para as vesículas relacionadas ao complexo de Golgi, e Cccp2 outra cupro- ATPase bombeia cobre para dentro das vesículas correspondentes ao complexo de Golgi. (Adaptado de (Pena, Lee et al. 1999).

Além do gene *ICS3*, também foram identificados em *S. cerevisiae* outros genes envolvidos no metabolismo do cobre, tais como *ICS1* e *Vps15p*, de função molecular e biológica conhecidas. O gene *ICS1/YIR004W* atua sobre a importação de proteínas do citossol para o peroxissomo (Hettema, Ruigrok et al. 1998; Walsh, Bursac et al. 2004). A proteína codificada pelo gene *ICS1* possui um domínio J.

Homólogos em *Escherichia coli* do gene *ICS1* atuam em conjunto com proteínas chaperonas da família das “*heat shock proteins*” Hsp70 em uma variedade de processos celulares (Hettema, Ruigrok et al. 1998; Qiu, Shao et al. 2006). O gene *Vps15p* é necessário para o recrutamento de *Vps34p* na membrana vacuolar e sua subsequente estimulação. *Vps34p* possui atividade fosfatidilinositol-3-quinase essencial para a correta localização de proteínas vacuolares (Stack and Emr 1994; Stack, DeWald et al. 1995; Kiel, Rechinger et al. 1999; Gunther, Nguyen et al. 2005; Obara, Sekito et al. 2006; Duennwald, Echeverria et al. 2012).

1.7 Resposta e adaptação para integridade celular, e triagem funcional em meio de cultura ácido

Kawahata e colaboradores (Kawahata, Masaki et al. 2006) utilizando dois tipos de análises, técnica de microarray de DNA para investigarem a resposta de choque ao ácido como primeiro passo para adaptação as condições ácidas e, após essa adaptação, para a manutenção da integridade em condições ácidas, e a outra técnica de triagem funcional, utilizando a deleção de genes não essenciais de *S. cerevisiae*.

Os resultados da análise de expressão demonstraram:

- 1) Genes envolvidos na reposta ao estresse: *YGP1*, *ATPS1* e *HPS150* foram induzidos sob condições de choque ácido;
- 2) Genes envolvidos no metabolismo de metais regulados por Aft1p: *FIT2*, *ARN1* e *ARN2* foram induzidos sob condição adaptação ácida;
- 3) Genes envolvidos na homeostase de metais: *ARN1*, *CCC2*, *FIT1-3*, *CUP1* e *SIT1* foram também induzidos sob condição de adaptação ácida. A maioria desses genes foram regulados por Aft1p, um fator de transcrição que responde á absorção intracelular de ferro.

A maioria dos genes regulados por Aft1p (*FRE1-3*, *FRE5*, *FIT1-3*, *COT1*, *SIT1* e *FET3*), que são do mesmo grupo não apresentaram mudanças significativas sob condição de choque ácido mas, foram induzidos na adaptação ao ácido.

A análise de expressão indicou que, a maioria dos genes envolvidos no metabolismo de metal regulados por Aft1p, foram induzidos sob condições de adaptação ácida. A análise funcional indicou que a perda das VATP-ases e das proteínas HOG MAPK causam sensibilidade ao ácido.

Ambas as análises indicam que a mudança da expressão de genes, relacionados com a parede celular em condições ácidas, resulta numa alteração da estrutura da parede celular (Kawahata, Masaki et al. 2006).

A depleção da parede celular da *SED1* demonstrou resistência ao ácido láctico, embora a expressão de *SED1* tenha sido induzida pela exposição ao ácido láctico. Portanto os genes envolvidos como componentes de parede celular foram identificados como sendo importantes á resposta a ácidos orgânicos e baixo pH.

Proteínas codificadas por *VMA2*, *VMA4* e *VMA6* cuja deleção causa sensibilidade a todos os ácidos, foram translocadas por complexos proteicos H⁺VATP-ase localizados no vacúolo, esses achados sugerem que o vacúolo é essencial para o crescimento na presença de meio ácido e baixo pH (Kawahata, Masaki et al. 2006).

A depleção da proteína de resposta á hiperosmolaridade codificadas por *HOG1*, *PBS2*, *SSK1* e *SSK2* as quais se encontram na via HOG MAPK, também resultam na sensibilidade ao ácido. Esses resultados sugerem que a condição ácida altera a estrutura da parede celular, de fato, o baixo pH tem demonstrado induzir alteração na estrutura da parede das células das leveduras (Lawrence, Botting et al. 2004). Além disso, estudos recentes demonstraram que Sed1p esta envolvido na manutenção do genoma mitocondrial assim como várias formas modificadas de

Sed1p são expressas e a maioria dessas formas interagem com a polimerase mitocondrial in vitro. A depleção das VATP-ases em mutantes exibiu sensibilidade aos ácidos, indicando que esta enzima é criticamente importante para a resistência ao ácido (Kawahata, Masaki et al. 2006).

Figura 5: Análise de expressão (microarray do DNA) do perfil de 277 genes que tiveram seus níveis de expressão modificados em pelo menos uma condição. A árvore está ligada a partir da coluna do fundo para a coluna seguinte e assim por diante (a-c) choque ácido, (d-f) adaptação ácida (a) e (d)

com ácido láctico, (b) e (e) com ácido acético e (c) e (f) com ácido clorídrico. Cada linha horizontal representa um único gene com seu respectivo nome à direita. A mudança de faixa é representada pela barra de cores. Os genes em negrito estão envolvidos no metabolismo dos metais, e as setas indicam os genes indicados em nosso trabalho. Adaptado de (Kawahata, Masaki et al. 2006).

Sensitivity to acids									
ORF name	Gene	Lactic acid	Acetic acid	Hydrochloric acid	ORF name	Gene	Lactic acid	Acetic acid	Hydrochloric acid
Other function									
YOR092W	<i>ECM3</i>	—			YOR001W	<i>RRP6</i>	—		
YNL148C	<i>ALF1</i>		—		YJL080C	<i>SCP160</i>			—
YMR116C	<i>ASC1</i>	—	—	—	YOR035C	<i>SHE4</i>	—	—	—
YLR399C	<i>BDF1</i>			—	YNL032W	<i>SIW14</i>		—	
YPL221W	<i>BOP1</i>			—	YMR216C	<i>SKY1</i>		—	
YEL029C	<i>BUD16</i>		—		YNL243W	<i>SLA2</i>	—		—
YCR047C	<i>BUD23</i>	—	—		YBL058W	<i>SHP1</i>		—	
YER014C-A	<i>BUD25</i>		—		YNR023W	<i>SNF12</i>	—	—	
YLR226W	<i>BUR2</i>	—			YAL047C	<i>SPC72</i>	—		—
YGR217W	<i>CCH1</i>	—		—	YOL091W	<i>SPD21</i>		—	
YDR364C	<i>CDC40</i>	—	—	—	YGR063C	<i>SPT4</i>		—	—
YLR418C	<i>CDC73</i>		—		YNL138W	<i>SRV2</i>	—	—	—
YLR087C	<i>CSF1</i>		—	—	YDR293C	<i>SSD1</i>			—
YKL046C	<i>DCW1</i>			—	YHR064C	<i>SSZ1</i>		—	
YKL054C	<i>DEF1</i>	—	—	—	YMR125W	<i>STO1</i>		—	—
YDL160C	<i>DHH1</i>			—	YMR039C	<i>SUB1</i>		—	
YEL019W	<i>EAF5</i>		—		YLR182W	<i>SWI6</i>		—	
YKL204W	<i>EAP1</i>			—	YPR163C	<i>TIF3</i>		—	—
YLR436C	<i>ECM30</i>		—		YOR295W	<i>UAF30</i>		—	
YLR443W	<i>ECM7</i>	—		—	YOR068C	<i>VAM10</i>	—		—
YNL133C	<i>FYV6</i>			—	YDR359C	<i>VID21</i>		—	
YGR196C	<i>FYV8</i>	—		—	YGR105W	<i>VMA21</i>		—	—
YGR163W	<i>GTR2</i>		—		YNL197C	<i>WHI3</i>	—		
YER057C	<i>HMF1</i>	—			YCL002C			—	
YMR032W	<i>HOF1</i>		—		YCR079W			—	
YJL159W	<i>HSP150</i>	—			YDL173W			—	
YCR020W-B	<i>HTL1</i>	—	—	—	YEL007W			—	
YOL012C	<i>HTZ1</i>		—		YGL007C-A		—	—	—
YGL168W	<i>HUR1</i>			—	YGL188C-A			—	
YJL077C	<i>ICS3</i>		—	—	YJL046W			—	
YEL044W	<i>IES6</i>			—	YKL077W			—	
YOL081W	<i>IRA2</i>	—	—		YKR041W		—		—

Tabela 2: A tabela relacionada á análise de triagem funcional, utilizando a deleção de genes não essenciais. O sinal (-) indica a deleção do gene da cepa tem sensibilidade ao ácido, a coluna em vermelho destaca o gene *ICS3*.

1.8 Microelementos no papel do metabolismo celular

Microelementos apresentam importante papel no metabolismo celular, aparentemente íons metálicos são vitais para todos os organismos, de forma que transportadores iônicos são fundamentais para a manutenção da sua homeostase. Leveduras tornaram-se um microrganismo modelo para o estudo de transportadores de metal e o acúmulo destes nas células (Cohen, Nelson et al. 2000). Íons de zinco, manganês e cobre são muito interessantes, pois apresentam um efeito positivo na atividade respiratória e na taxa de crescimento de *Saccharomyces cerevisiae*. As leveduras têm habilidade de acumular íons metálicos através de soluções aquosas por diferentes interações físico-químicas (adsorção e absorção ou através de um mecanismo dependente do metabolismo). Os processos de sorção são dependentes de grupos funcionais disponíveis na superfície da célula e da natureza dos íons metálicos, portanto a concentração de íons livres, a eletronegatividade do ligante, cátions metálicos, o tamanho da cavidade, apresentam grande influência na seletividade da aquisição de metal (Chen, Chen et al. 2011).

Leveduras são organismos quimiorganotróficos, ou seja, obtêm carbono e energia a partir de compostos orgânicos. Esses compostos são na maioria açúcares dos quais a glicose é amplamente utilizada pela levedura. As leveduras requerem uma vasta gama de minerais como suprimento para seu crescimento adequado. Potássio (K) e magnésio (Mg), são considerados macroelementos e são requeridos em concentrações milimolar para estabelecer o principal local metálico catiônico na célula de levedura. Outros minerais são geralmente requeridos na ordem de micromolar ou até mesmo nanomolar, referidos como micro ou elementos traço e incluem: Mn (manganês), Ca (cálcio), Fe (ferro), Zn (zinco), Cu (cobre), Ni (níquel), Co (cobalto) e Mo (molibdênio) (Walker, Graeme M. 1998).

1.9 Espectrometria de Emissão Atômica com Plasma Induzido Acoplado (ICP-AES)

A técnica de emissão atômica mede a energia que é perdida quando um elétron passa de um nível superior para um nível mais baixo de energia. A quantidade de energia é transferida para o átomo através de mecanismos de colisão com outra partícula, resultando na excitação do átomo. Um elétron da camada de valência é excitado para um nível energético superior e posteriormente o elétron retorna ao estado fundamental (Aucélio, et al., 2007).

O ICP-AES é uma técnica bastante utilizada para a determinação de metais em quantidades diversas, maiores (%) ou menores (mg/L ou µg/L), numa grande variedade de amostras, tais como, amostras orgânicas, águas, alimentos dentre outros. A técnica de ICP-AES tem sido utilizada como um método de análise quantitativa devido as suas excelentes capacidades analíticas como, capacidade multi-elementar, alto poder de detecção, elevada precisão e pequeno tempo de análise (Edlund, M., Visser, H., and Heitland, 2002).

Os metais de transição são essenciais para muitos processos metabólicos, sua homeostase é de fundamental importância para a manutenção dos principais mecanismos fisiológicos e, conseqüentemente, para a viabilidade celular (Wang, Okonkwo et al. 2013). Entretanto, a armazenagem ou o excesso desses íons metálicos podem causar doenças genéticas (Prohaska, Pomazal et al. 2000). Por exemplo, excesso na captação de ferro está relacionado com a doença hereditária hemocromatose, a anemia e a arteriosclerose. O Mn^{2+} é uma neurotoxina potente e o uso industrial de Mn^{2+} tem acarretado casos de “manganismos” que é caracterizado por distúrbios em processos mentais e sintomas semelhantes ao da doença de Parkinson (Pal, Samii et al. 1999) Os íons metálicos estão sendo

associados a doenças neurodegenerativas como, doença de Parkinson (PD), e Alzheimer (AD) (Pal, Samii et al. 1999).

Os íons mais estudados são os cátions divalentes, como Cu^{2+} , Mn^{2+} , Fe^{2+} e Zn^{2+} devido a importância no papel do metabolismo celular. Esses diferentes íons metálicos podem ser agrupados de acordo com sua atividade de oxido-redução:

(1) redox ativa: inclui os íons Fe^{2+} , Cu^{2+} , Co^{2+} , funcionam, geralmente, em enzimas que participam de reações redox e na conversão de componentes ativos que contêm oxigênio;

(2) redox ativa em menor extensão, inclui o íon Mn^{2+} ;

(3) redox não-ativa: inclui os íons Ca^{2+} e Zn^{2+} , são co-fatores de transcrição e de outras enzimas envolvidas no metabolismo do DNA (Pittman, Valente et al. 2006; Hicsonmez, Erees et al. 2009; Wu, O'Doherty et al. 2011). Porém, pouco ainda se conhece sobre os transportadores e a homeostase da maioria dos íons metálicos.

Nos últimos anos foi descrita uma família de transportadores de íons metálicos, NRAMP (proteína natural de macrófago associado à resistência – “Natural Resistance-Associated Macrophage Protein”) aparentemente bem conservada de bactérias a humanos. A família NRAMP funciona como transportadora de íons em geral, podendo transportar Mn^{2+} , Zn^{2+} , Cu^{2+} , Cd^{2+} , Ni^{2+} e Co^{2+} (Culotta, Yang et al. 2005; Leary, Cobine et al. 2007).

O vacúolo de leveduras serve como principal organela capaz de estocar íons e possui um papel importante na homeostase de Ca^{2+} e de outros cátions como o Mn^{2+} , Zn^{2+} , Mg^{2+} e Cd^{2+} (Okorokov, Kulakovskaya et al. 1985; Pena, Lee et al. 1999; Avery 2001; Culotta, Yang et al. 2005).

1.9.1 Espectrometria de Emissão Atômica com Plasma Induzido Acoplado (ICP-AES), análise de cobre intracelular

Pelo fato de ser um elemento traço das células, a concentração de cobre testada, 10mM é muito elevada, mesmo em estados fisiopatológicos. O cobre é um elemento fundamental no organismo humano e apresenta relação direta em diversas funções orgânicas, tais como: atividade mitocondrial, biossíntese da melanina, metabolismo da dopamina, homeostase do ferro, ação antioxidante, formação do tecido conectivo, entre outras (Culotta, Lin et al. 1999). Com o objetivo de evitar a toxicidade celular ocasionada pelo cobre, diversos mecanismos permitem a circulação, compartimentalização e excreção deste elemento no organismo, de modo a assegurar que todas as reações que tenham sua dependência ocorram de maneira harmônica (Rees, Lee et al. 2004).

Em uma abordagem realizada por Schuller e colaboradores (Schuller, Auffermann et al. 2013) utilizando cepas *Saccharomyces cerevisiae* como modelo experimental para análise de absorção intracelular de cobre através da superexpressão do transportador específico de cobre *CTR1* e uma variante de *CTR1* com truncamento no C-terminal após o 300º aminoácido (*ctr1 Δ300*). Para determinarem a sensibilidade gerada por essas linhagens ao cobre, utilizaram a análise Espectrometria de Emissão Atômica com Plasma Induzido Acoplado (ICP-AES), e investigaram os efeitos da super-expressão de ambas as construções sob condições de excesso de cobre no meio celular de diferentes elementos de *Saccharomyces cerevisiae*.

1.9.2 Espectrometria de Emissão Atômica com Plasma Induzido Acoplado (ICP-AES), análise de ferro intracelular

Cepas de *S. cerevisiae* com falta de um gene transportador de ferro não podem crescer sob condições de estresse alcali. Muitos genes cuja ausência resulta em redução do crescimento em pH levemente alcalino, incluem vários genes essenciais para a homeostase do cobre e do ferro (Serrano, Bernal et al. 2004), portanto a mudança de pH, tanto ácido ou levemente alcalino, afetam o metabolismo do ferro, embora nenhuma das cepas com falta de genes do metabolismo do ferro apresentaram resistência ou sensibilidade aos ácidos. Estudos recentes tem demonstrado que a ativação da Aft1p não é uma resposta direta do ferro citossólico, mas a ativação dos regulões de ferro são controladas pela síntese dos agregados de Fe-S os quais na levedura são localizados dentro da mitocôndria (Chen, Crisp et al. 2004; Phadnis and Ayres Sia 2004; Kawahata, Masaki et al. 2006). Estudos tem demonstrado que Aft1p esta envolvida na mudança diáuxica e no estresse oxidativo como Aft1p apresenta funções diversas, serão necessários mais estudos para a compreensão do porque a expressão dos genes envolvidos no metabolismo dos metais é induzida e porque Aft1p fica localizada no núcleo apenas sob condições de depleção de ferro (Cohen, Nelson et al. 2000; Haurie, Boucherie et al. 2003; Puig, Askeland et al. 2005; Kawahata, Masaki et al. 2006).

2. JUSTIFICATIVA

Estudos em larga-escala relacionaram o gene *ICS3* ao metabolismo do cobre (Entian, Schuster et al. 1999). No entanto, pouco se sabe sobre sua função e seu envolvimento com o metabolismo oxidativo e o porquê a linhagem mutante é mais sensível ao cobre. Diversas doenças humanas estão relacionadas a perturbações na homeostase do Cobre, dentre elas:

2.1 Doença de Menkes

Doença autossômica recessiva. Os pacientes possuem uma deficiência sistêmica profunda de cobre acompanhada por anormalidades neurológicas severas devido a uma ausência de bombeamento deste elemento das células intestinais para a corrente sanguínea (Kaler 1996; Kaler 1998; Samimi, Katano et al. 2004).

2.2 Doença de Wilson (WD)

É um distúrbio herdado com caráter autossômico recessivo. Envolve uma pobre incorporação de cobre na ceruloplasmina e um defeito na excreção biliar. O cobre acumula-se nos hepatócitos, e quando ocorre a lise, o metal liberado difunde-se para o sangue e acumula-se nos tecidos extra-hepáticos (Schilsky, Stockert et al. 1998). Os órgãos mais predominantemente afetados pela toxicidade do cobre são o fígado e o cérebro (Roberts and Cox 1998; Babich, Skvortsov et al. 2013).

2.3 Doenças Hematológicas

Pacientes com deficiência de cobre podem desenvolver déficits hematopoiéticos, resultando em anemia hipocrômica microcítica (Wu, Ricker et al. 2006; Yasokawa, Murata et al. 2008), que é caracterizada por uma deficiência

parcial ou completa da síntese de cadeias globínicas, e distúrbios no metabolismo do ferro (Culotta, Lin et al. 1999; Halfdanarson, Kumar et al. 2008). O cobre atua como cofator da ceruloplasmina, enzima que oxida o ferro, permitindo assim, sua mobilização e seu transporte de estoques hepáticos para a medula óssea a fim de ser usado na eritropoiese (Nagano, Toyoda et al. 2005).

2.4 Doenças Neurodegenerativas

Todos os tecidos humanos sofrem lesões oxidativas, porém, o sistema nervoso central é especialmente sensível por apresentar capacidade reduzida de regeneração celular em comparação com outros órgãos. Isto porque os neurônios de um indivíduo adulto são células pós-mitóticas, ou seja, não se replicam mais (Rivera-Mancia, Perez-Neri et al. 2010). A reposição de um neurônio é um processo muito mais lento que a regeneração de outros tipos celulares. A morte de neurônios induzida por toxinas ou pelo processo normal de envelhecimento, pode causar vários comprometimentos ao sistema nervoso e, o consumo de oxigênio (O_2) pelo cérebro é muito elevado (Lee, Giordano et al. 2012; Pimentel, Batista-Nascimento et al. 2012). Doenças neurodegenerativas associadas com o rompimento da homeostase de metais no cérebro (Fox, Kama et al. 2007; Kwon, Heo et al. 2011) incluem:

- Doença de Alzheimer (AD)
- Doença de Parkinson (PD)
- Doença de Huntington (HD)
- Esclerose Lateral Amiotrófica (ALS) (Rivera-Mancia, Perez-Neri et al. 2010; Bin, Li et al. 2013).

As doenças neurodegenerativas estão relacionadas com o acúmulo de proteínas que podem ser oxidadas por diferentes vias, resultando na formação de

carbonilas proteicas, produtos estes irreversíveis que tendem a formar agregados proteicos, que não são possíveis de serem desagregados por vias proteolíticas normais das células (Cabisco, Piulats et al. 2000; Rodriguez-Rocha, Garcia-Garcia et al. 2013). Importantes enzimas como, e CuZnSOD são facilmente inativadas pela indução da carbonilação. O acúmulo de proteínas oxidadas está associado ao envelhecimento e algumas patologias como ALS (Esclerose Amiotrófica Lateral) e AD (Doença de Alzheimer) (Rivera-Mancia, Perez-Neri et al. 2010).

A doença de Parkinson é caracterizada pelo acúmulo de inclusões proteicas denominadas corpos de Lewy contendo proteínas mal dobradas e agregados de α -sinucleína. Foi demonstrado em leveduras que a remoção de agregados de α -sinucleína é mais dependente de autofagia e degradação de proteínas vacuolares do que via proteassoma (Petroi, Popova et al. 2012; Rodriguez-Rocha, Garcia-Garcia et al. 2013).

3. Cistina/Cisteína e Cistinosina

A cistina é um dissulfeto formado pela oxidação de duas moléculas de aminoácido cisteína. Cistina e cisteína participam de reações reversíveis de oxirredução. Na presença de oxigênio (O_2), a cisteína é rapidamente oxidada à cistina (CysS-SCys) (Gahl, Balog et al. 2007). Em leveduras, o gene *ERS1* codifica uma proteína transportadora de cistina do vacúolo para o citossol (Gao, Wang et al. 2005). Foi identificado em humanos o ortólogo de *ERS1*, o gene *CTNS*. Este gene está mutado em pacientes com cistinose nefropática, cuja principal consequência é a síndrome renal de Fanconi (Town, Jean et al. 1998; Eskelinen, Tanaka et al. 2003; Goodyer 2011). A cistinose nefropática é uma doença autossômica recessiva, sistêmica e progressiva caracterizada pelo acúmulo intralisossomal de cistina

(Kalatzis, Cherqui et al. 2001). O armazenamento de cistina na cistinose é intracelular, com formação de cristais nos rins, pulmões, apêndice vermiforme, conjuntiva, córnea, retina, linfonodos, tireóide, timo, placenta, cérebro entre outros (Gahl, Balog et al. 2007).

Estudos demonstraram um aumento da autofagia e disfunção mitocondrial em fenótipos de cistinose nefropática. Além disso, uma inibição específica da autofagia resultou em uma significativa atenuação de morte celular nessa patologia. Esses estudos evidenciaram que a autofagia mitocondrial anormal possivelmente seja o mecanismo crítico para a síndrome renal de Fanconi e danos renais progressivos (Sansanwal and Sarwal 2010; Sansanwal, Yen et al. 2010).

O presente trabalho tem como propósito caracterizar a função biológica e molecular do gene *ICS3* em relação ao estresse oxidativo e metabolismo do cobre. Nossos estudos poderão esclarecer a participação desse gene em mecanismos importantes através do uso de um modelo simples e de fácil acesso como a levedura.

4. Higromicina B

Higromicina B é um antibiótico aminoglicosídeo que inibe o crescimento de células procarióticas e eucarióticas, especificamente inibindo a síntese de proteínas. Sensibilidade à higromicina B tem sido descrita para diversas linhagens mutantes de leveduras, incluindo aquelas com defeitos em biossíntese de parede celular, glicosilação, transporte de íons e função vacuolar. A linhagem $\Delta ers1$ apresenta um fenótipo de hipersensibilidade à higromicina B, que é totalmente revertido com a expressão de *CTNS* humano, mas não de suas isoformas mutantes, relacionadas com a cistinose (Gao, Wang et al. 2005)

5.OBJETIVO

O presente trabalho teve como foco estudar a função molecular do gene *ICS3/YJL077C* na resposta antioxidante e no metabolismo do cobre, através do efeito da adição de CuSO_4 sobre a cinética de crescimento, viabilidade e acúmulo de ferro e cobre da linhagem mutante de *S. cerevisiae* Δics3 .

5.1 Objetivos específicos

- Avaliar a tolerância de mutantes Δics3 frente ao estresse oxidativo.
- Avaliar a tolerância de mutantes Δics3 frente ao estresse por cobre.
- Avaliar a tolerância de mutantes Δics3 frente ao estresse por cisteína e cistina.
- Análise de espectrometria de emissão atômica (ICP-AES) para medição quantitativa de níveis intracelulares de cobre e ferro.

6. Materiais E Métodos

6.1 Meios de cultura

- Meio YPD: 1% extrato de levedura (AMRESCO®), 2% peptona (Fischer Bioreagents®) e 2% dextrose (Fischer Bioreagents®).
- Meio SD completo: 0,7% de Yeast Nitrogen Base sem aminoácidos (AMRESCO®) + 2% de Glicose (AMRESCO®), 0,12% de DROPOUT (Mistura de todos os aminoácidos com exceção de Leucina, Uracila, Triptofano e Histidina), 0,8% de Histidina (USB®), 0,8% de Leucina (USB®), 0,8% de Triptofano (USB®), 0,8% de uracila (USB®), e adição de 2% de ágar (Fisher Bioreagents®) quando o meio for sólido.

- Meio YPAD: 1% de extrato de levedura (AMRESCO®), 2% de peptona (Fischer Bioreagents®), 2% dextrose (Fischer Bioreagents®), 50 mg de sulfato de adenina (USB®) por litro suplementado com 0,5M de KCl (Synth®) (ótimo crescimento), 2% de ágar (Fischer Bioreagents®), e diferentes concentrações de Higromicina B (Sigmaldrich®) (Gao, Wang et al. 2005).

6.2 Linhagens utilizadas

- Linhagem BY4741 de *Saccharomyces cerevisiae* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), além da coleção Knock-out (YKO) deletion collection (Invitrogen), que contém 5180 clones de leveduras mutantes para genes não-essenciais, divididos em 54 placas com 96 clones cada.
- Linhagem YJL077C coleção Knock-out (YKO) deletion collection (Invitrogen).

6.3 Cultivos

- a) **Para experimento em YPD com cobre:** foram adicionadas as concentrações de 5mM, 7mM e 10mM de CuSO₄ (Sigmaldrich®). Após a adição de sulfato de cobre ao meio YPD líquido o pH foi aferido utilizando-se tiras de papel tornassol (Merck®) como indicador de pH.
- b) **Para experimento com cobre em pH 6.0:** foram adicionadas as concentrações de 7mM e 10mM de CuSO₄ (Sigmaldrich®) em meio YPD e o pH foi aferido com tiras de papel tornassol (Merck®) e foi ajustado para pH 6.0 com a adição de 0,003mL de NaOH 5M (Merck®).
- c) **Para experimento em meio ácido:** o pH do meio foi ajustado para pH 4.0 com adição de 0,007mL de HCl 1M (Merck®).

- d) **Para experimento com cistina e cisteína:** foram adicionadas as concentrações de 5mM, 7mM e 10mM desses aminoácidos (cistina e; cisteína – (Merck®).
- e) **Para experimentos com cobalto e sulfato de ferro:** foram adicionadas as concentrações de 7mM e 10mM desses reagentes diretamente ao meio de cultura (cobalto e sulfato de ferro - Merck®) líquido YPD.
- a) Para experimentos com PMSF (Fenil Metil Sulfonil Fedrila – Sigmaldrich): foram adicionadas as concentrações de 1mM e 2mM de PMSF (Sigmaldrich®) diluídas em álcool etílico absoluto (Synth®).
- A placa de YPD com peróxido de hidrogênio foi preparada espalhando-se o peróxido de hidrogênio (Merck®) a temperatura ambiente, diluído em água estéril (MilliPore®), a partir de uma solução estoque 10M , nas placas contendo meio sólido frio e usadas imediatamente.
 - As células foram crescidas em meio YPD líquido as condições de análise foram de 0,2 de 600nm (BioPhotometer Plus eppendorf®). Foram adicionadas ao meio de cultura líquido YPD as concentrações de 7mM e 10mM de Co e FeSO₄ retiradas em diferentes tempos e plaqueadas em diluições de 5 vezes de 5 µl em placa de YPD sólido.
 - A placa de YPAD foi preparada espalhando-se a Higromicina B (Invitrogen®) a temperatura ambiente, diluída em água estéril a partir de uma solução estoque 50mg/ml, nas placas contendo meio sólido frio.

6.4 Quantificação de massa seca

Para a quantificação de massa seca foi inicialmente construída uma curva de calibração. Para isso, preparou-se o pré-inóculo. As células foram crescidas em 100 ml de meio líquido YPD a 30°C a 175 rpm por 16 horas em agitador rotativo (New Brunswick® Scientific Excella E24 Incubator Shaker Series). O pré-inóculo foi submetido à centrifugação (Eppendorf Centrifuge 5810R) a frio (8°C) por 10 minutos a uma velocidade de 8000 G. Após este procedimento, descartou-se o sobrenadante dos dois tubos Falcon (50mL) e as células foram ressuspensas em 40 ml de água ultrapura (MilliQ®). Para a padronização dos pontos de diluição utilizados na curva de calibração foram realizadas várias diluições testes iniciais variando de 1:3 até 1:100. A partir desse experimento inicial, estabeleceu-se como pontos de diluição referência as proporções 1:10, 1:20, 1:40, 1:60, 1:80 e 1:100. As amostras diluídas tiveram sua absorbância medida com o uso do espectrofotômetro (ÚNICO® SQ-4802 Double Beam Scanning UV/Visible). Paralelamente às medidas de absorbância, a massa seca de cada diluição foi obtida para a construção da curva de calibração. A pesagem da massa seca foi realizada utilizando-se membranas Millipore® de 0,22 µm. Para isso, 20 ml de cada diluição foram filtradas à vácuo (Millipore®) através das membranas. Após filtração, estas foram colocadas em estufa (Estufa 502 FANEM®) a 70°C até secagem. Em seguida, as membranas foram pesadas em balança analítica (AS220/x RADWAG®) e o cálculo da massa seca foi realizado subtraindo-se a respectiva massa inicial de cada membrana (virgem) da massa final medida após a filtração e secagem das amostras (tabela 2).

Para o cálculo da variação da massa seca entre as diferentes diluições, utilizou-se a seguinte fórmula:

$$\text{MASSA SECA} = \Delta M = \text{massa final} - \text{massa inicial}$$

$$\Delta V = \text{volume final} - \text{volume inicial}$$

Tabela 3: Medidas de massa inicial e massa seca final.

Amostra	Diluição	OD 600 (unidades arbitrárias)	Massa Inicial (Membrana virgem(g))	Massa Final (Membrana com adição da diluição filtrada(g))	Massa Seca (g/l)
1	1:10	0,729	0,1227	0,1273	0,230
2	1:20	0,327	0,1228	0,1248	0,100
3	1:40	0,165	0,122	0,1229	0,045
4	1:60	0,119	0,122	0,1226	0,030
5	1:80	0,094	0,1215	0,1218	0,015
6	1:100	0,073	0,1224	0,1225	0,005

A partir dos dados mostrados na tabela acima, foi plotado o gráfico (figura 6).

Figura 6: Curva de calibração obtida durante a padronização da metodologia para a obtenção da produção de biomassa (linhagem BY4741).

Após estabelecermos a metodologia para padronização da curva de calibração, foram preparados pré-inóculos de ambas as linhagens selvagem e mutante, para construirmos a curva de calibração das respectivas linhagens.

6.5 Testes de tolerância

Figura 7: Figura esquemática dos testes de tolerância.

Inicialmente, pré-inóculos das linhagens selvagem e mutante ($\Delta ics3$) foram crescidos em meio YPD a 30 °C em agitador rotativo 175 rpm por 24 horas. Após este período de tempo as culturas foram diluídas para uma OD_{600nm} (eppendorf® BioPhotometer *plus*) de 0,2 (inóculo). Esta correção foi feita com a finalidade de se iniciar igualmente o crescimento de ambas as culturas (selvagem e mutante).

Para avaliação da tolerância ao peróxido de hidrogênio (H_2O_2) as leveduras *Saccharomyces cerevisiae* (inóculo) foram plaqueadas em meio YPD sólido contendo H_2O_2 (Merck®) ou não (controle). Os inóculos foram incubados e retirados em tempos diferentes. A densidade óptica foi corrigida para 0.2 e 5 μL de cada uma das diluições seriadas de 5x foram plaqueadas em YPD contendo ou não (controle) peróxido de hidrogênio (H_2O_2 3mM). As placas foram incubadas a 30°C (Estufa 502 Fanem®) por 24 horas. Foram testadas as concentrações de 1mM e 3mM de H_2O_2

(Merck®) espalhadas nas placas de cultura. Todos os ensaios foram realizados em triplicata.

Para teste de tolerância ao cobre foi adicionado ao meio de cultura líquido (YPD e SD), as concentrações 5, 7 e 10mM de sulfato de cobre (CuSO_4) e após 4, 8, 16 e 24 horas alíquotas foram retiradas para avaliação da viabilidade por testes de diluição seriada em meios YPD e, SD sólidos, exatamente como descrito acima.

Para teste de tolerância ao cobre com pH do meio de cultura corrigido para pH 6.0, foi adicionado ao meio de cultura líquido (YPD e SD), as concentrações 7 e 10mM de sulfato de cobre (CuSO_4) e 0,003 ml de NaOH a 5M, e após 4, 8 e 24 horas alíquotas foram retiradas para avaliação da viabilidade por testes de diluição seriada em meios YPD e, SD sólidos.

Para teste de tolerância ao meio YPD ácido (pH 4.0), foi adicionado ao meio de cultura YPD líquido, 0,007 ml de HCl a 1M e, após 4, 8 e 24 horas alíquotas foram retiradas para avaliação da viabilidade por testes de diluição seriada em meio YPD sólido.

Para o teste de resistência à cistina e à cisteína, as mesmas foram adicionadas diretamente ao meio de cultura líquido YPD em diferentes concentrações (3mM, 5mM, 7mM e 10mM). Alíquotas foram retiradas para ensaio de viabilidade por diluição seriada após 4, 8, 10, 12, 16 e 24 horas.

Para o teste de resistência à higromicina B (Sigma®), as linhagens mutantes Δics3 e Δers1 , e a linhagem selvagem BY4741 foram estriadas em placas sólidas de YPAD contendo Higromicina B nas concentrações de 150 μM e 250 μM e foram, incubadas a 30°C por 24 horas para que avaliássemos se haveria ou não reversão ou alívio dos fenótipos das linhagens da levedura. Após 24 horas de incubação á 30°C as placas foram retiradas e fotografadas.

Para teste de tolerância a FeSO_4 e Co II , os metais foram adicionados ao meio de cultura líquido YPD, nas concentrações 5mM, 7mM e 10mM de cada elemento e após 4, 8, 16 e 24 horas alíquotas foram retiradas para avaliação da viabilidade por testes de diluição seriada em meios YPD sólido.

6.6 Espectrometria de Emissão Atômica com Plasma Induzido Acoplado (ICP-AES)

Para o teste de ICP-AES, os pré inóculos das linhagens selvagem e mutante (*Δics3*) foram crescidos em 80 ml meio líquido YPD a 30 °C em agitador rotativo a 175 rpm por 20 horas. Após este período de tempo as culturas foram diluídas para uma $\text{OD}_{600\text{nm}}$ (eppendorf® BioPhotometer *plus*) de 0,6 (inóculo). Esta correção foi feita com a finalidade de se iniciar igualmente o crescimento de ambas as culturas (selvagem e mutante). O volume de cada amostra do inóculo foi de 250 ml e, todas as análises seguiram o mesmo padrão. Quando utilizado o sulfato de cobre esse foi adicionado ao meio YPD líquido na concentração de 10mM; para o experimento com sulfato de cobre 10mM em pH 6.0, após a adição do CuSO_4 o meio de cultura teve seu pH ajustado com 0,003 ml de NaOH 5M (Merck®); para análise do meio líquido YPD ácido, o pH do meio de cultura foi ajustado para 4.0 com 0,007ml de HCl 1M (Merck®). As amostras foram colocadas em agitador rotativo a 30°C 175 rpm (e após 20 horas de incubação em agitador rotativo, os inóculos foram retirados e as amostras foram centrifugadas (centrifuga eppendorf® Centrifuge 5810 R) a 3000 x G por 15 minutos, o meio de cultura residual foi totalmente removido. Após esse procedimento, as células foram lavadas com água ultrapura (MilliQ®) e centrifugadas novamente a 3000 x G, o sobrenadante foi descartado. O restante de massa celular foi transferido para eppendorfs de 2ml e centrifugados por 10 minutos a 3000 x G, o sobrenadante foi totalmente removido. Para a secagem, as amostras,

foram colocadas em Speed Vac (eppendorf® Concentrator *plus*) por um período de 20 horas a 30°C. Após essa etapa, os pellets foram pesados a fim de obtermos a massa final. Os valores estão na tabela abaixo: (Schuller, Auffermann et al. 2013).

Linhagem	Ods	Massa (g)
BY4741 Controle	9,37	0,4445g
$\Delta ics3$ Controle	1,504	0,5065g
BY4741 Ácido (pH4.0)	9,98	0,4602g
$\Delta ics3$ Ácido (pH4.0)	12,86	0,4642g
BY4741 CuSo ₄ (pH4.0)	1,61	0,0959g
$\Delta ics3$ CuSo ₄ (pH4.0)	1,12	0,0547g
BY4741 CuSo ₄ (pH6.0)	6,38	0,3581g
$\Delta ics3$ CuSo ₄ (pH6.0)	6,48	0,4007g

Tabela 4: Medidas de massa e densidade óptica das linhagens BY4741 e $\Delta ics3$ de *Saccharomyces cerevisiae* para análise de ICP-AES.

Após a pesagem, as amostras foram entregues ao departamento de Química Analítica, do Instituto de Química da Universidade de São Paulo, para a análise de ICP-AES.

7. RESULTADOS E DISCUSSÃO

7.1. Quantificação de Massa Seca

A quantificação de massa seca das linhagens BY4741 e $\Delta ics3$ foram realizadas utilizando-se as curvas de calibração (figura 8, A e B) construídas a partir dos dados obtidos e apresentados nas tabelas 5 e 6, respectivamente.

Linhagem BY4741 Controle			
OD	M. inicial	M. final	Massa Seca (g/l)
0,729	0,1227 g	0,1273 g	0,230 g/l
0,618	0,0861 g	0,0892 g	0,207 g/l
0,327	0,1228 g	0,1248 g	0,100 g/l
0,165	0,1220 g	0,1229 g	0,045 g/l
0,119	0,1220 g	0,1226 g	0,030 g/l
0,094	0,1215 g	0,1218 g	0,015 g/l
0,073	0,1224 g	0,1225 g	0,005 g/l

Tabela 5: Medidas de massa inicial e massa seca final para a construção da curva de calibração para a linhagem BY4741.

Linhagem $\Delta ics3$ Controle			
OD	M. inicial	M. final	Massa Seca (g/l)
0,722	0,0860 g	0,0901 g	0,273 g/l
0,626	0,0860 g	0,0892 g	0,213 g/l
0,555	0,0862 g	0,0892 g	0,200 g/l
0,473	0,0831 g	0,0853 g	0,147 g/l
0,422	0,0829 g	0,0845 g	0,120 g/l
0,331	0,0853 g	0,0871 g	0,107 g/l
0,202	0,0830 g	0,0840 g	0,067 g/l
0,149	0,0846 g	0,0850 g	0,027 g/l
0,11	0,0849 g	0,0852 g	0,020 g/l
0,072	0,0836 g	0,0837 g	0,007 g/l

Tabela 6: Medidas de massa inicial e massa seca final para a construção da curva de calibração para a linhagem $\Delta ics3$.

Figura 8: Curva de calibração obtida para a obtenção da produção de biomassa. (A) linhagem BY4741 e (B) linhagem Δ ics3.

Essas curvas permitem a avaliação da produção celular pela medida simples de absorbância por espectrofotometria.

7.2 Cinética de crescimento das linhagens BY4741 e Δ ics3

Para observarmos o efeito da deleção do gene *ICS3* sobre a cinética de crescimento, as linhagens BY4741 e Δ ics3 foram incubadas em 20 ml de meio YPD líquido a 30 °C em agitador rotativo a 175 rpm em erlenmeyer de 250 ml. Após 16 horas, os pré inóculos foram retirados e suas OD_{600nm} foram corrigidas para 0,1. Os inóculos foram colocados novamente em agitador rotativo por até 107 horas. Amostras desse inóculo foram coletadas em diferentes tempos e as medidas de absorbância (OD_{600nm}) foram realizadas.

Foi plotado o gráfico da cinética de crescimento de ambas as linhagens (figura 16).

Figura 9: Cinética de crescimento das linhagens selvagem e mutante.

As células de ambas as linhagens (BY4741 e Δ ics3) foram crescidas em meio YPD a 30° C por 16 horas, diluídas para uma OD_{600nm} de 0,1 em YPD fresco . As amostras foram colocadas em *shaker* por até 107 horas e retiradas em diferentes tempos para leitura de absorbância.

A partir do gráfico acima verificou-se que as linhagens mutante para Δ ics3 e selvagem apresentam pouca ou nenhuma diferença em sua cinética de crescimento. Esses dados sugerem que a deleção do gene *ICS3*, não é essencial para a condição de crescimento testada (meio rico YPD, temperatura ideal para levedura 30°C).

7.3 Teste de Tolerância na Presença de Peróxido de Hidrogênio

Estudos prévios realizados pelo nosso grupo em larga escala com 217 mutantes viáveis sugeriu que a linhagem Δ ics3 é sensível ao estresse oxidativo. Para confirmar esses resultados iniciais foram realizados testes de tolerância especificamente para a linhagem Δ ics3. Foi testada a tolerância desta linhagem em duas concentrações de peróxido de hidrogênio, 1 mM e 3 mM (figura 10).

Figura 10: Teste de tolerância ao peróxido de hidrogênio. As células de ambas as linhagens (BY4741 e $\Delta ics3$) foram crescidas em meio líquido YPD, a 30°C, por 24 horas e, a partir de uma OD_{600nm} de 0,2 uma alíquota de 5μL de cada diluição seriada de 5x foi aplicada em placa de YPD sólido, (placa controle) ou contendo peróxido de hidrogênio nas concentrações 1 mM e 3mM. As placas foram incubadas a 30°C por 24 horas e fotografadas. (Ensaio realizado em triplicata).

Os resultados obtidos demonstram que a concentração de 3 mM de peróxido de hidrogênio é letal para a linhagem $\Delta ics3$ uma vez que não se observa formação de colônias. Esta aumentada sensibilidade ao agente oxidante sugere que a proteína Ics3p é importante para o processo de proteção e/ou manutenção celular durante o estresse oxidativo.

7.4 Teste de Tolerância na Presença de Sulfato de Cobre

Outro estudo em larga escala, relacionou a mutação do gene *ICS3* ao metabolismo intracelular de cobre (Entian, Schuster et al. 1999). Com o objetivo de confirmar este resultado, realizamos testes de tolerância da linhagem $\Delta ics3$ em concentrações de 7 mM e 10 mM de sulfato de cobre (figura 11).

Incubadas 30°C por 48 horas

Figura 11: Testes de tolerância ao cobre. As células de ambas as linhagens (BY4741 e $\Delta ics3$) foram crescidas em meio YPD a 30° C por 16 horas, diluídas para uma OD_{600nm} de 0,2 em YPD fresco no qual foi adicionado ou não (controle) $CuSO_4$ nas concentrações indicadas. As amostras foram colocadas em *shaker* e retiradas após 4h (A), 6h (B) e 24h (C) horas; foram feitas diluições a partir de uma OD_{600nm} de 0,2 e uma alíquota de 5 μ L de cada diluição seriada de 5x foi aplicada em placa de YPD sólido. As placas foram incubadas a 30°C por 24 horas e fotografadas (Ensaio realizado em triplicata).

Para os experimentos apresentados na figura 11, as medidas de sensibilidade ao metal cobre das linhagens selvagem e mutante foram realizadas com sulfato de cobre ($CuSO_4$) sem correção de pH do meio de cultivo YPD. No entanto, observamos que o pH dos meios YPD com e sem adição de $CuSO_4$ apresentam importantes diferenças de pH (pH = 6.0 para YPD e pH = 4.0 para YPD + $CuSO_4$). Dessa forma, realizamos novos experimentos onde o pH de ambos os meios foram igualmente corrigidos (pH = 6.0). Após as triplicatas (imagem seguinte) realizadas com correção de pH do meio YPD + $CuSO_4$, os resultados mostram que

as linhagens $\Delta ics3$, $\Delta ics1$ e também a selvagem não apresentaram a mesma sensibilidade ao cobre quando o pH do meio de cultura é corrigido (figura 13).

7.5 Teste de Tolerância na Presença de Sulfato de Cobre no meio de cultura YPD pH 4.0 utilizando-se as linhagens BY4741, $\Delta ics3$ e $\Delta ics1$

Linhagens BY4741, $\Delta ics3$ e $\Delta ics1$ em Meio YPD 7mM e 10mM sem correção de pH

Figura 12: Testes de tolerância ao cobre. As células das linhagens BY4741, $\Delta ics3$ e $\Delta ics1$, foram crescidas em meio YPD a 30° C por 16 horas, diluídas para uma OD_{600nm} de 0,2 em YPD fresco no qual foi adicionado ou não (controle) CuSO₄ nas concentrações indicadas. As amostras foram colocadas em *shaker* e retiradas após 4 horas e 8 horas; foram feitas diluições a partir de uma OD_{600nm} de 0,2 e uma alíquota de 5μL de cada diluição seriada de 5x e foi aplicada em placa de YPD sólido. As placas foram incubadas a 30°C por 24 horas e fotografadas (Ensaio realizado em triplicata).

Para os resultados obtidos na presença de CuSO₄ a 10mM após um período de 8 horas de incubação foi possível observarmos que a concentração deste metal

afeta a linhagem Δics1 aproximadamente 100 vezes mais do que para a Δics3 , estes resultados corroboram com os dados publicados por *Entian* e colaboradores (Entian, Schuster et al. 1999).

7.6 Teste de Tolerância na Presença de Sulfato de Cobre no meio de cultura YPD pH 6.0 utilizando-se as linhagens BY4741, Δics3 e Δics1

Linhagens BY4741, Δics3 e Δics1 Meio YPD pH Corrigido CuSO_4 10mM

Figura 13: Testes de tolerância ao cobre, utilizando-se a correção de pH (pH 6.0 ambos os meios). As células de ambas as linhagens (BY4741, Δics3 e Δics1) foram crescidas em meio YPD a 30° C por 16 horas, diluídas para uma $\text{OD}_{600\text{nm}}$ de 0,1 em YPD fresco no qual foi adicionado ou não (controle) CuSO_4 na concentração de 10mM. As amostras foram colocadas em *shaker* e retiradas em diferentes tempos para leitura das ODs, após 4, 8 e 24 horas de incubação no agitador rotativo foram feitas diluições a partir de uma $\text{OD}_{600\text{nm}}$ de 0,2 e uma alíquota de 5 μL de cada diluição seriada de 5x foi aplicada em placa de meio sólido YPD. As placas foram incubadas a 30°C por 24 horas e fotografadas. Os ensaios foram realizados em triplicata.

Na literatura foram observados fenótipos sensíveis ao cobre, porém sem menção ao pH do meio, utilizamos também a linhagem mutante Δics1 , de função molecular e biológica conhecidas, sendo que essa linhagem é mais sensível ao

cobre do que a isogênica $\Delta ics3$, conforme dados publicados por Entian e colaboradores (Entian, Schuster et al. 1999). Nos resultados de sensibilidade ao cobre com pH corrigido para pH 6.0, não ocorre o mesmo fenômeno quando o pH do meio de cultura não tem seu pH ajustado, demonstrando que, tanto a linhagem $\Delta ics3$ quanto a $\Delta ics1$ apresentam sensibilidade ao cobre apenas quando o pH do meio de cultura está ácido, relacionando ambas as linhagens com a disfunção da VATP-ase.

Assim, avaliamos a sensibilidade das linhagens selvagem e mutante em meios YPD com pH = 6.0 e 4.0. Estes experimentos foram importantes para avaliarmos a real contribuição do CuSO_4 e/ou pH para a sensibilidade observada na linhagem $\Delta ics3$ observada anteriormente (figura 13).

7.7 Cinética de Crescimento das Linhagens BY4741 E $\Delta ics3$ na Presença de Sulfato de Cobre

Foi realizada uma cinética de crescimento de ambas as linhagens em meio YPD líquido com pH corrigido (pH = 6.0) contendo ou não (controle) 10 mM de sulfato de cobre. Como pode-se observar na figura 14 o cobre não influenciou o crescimento da linhagem BY4741. Já para a linhagem $\Delta ics3$ podemos observar a partir da linha de tendência plotada no gráfico, que o tratamento com 10 mM de sulfato de cobre alterou sua cinética de crescimento no período de tempo aproximado entre 30 e 65 horas (figura 14). Foi possível observar que após 72 horas de tratamento com 10 mM de sulfato de cobre as cinéticas de crescimento de ambas as linhagens (figura 14) corroboram com o plaqueamento apresentado na figura 13. Este resultado preliminar demonstra que neste período não há diferença expressiva de crescimento das linhagens tratadas ou não (controle).

Figura 14: Cinética de crescimento das linhagens selvagem e mutante em meio YPD contendo ou não sulfato de cobre (10 mM) com pH corrigido (pH = 6.0). As células da linhagens **BY4741** e **Δics3** foram crescidas em meio YPD a 30° C por 16 horas, diluídas para uma OD_{600nm} de 0,1 em YPD fresco no qual foi adicionado ou não (controle) CuSO₄ na concentração de 10mM. As amostras foram colocadas em *shaker* e retiradas em diferentes tempos para leitura das absorvâncias. As barras de

erro representam o desvio padrão em relação à média das 3 réplicas biológicas representada nos gráficos.

7.8 Teste de Tolerância ao Meio de Cultura YPD Ácido (pH 4.0)

Linhagens: BY4741 e $\Delta ics3$ em Meio de Cultura YPD, Controle e YPD Ácido (pH 4.0)

Figura 15: Testes de tolerância ao meio YPD Ácido (pH 4.0). As células de ambas as linhagens (BY4741 e $\Delta ics3$) foram crescidas em meio YPD utilizando-se a correção de pH (pH 4.0) e Controle (pH 6.0) a 30° C por 16 horas, diluídas para uma OD_{600nm} de 0,2 em YPD fresco. As amostras foram colocadas em *shaker* e retiradas após 4horas e 8horas; foram feitas diluições a partir de uma OD_{600nm} de 0,2 e uma alíquota de 5 μ L de cada diluição seriada de 5x e aplicadas em placa de YPD sólido. As placas foram incubadas a 30°C por 24 horas e fotografadas (Ensaio realizado em triplicata).

As leveduras geralmente crescem muito bem em um meio de cultura inicial com pH entre 4.0 - 6.0, mas muitas leveduras são capazes de crescer em uma vasta gama de pH. Esta habilidade que elas possuem de crescer em baixo pH fazem com que ocupem diferentes nichos ecológicos e também consumam alimentos ácidos e geralmente não apresentam um bom crescimento na presença de pH alcalino (Walker, Graeme M. 1998). Nossos resultados estão em concordância, é possível observarmos nas imagens, que o pH ácido (pH 4.0) (causado pela adição de HCl ao

meio de cultura) não altera a viabilidade das linhagens mutante e selvagem, no período de 24 horas, pois ambas as linhagens apresentaram a mesma cinética de crescimento em meio YPD controle (pH 6.0) e ácido (pH 4.0). Entretanto, a linhagem $\Delta ics3$, não apresenta a mesma resposta em sua cinética de crescimento na presença de cobre em pH ácido, indicando que $\Delta ics3$ apresenta sensibilidade ao ácido quando em presença de íons metálicos de cobre.

7.9 Testes de Tolerância a diferentes metais, Cobalto e Ferro nas Concentrações 7mM e 10mM

Linhagens BY4741 e $\Delta ics3$ em meio YPD com Cobalto a 7mM e 10 mM

Linhagens BY4741 e Δ ics3 em meio YPD com Sulfato de Ferro a 7mM e 10 mM

Figura 16: Testes de tolerância aos metais cobalto e ferro. As células de ambas as linhagens (BY4741 e Δ ics3) foram crescidas a 30° C por 16 horas, diluídas para uma OD_{600nm} de 0,2 em YPD fresco no qual foi adicionado ou não (controle) CuSO₄ nas concentrações indicadas. As amostras foram colocadas em *shaker* e retiradas após 4 horas e 8 horas; foram feitas diluições a partir de uma OD_{600nm} de 0,2 e uma alíquota de 5μL de cada diluição seriada de 5x e aplicadas em placa de YPD sólido. As placas foram incubadas a 30°C por 24 horas e fotografadas (Ensaio realizado em triplicata).

De acordo com nossos resultados foi possível concluir que o sulfato de ferro e o cobalto nas concentrações de 7mM e 10mM após 8 horas de incubação, não são letais para ambas as linhagens, selvagem e mutante, sugerindo que estes metais estejam sendo utilizados como fonte de nutrientes para a levedura. Podemos observar que, o efeito de letalidade, observado em Δ ics3 quando exposta ao cobre nas mesmas condições, é específico para esse metal, visto que não há diferença significativa na viabilidade dessas células quando expostas as mesmas concentrações de ferro ou cobalto.

7.10 Teste de ICP-AES para Análise de Absorção Intracelular de Cobre em pH 4.5 e pH 6.0

Figura 17: Gráficos de ICP-AES para análises de absorção intracelular de cobre em *Sacharomyces cerevisiae* com e sem adição de sulfato de cobre na concentração de 10Mm, em pH4.5 e pH 6.0 em meio de cultura líquido YPD.

De acordo com os resultados da análise de ICP-AES, é possível observarmos nos gráficos acima, que a absorção de cobre na linhagem $\Delta ics3$ é aproximadamente 3,5 vezes maior em pH 4.5 do que em pH 6.0. Esses dados sugerem que grandes quantidades de cobre estão sendo importadas para o meio intracelular devido á protonação, causado pela acidificação do meio de cultura, às membranas vacuolares e consequentemente um comprometimento da funcionalidade da atividade da VATP-ase, refletindo o papel critico do vacúolo na detoxificação de íons metálicos, propondo o envolvimento de $\Delta ics3$ às ações das VATP-ases.

TABELA 7: Concentrações ótimas de cátions na levedura

Cation	Concentration
H ⁺	1.0 μ M (pH 6.0)
K ⁺	2-4 mM
Mg ²⁺	2-4 mM
Mn ²⁺	2-4 μ M
Ca ²⁺	< μ M
Cu ²⁺	1.5 μ M
Fe ²⁺	1-3 μ M
Zn ²⁺	4-8 μ M
Ni ²⁺	10-90 μ M
Mo ²⁺	1.5 μ M
Co ²⁺	0.1 μ M
B ⁺	0.4 μ M

(Adaptado de Walk, Graeme M. –“Yeast Physiology and Byotechnology”)

O cobre também é um cátion divalente vital para células de leveduras (Stehlik-Tomas *et al.*, 2004). A concentração ótima no meio nutritivo para crescimento da levedura e atividade fermentativa está na faixa de 1-10 μ M. Segundo JONES & GREENFIELD (1984), a concentração de 10 μ M já pode inibir o crescimento, sendo a concentração ótima entre 1-1,5 mM.

7.11 Teste de ICP-AES para Análise de Absorção Intracelular de Ferro em pH 4.5 e pH 6.0

Figura 18: Gráfico de ICP-AES para análises de absorção intracelular de ferro em *Sacharomyces cerevisiae* com e sem adição de sulfato de cobre na concentração de 10mM, e em pH4.5 e pH 6.0 em meio de cultura líquido YPD.

Ferro e cobre são metais de transição e estão envolvidos em reações redox que é essencial para todos os eucariotos, entretanto a concentração intracelular desses íons deve ser cuidadosamente monitorada, pois são potencialmente tóxicos. Os genes *CTR1* e *CTR3* são mediadores de captura de alta afinidade ao cobre, enquanto a captura de baixa afinidade ao cobre é mediada por transportadores de membrana incluindo Fet4 cujos transportadores são similares aos de afinidade ao ferro, zinco e outros cátions, e Smf1 um membro da família de transportadores metálicos (NRAMP), que apresenta uma ampla especificidade a metais. O fato de não existir cobre livre no citossol depende da existência de transportadores intracelulares chamadas metalochaperonas, que entregam o cobre a diferentes

compartimento intracelulares nos quais este metal será incorporado á enzimas alvo(Serrano, Bernal et al. 2004).

De acordo com os resultados da análise de absorção intracelular de ferro, ambas as linhagens BY4741 e $\Delta ics3$, em meio de cultura liquido YPD, controle (pH 6.0), apresentam uma menor absorção de ferro quando comparadas a absorção deste mesmo metal em meio de cultura liquido YPD ácido (pH 4,5), esses dados estão de acordo com os resultados publicados por Kawahata e colaboradores (Kawahata, Masaki et al. 2006), demonstrando que o pH do meio de cultura interfere diretamente na absorção de íons metálicos.

Em meio de cultura líquido YPD com adição de CuSO_4 10 mM, em pH 4,5 e pH 6.0, é possível observarmos que a linhagem $\Delta ics3$, em condições de pH 6.0, absorve aproximadamente 3 vezes menos a quantidade de ferro do que quando em pH 4.5, sugerindo que a acidificação do meio, causada pela adição do sulfato de cobre, leve a uma absorção maior de cobre e consequente aumento na absorção de ferro, visto que, a absorção de ferro é diretamente ligada ao equilíbrio dos níveis de cobre.

7.12 Teste de Resistência à Cistina e Cisteína

Uma vez que observamos *in silico* uma interação física e genética entre os genes *ICS3* e *ERS1*, e que este último codifica para uma proteína transportadora de cistina do vacúolo para o citossol, realizamos experimentos de resistência da linhagem $\Delta ics3$ frente aos estresses com 5 mM de cisteína e 5 mM de cistina (figura 19). Se *ICS3* possuir função similar a *ERS1*, o transporte desses aminoácidos estará prejudicado levando a uma toxicidade quando a levedura mutante for exposta a altas doses dos mesmos. Assim, avaliamos se a cisteína ou a cistina possuem algum

efeito tóxico sobre a linhagem $\Delta ics3$, além da mudança na cinética já mostrada preliminarmente.

Figura 19: Cinética de crescimento das linhagens BY4741 e $\Delta ics3$ submetidas ao estresse com 5 mM de cisteína ou 5 mM de cistina. As células de ambas as linhagens (BY4741 e $\Delta ics3$) foram crescidas em meio YPD a 30° C por 16 horas. Após esse período, amostras foram diluídas para uma OD_{600nm} de 0,2 em YPD fresco no qual foi adicionado ou não (controle) cistina ou cisteína na concentração final de 5mM. As amostras foram colocadas em *shaker* e retiradas em diferentes tempos para leitura das absorbâncias plotadas no gráfico.

Observamos que a linhagem mutante é sensível ao estresse tanto com cisteína quanto com cistina. Essa mesma sensibilidade não foi observada para a linhagem selvagem BY4741. Pode-se observar também no gráfico acima, que em condições não estressantes (meio YPD sem cisteína e cistina) ambas as linhagens apresentam uma cinética de crescimento parecidas, reconfirmando que a deleção do gene *ICS3* não altera o crescimento das leveduras na condição testada (meio rico YPD, temperatura ideal para levedura 30°C).

8. Testes Realizados Durante o Período de Desenvolvimento do Projeto

8.1 Teste de Sensibilidade a Higromicina B

Figura 20: Testes de tolerância a Higromicina B. As células de ambas as linhagens (BY4741 e Δ ics3) foram retiradas diretamente do meio sólido YPD e estriadas em placas sólidas YPAD, contendo ou não (controle) Higromicina B. As placas foram incubadas a 30°C por 24 horas e fotografadas.

Testamos a tolerância de Δ ics3 na presença de higromicina B adicionado ao meio sólido YPAD. Caso fosse observada a sensibilidade a esse antibiótico, realizaríamos o ensaio de complementação de função de Δ ers1 com o vetor de expressão pYES2/CT (Invitrogen) contendo o gene *lcs3p* em levedura. Essa construção seria transformada em levedura e essas células seriam expostas à higromicina B para que analisássemos se haveria uma reversão ou alívio de fenótipo. Isso nos ajudaria a estabelecer quão estreita poderia ser a relação de funcionalidade entre *ICS3* x *ERS1*. Entretanto *ICS3* não apresentou sensibilidade a este antibiótico.

8.2 Teste de Tolerância ao PMSF na Presença de 10mM de CuSO_4 em pH 4.0 e pH 6.0

Linhagens BY4741 e Δics3 com CuSO_4 10mM na presença de PMSF 1mM e 2mM em meio YPD, após 4 horas

Figura 21: Testes de tolerância ao cobre em meio ácido (pH 4.0) e meio com pH corrigido para pH 6.0 na presença de PMSF. As células das linhagens BY4741 e Δics3 foram crescidas em meio YPD a 30° C por 16 horas, diluídas para uma $\text{OD}_{600\text{nm}}$ de 0,2 em YPD fresco no qual foi adicionado CuSO_4 nas concentrações indicadas. As amostras foram colocadas em *shaker* e retiradas após 4 horas; foram feitas diluições a partir de uma $\text{OD}_{600\text{nm}}$ de 0,2 e uma alíquota de 5 μL de cada diluição seriada de 5x e aplicadas em placa de YPD sólido. As placas foram incubadas a 30°C por 24 horas e fotografadas (Ensaio realizado em triplicata).

Podemos observar nas imagens acima que a adição de diferentes concentrações (1mM e 2mM) de PMSF na presença de CuSO_4 , alteram a cinética de crescimento das linhagens selvagem e mutante em pH 4.0, o mesmo não ocorre em pH 6.0 nas mesmas condições. Para as placas controle os resultados obtidos demonstram que o cobre em pH 4.0 inibe a cinética de crescimento das linhagens BY4741 e Δics3 .

8.3 Teste de Tolerância ao PMSF na Presença de Meio de Cultura Ácido (pH 4.0) e sem Controle (pH 6.0)

Linhagem BY4741 e $\Delta ics3$ em meio YPD Controle e Ácido(pH 4.0) na presença de PMSF 1mM e 2mM, após 4 e 8 horas

Figura 22: Testes de tolerância ao meio YPD Ácido (pH4.0) e Controle na presença de PMSF nas concentrações indicadas. As células das linhagens BY4741 e $\Delta ics3$ foram crescidas em meio YPD controle e ácido a 30° C por 16 horas, diluídas para uma OD_{600nm} de 0,2 em YPD fresco no qual foi adicionado $CuSO_4$ nas concentrações indicadas. As amostras foram colocadas em *shaker* e retiradas após 4 horas e 8 horas; foram feitas diluições a partir de uma OD_{600nm} de 0,2 e uma alíquota de 5 μ L de cada diluição seriada de 5x e aplicadas em placa de YPD sólido. As placas foram incubadas a 30°C por 24 horas e fotografadas (Ensaio realizado em triplicata)

Nas imagens acima os resultados demonstram que o PMSF nas condições testadas, não interfere na cinética de crescimento de ambas as linhagens, selvagem

e mutante, para os meios de cultura pH 4.0 e pH 6.0, demonstrando o papel crítico do pH apenas na presença de íons metálicos.

9. Considerações finais

- 1) Análises da cinética de crescimento em meio rico YPD, em temperatura ótima para a levedura 30°C, mostraram que a mutação em *YJL077C* não leva a uma mudança no perfil de crescimento da levedura quando comparada a sua isogênica selvagem.
- 2) Através dos resultados obtidos em nossos experimentos realizados com as linhagens BY4741 e $\Delta ics3$ foi possível concluir que a linhagem $\Delta ics3$ apresenta maior sensibilidade ao peróxido de hidrogênio, sugerindo a importância desse gene no processo de manutenção/proteção contra o estresse oxidativo.
- 3) Testes de tolerância na presença de cobre em diferentes concentrações (7mM e 10mM) e tempos (4, 6 e 24 horas), confirmaram os resultados de Entian *et al* (1999), demonstrando que a linhagem $\Delta ics3$ apresenta maior sensibilidade a altas concentrações desse metal.
- 4) Teste de tolerância ao cobre utilizando-se as linhagens BY4741, $\Delta ics1$ e $\Delta ics3$ confirmaram os resultados de Entian e colaboradores (Entian, Schuster *et al*. 1999), demonstrando que a linhagem $\Delta ics1$ é mais sensível ao cobre do que sua isogênica $\Delta ics3$.
- 5) Testes de tolerância ao cobre com correção de pH foram realizados pois verificou-se uma importante diferença de pH entre os meios YPD com e sem cobre, pH = 4.0 e pH = 6.0, respectivamente. Os resultados obtidos mostram que a linhagem $\Delta ics3$ não apresenta a mesma sensibilidade a este metal quando comparada à linhagem selvagem como demonstrado nos testes anteriores, onde não foram realizadas as correções de pH do meio, de forma que o pH, interfere diretamente na homeostase dos íons metálicos.
- 6) Testes de tolerância com pH ácido em meio YPD, demonstraram que, a mudança de pH não interfere na cinética de crescimento das linhagens selvagem e mutante. A interferência de pH causa alterações na cinética de crescimento das linhagens apenas na presença de íons metálicos de cobre.

- 7) Testes de tolerância a outros metais utilizando as linhagens mutantes $\Delta ics1$ e $\Delta ics3$ e selvagem BY441, demonstram que as linhagens não apresentam sensibilidade ao cobalto nem ao sulfato de ferro sendo sua sensibilidade específica para o cobre.
- 8) Resultados das análises de ICP-AES para absorção intracelular de cobre, demonstraram que a absorção de cobre na linhagem $\Delta ics3$ é aproximadamente 3,5 vezes maior em pH 4.5 do que em pH 6.0. Esses dados sugerem que grandes quantidades de cobre estão sendo importadas para o meio intracelular devido à protonação, causada pela acidificação do meio de cultura, às membranas vacuolares e consequentemente um comprometimento da funcionalidade da atividade da VATP-ase
- 9) Resultados das análises de ICP-AES para absorção intracelular de ferro, demonstraram que ambas as linhagens BY4741 e $\Delta ics3$, em meio de cultura líquido YPD, controle (pH 6.0), apresentam uma menor absorção de ferro quando comparadas a absorção deste mesmo metal em meio de cultura líquido YPD ácido (pH 4,5), esses dados estão de acordo com os resultados publicados por Kawahata e colaboradores (Kawahata, Masaki et al. 2006), demonstrando que o pH do meio de cultura interfere diretamente na absorção de íons metálicos.
- 10) Resultados preliminares demonstraram que cistina ou cisteína interferem na cinética de crescimento da linhagem $\Delta ics3$ em uma concentração de 5mM, quando comparadas com a linhagem BY4741.
- 11) Testes Realizados Durante o Período de Desenvolvimento do Projeto
 - 11.1) Experimentos realizados com Higromicina B demonstraram que as concentrações de 150 $\mu\text{g/ml}$ e 250 $\mu\text{g/ml}$, após um período de 24 horas de incubação, não afetam o crescimento da linhagem $\Delta ics3$, já a linhagem *ers1*, a concentração de 250 $\mu\text{g/ml}$ após 24 horas de incubação, apresentou sensibilidade a este antibiótico.
 - 11.2) Teste de Tolerância ao PMSF na Presença de Meio de Cultura Ácido (pH 4.0) e sem Controle (pH 6.0). A adição de diferentes concentrações (1mM e 2mM) de PMSF na presença de CuSO_4 , alteraram a cinética de crescimento das linhagens selvagem e mutante em pH 4.0, o mesmo não ocorre em pH 6.0 nas mesmas condições. Sugerindo que há uma interferência do PMSF na presença de cobre em pH 4.0

11.3) os resultados demonstram que o PMSF nas condições testadas, não interfere na cinética de crescimento de ambas as linhagens, selvagem e mutante, para os meios de cultura YPD pH 4.0 e pH 6.0, demonstrando o papel crítico do pH apenas na presença de íons metálicos. Todos esses dados reforçam a importância crítica do papel do pH na presença de metais.

10. Bibliografia

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The clearance of malfunctioning mitochondria is an important housekeeping function in respiring eukaryotic cells and plays a role in physiological homeostasis as well as in the progression of late-onset diseases. This clearance is thought to occur by a specific form of autophagic degradation called mitophagy. Although the mechanism of nonspecific macroautophagy is relatively well established, the selective autophagic degradation of mitochondria has only recently begun to receive significant attention. An important step toward elucidating the mechanism by which defective mitochondria are selected and degraded is the establishment of conditions under which mitophagy is induced. This review covers our current understanding of mitophagy in the model organism *Saccharomyces cerevisiae* and its modes of activation, with a focus on stationary-phase mitophagy—a form of mitophagy that holds promise as a potential quality control mechanism.

Adamo, G. M., S. Brocca, et al. (2012). "Laboratory evolution of copper tolerant yeast strains." Microb Cell Fact 11: 1.

BACKGROUND: Yeast strains endowed with robustness towards copper and/or enriched in intracellular Cu might find application in biotechnology processes, among others in the production of functional foods. Moreover, they can contribute to the study of human diseases related to impairments of copper metabolism. In this study, we investigated the molecular and physiological factors that confer copper tolerance to strains of baker's yeasts. **RESULTS:** We characterized the effects elicited in natural strains of *Candida humilis* and *Saccharomyces cerevisiae* by the exposure to copper in the culture broth. We observed that, whereas the growth of *Saccharomyces* cells was inhibited already at low Cu concentration, *C. humilis* was naturally robust and tolerated up to 1 g . L-1 CuSO₄ in the medium. This resistant strain accumulated over 7 mg of Cu per gram of biomass and escaped severe oxidative stress thanks to high constitutive levels of superoxide dismutase and catalase. Both yeasts were then "evolved" to obtain hyper-resistant cells able to proliferate in high copper medium. While in *S. cerevisiae* the evolution of robustness towards Cu was paralleled by the increase of antioxidative enzymes, these same activities decreased in evolved hyper-resistant *Candida* cells. We also characterized in some detail changes in the profile of copper binding proteins, that appeared to be modified by evolution but, again, in a different way in the two yeasts. **CONCLUSIONS:** Following evolution, both *Candida* and *Saccharomyces* cells were able to proliferate up to 2.5 g . L-1 CuSO₄ and to accumulate high amounts of intracellular copper. The comparison of yeasts differing in their robustness, allowed highlighting physiological and molecular determinants of natural and acquired copper tolerance. We observed that different mechanisms contribute to confer metal tolerance: the control of copper uptake, changes in the levels of enzymes involved in oxidative stress response and changes in the copper-binding proteome. However, copper elicits different physiological and molecular reactions in yeasts with different backgrounds.

Alibhoy, A. A. and H. L. Chiang (2011). "Vacuole import and degradation pathway: Insights into a specialized autophagy pathway." World J Biol Chem 2(11): 239-245.

Glucose deprivation induces the synthesis of pivotal gluconeogenic enzymes such as fructose-1,6-bisphosphatase, malate dehydrogenase, phosphoenolpyruvate carboxykinase and isocitrate lyase in *Saccharomyces cerevisiae*. However, following glucose replenishment, these gluconeogenic enzymes are inactivated and degraded. Studies have characterized the mechanisms by which these enzymes are inactivated in response to glucose. The site of degradation of these proteins has also been ascertained to be dependent on the duration of starvation. Glucose replenishment of short-term starved cells results in these proteins being degraded in the proteasome. In contrast, addition of glucose to cells starved for a prolonged period results in these proteins being degraded in the vacuole. In the vacuole dependent pathway, these proteins are sequestered in specialized vesicles termed vacuole import and degradation (Vid). These vesicles converge with the endocytic pathway and deliver their cargo to the vacuole for degradation. Recent studies have identified that internalization, as mediated by actin polymerization, is essential for delivery of cargo proteins to the vacuole for degradation. In addition, components of the target of rapamycin complex 1 interact with cargo proteins during glucose starvation. Furthermore, Tor1p dissociates from cargo proteins following glucose replenishment. Future studies will be needed to elaborate on the importance of internalization at the plasma membrane and the subsequent import of cargo proteins into Vid vesicles in the vacuole dependent degradation pathway.

Arguello, J. M., D. Raimunda, et al. (2012). "Metal transport across biomembranes: emerging models for a distinct chemistry." *J Biol Chem* **287**(17): 13510-13517.

Transition metals are essential components of important biomolecules, and their homeostasis is central to many life processes. Transmembrane transporters are key elements controlling the distribution of metals in various compartments. However, due to their chemical properties, transition elements require transporters with different structural-functional characteristics from those of alkali and alkali earth ions. Emerging structural information and functional studies have revealed distinctive features of metal transport. Among these are the relevance of multifaceted events involving metal transfer among participating proteins, the importance of coordination geometry at transmembrane transport sites, and the presence of the largely irreversible steps associated with vectorial transport. Here, we discuss how these characteristics shape novel transition metal ion transport models.

Auesukaree, C., A. Damnernsawad, et al. (2009). "Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae*." *J Appl Genet* **50**(3): 301-310.

During fermentation, yeast cells are exposed to a number of stresses -- such as high alcohol concentration, high osmotic pressure, and temperature fluctuation - so some overlap of mechanisms involved in the response to these stresses has been suggested. To identify the genes required for tolerance to alcohol (ethanol, methanol, and 1-propanol), heat, osmotic stress, and oxidative stress, we performed genome-wide screening by using 4828 yeast deletion mutants. Our screens identified 95, 54, 125, 178, 42, and 30 deletion mutants sensitive to ethanol, methanol, 1-propanol, heat, NaCl, and H₂O₂, respectively. These deleted genes were then classified based on their cellular functions, and cross-sensitivities between stresses were determined. A large number of genes involved in vacuolar H⁽⁺⁾-ATPase (V-ATPase) function, cytoskeleton biogenesis, and cell wall integrity, were required for tolerance to alcohol, suggesting their protective role against alcohol stress. Our results revealed a partial overlap between genes required for alcohol tolerance and those required for thermotolerance. Genes involved in cell wall integrity and the actin cytoskeleton are

required for both alcohol tolerance and thermotolerance, whereas the RNA polymerase II mediator complex seems to be specific to heat tolerance. However, no significant overlap of genes required for osmotic stress and oxidative stress with those required for other stresses was observed. Interestingly, although mitochondrial function is likely involved in tolerance to several stresses, it was found to be less important for thermotolerance. The genes identified in this study should be helpful for future research into the molecular mechanisms of stress response.

Avery, S. V. (2001). "Metal toxicity in yeasts and the role of oxidative stress." *Adv Appl Microbiol* **49**: 111-142.

Babich, P. S., A. N. Skvortsov, et al. (2013). "Non-hepatic tumors change the activity of genes encoding copper trafficking proteins in the liver." *Cancer Biol Ther* **14**(7).

To assess the statistical relationship between tumor growth and copper metabolism, we performed a meta-analysis of studies in which patients with neoplasms were characterized according to any of the copper status indexes (atomic copper serum concentration, serum oxidase activity, ceruloplasmin protein content). Our meta-analysis shows that in the majority of cases (more than 3100 patients), tumor growth positively correlates with the copper status indexes. Nude athymic CD-1 nu/nu mice with subcutaneous tumors of human origin, C57Bl/6J mice with murine melanoma and Apc (Min) mice with spontaneously developing adenomas throughout the intestinal tract were studied to experimentally determine the relationship between tumor progression, liver copper metabolism and copper status indexes. We showed that the copper status indexes increased significantly during tumor growth. In the liver tissue of tumor-bearing mice, ceruloplasmin gene expression, as well as the expression of genes related to ceruloplasmin metallation (CTR1 and ATP7B), increased significantly. Moreover, the presence of an mRNA splice variant encoding a form of ceruloplasmin anchored to the plasma membrane by glycosylphosphatidyl inositol, which is atypical for hepatocytes, was also detected. The ATP7A copper transporter gene, which is normally expressed in the liver only during embryonic copper metabolism, was also activated. Depletion of holo-ceruloplasmin resulted in retardation of human HCT116 colon carcinoma cell growth in nude mice and induced DNA fragmentation in tumor cells. In addition, the concentration of cytochrome C increased significantly in the cytosol, while decreasing in the mitochondria. We discuss a possible trans-effect of developing tumors on copper metabolism in the liver.

Babior, B. M., R. S. Kipnes, et al. (1973). "Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent." *J Clin Invest* **52**(3): 741-744.

As a highly reactive substance produced in biological systems by the one-electron reduction of oxygen, superoxide ($O(2)^{-}$) seemed a likely candidate as a bactericidal agent in leukocytes. The reduction of cytochrome c, a process in which $O(2)^{-}$ may serve as an electron donor, was found to occur when the cytochrome was incubated with leukocytes. $O(2)^{-}$ was identified as the agent responsible for the leukocyte-mediated reduction of cytochrome c by the demonstration that the reaction was abolished by superoxide dismutase, an enzyme that destroys $O(2)^{-}$, but not by boiled dismutase, albumin, or catalase. Leukocyte $O(2)^{-}$ production doubled in the presence of latex particles. The average rate of formation of $O(2)^{-}$ in the presence of these particles was 1.03 nmol/10(7) cells per 15 min. This rate, however, is only a lower limit of the true rate of $O(2)^{-}$ production, since any $O(2)^{-}$ which reacted with constituents other than cytochrome c

would have gone undetected. Thus, O(2) (-) is made by leukocytes under circumstances which suggest that it may be involved in bacterial killing.

Batista-Nascimento, L., C. Pimentel, et al. (2012). "Iron and neurodegeneration: from cellular homeostasis to disease." Oxid Med Cell Longev **2012**: 128647.

Accumulation of iron (Fe) is often detected in the brains of people suffering from neurodegenerative diseases. High Fe concentrations have been consistently observed in Parkinson's, Alzheimer's, and Huntington's diseases; however, it is not clear whether this Fe contributes to the progression of these diseases. Other conditions, such as Friedreich's ataxia or neuroferritinopathy are associated with genetic factors that cause Fe misregulation. Consequently, excessive intracellular Fe increases oxidative stress, which leads to neuronal dysfunction and death. The characterization of the mechanisms involved in the misregulation of Fe in the brain is crucial to understand the pathology of the neurodegenerative disorders and develop new therapeutic strategies. *Saccharomyces cerevisiae*, as the best understood eukaryotic organism, has already begun to play a role in the neurological disorders; thus it could perhaps become a valuable tool also to study the metalloneurobiology.

Beas-Zarate, C., S. V. Rivera-Huizar, et al. (2001). "Changes in NMDA-receptor gene expression are associated with neurotoxicity induced neonatally by glutamate in the rat brain." Neurochem Int **39**(1): 1-10.

The N-methyl-D-aspartate receptor (NMDA-R) is fully functional in the rat early in embryogenesis, and diverse neuronal plasticity events are regulated through its activation later in postnatal development. On the other hand, systemic administration of glutamate (Glu) to rats at birth induces neuronal degeneration in glutamatergic central nervous system regions via Glu receptor activation. However, it is not known whether an increase in neonatal Glu levels modifies the gene expression of NMDA-R subunits, or if these putative changes are related to gamma-aminobutyric acid-mediated (GABAergic) neurotransmission. We measured, by means of semi-quantitative reverse transcriptase polymerase chain reaction, changes in gene expression of the NMDA-R subunits: NMDA-R1, NMDA-R 2A and NMDA-R 2B in cerebral cortex (CC), striatum (ST) and hippocampus (HP) in the brains of rats treated neonatally with monosodium L-glutamate (MSG). These studies were supported by histological and quantitative analysis of the glia. Our results showed histological evidence of neuronal damage, and increased glial cell number and activity were detected. This was seen mainly in the ST and HP of MSG-treated animals. Significant increases in NMDA-R1, 2A and 2B subunits gene expression was also observed in ST and HP but not in CC, where only NMDA-R 2B was increased in MSG-treated rats. Our data suggest that increases in Glu levels and activation of Glu-receptors after neonatal administration of MSG induce an increase in glial cell reactivity and important changes in NMDA-R molecular composition, with signs of neuronal damage.

Berlett, B. S. and E. R. Stadtman (1997). "Protein oxidation in aging, disease, and oxidative stress." J Biol Chem **272**(33): 20313-20316.

Bin, Y., X. Li, et al. (2013). "Amyloid-beta peptide (1-42) aggregation induced by copper ions under acidic conditions." Acta Biochim Biophys Sin (Shanghai) **45**(7): 570-577.

It is well known that the aggregation of amyloid-beta peptide (A β) induced by Cu(2+) is related to incubation time, solution pH, and temperature. In this work, the aggregation of

Abeta1-42 in the presence of Cu(2+) under acidic conditions was studied at different incubation time and temperature (e.g. 25 and 37 degrees C). Incubation temperature, pH, and the presence of Cu(2+) in Abeta solution were confirmed to alter the morphology of aggregation (fibrils or amorphous aggregates), and the morphology is pivotal for Abeta neurotoxicity and Alzheimer disease (AD) development. The results of atomic force microscopy (AFM) indicated that the formation of Abeta fibrous morphology is preferred at lower pH, but Cu(2+) induced the formation of amorphous aggregates. The aggregation rate of Abeta was increased with the elevation of temperature. These results were further confirmed by fluorescence spectroscopy and circular dichroism spectroscopy and it was found that the formation of beta-sheet structure was inhibited by Cu(2+) binding to Abeta. The result was consistent with AFM observation and the fibrillation process was restrained. We believe that the local charge state in hydrophilic domain of Abeta may play a dominant role in the aggregate morphology due to the strong steric hindrance. This research will be valuable for understanding of Abeta toxicity in AD.

Bonilla, E., K. Tanji, et al. (1999). "Mitochondrial involvement in Alzheimer's disease." Biochim Biophys Acta **1410**(2): 171-182.

Borrelly, G., J. C. Boyer, et al. (2001). "The yeast mutant vps5Delta affected in the recycling of Golgi membrane proteins displays an enhanced vacuolar Mg2+/H+ exchange activity." Proc Natl Acad Sci U S A **98**(17): 9660-9665.

Bustos, R. I., E. L. Jensen, et al. (2013). "Copper deficiency alters cell bioenergetics and induces mitochondrial fusion through up-regulation of MFN2 and OPA1 in erythropoietic cells." Biochem Biophys Res Commun.

Copper is essential in cell physiology, participating in numerous enzyme reactions. In mitochondria, copper is a cofactor for respiratory complex IV, the cytochrome c oxidase. Low copper content is associated with anemia and the appearance of enlarged mitochondria in erythropoietic cells. These findings suggest a connection between copper metabolism and bioenergetics, mitochondrial dynamics and erythropoiesis, which has not been explored so far. Here, we describe that bathocuproine disulfonate-induced copper deficiency does not alter erythropoietic cell proliferation nor induce apoptosis. However it does impair erythroid differentiation, which is associated with a metabolic switch between the two main energy-generating pathways. That is, from mitochondrial function to glycolysis. Switching off mitochondria implies a reduction in oxygen consumption and ROS generation along with an increase in mitochondrial membrane potential. Mitochondrial fusion proteins MFN2 and OPA1 were up-regulated along with the ability of mitochondria to fuse. Morphometric analysis of mitochondria did not show changes in total mitochondrial biomass but rather bigger mitochondria because of increased fusion. Similar results were also obtained with human CD34+, which were induced to differentiate into red blood cells. In all, we have shown that adequate copper levels are important for maintaining proper mitochondrial function and for erythroid differentiation where the energy metabolic switch plus the up-regulation of fusion proteins define an adaptive response to copper deprivation to keep cells alive.

Cabiscol, E., E. Piulats, et al. (2000). "Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*." J Biol Chem **275**(35): 27393-27398.

We have analyzed the proteins that are oxidatively damaged when *Saccharomyces cerevisiae* cells are exposed to stressing conditions. Carbonyl groups generated by hydrogen peroxide or menadione on proteins of aerobically respiring cells were detected by Western blotting, purified, and identified. Mitochondrial proteins such as E2 subunits of both pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase, aconitase, heat-shock protein 60, and the cytosolic fatty acid synthase (alpha subunit) and glyceraldehyde-3-phosphate dehydrogenase were the major targets. In addition we also report the in vivo modification of lipamide present in the above-mentioned E2 subunits under the stressing conditions tested and that this also occurs with the homologous enzymes present in *Escherichia coli* cells that were used for comparative analysis. Under fermentative conditions, the main protein targets in *S. cerevisiae* cells treated with hydrogen peroxide or menadione were pyruvate decarboxylase, enolase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase. Under the stress conditions tested, fermenting cells exhibit a lower viability than aerobically respiring cells and, consistently, increased peroxide generation as well as higher content of protein carbonyls and lipid peroxides. Our results strongly suggest that the oxidative stress in prokaryotic and eukaryotic cells shares common features.

Castillo-Duran, C. and R. Uauy (1988). "Copper deficiency impairs growth of infants recovering from malnutrition." *Am J Clin Nutr* **47**(4): 710-714.

Castino, R., J. Davies, et al. (2005). "Autophagy is a prosurvival mechanism in cells expressing an autosomal dominant familial neurohypophyseal diabetes insipidus mutant vasopressin transgene." *FASEB J* **19**(8): 1021-1023.

Autosomal dominant familial neurohypophyseal diabetes insipidus (adFNDI) is a progressive, inherited neurodegenerative disorder that presents as polydipsia and polyuria as a consequence of a loss of secretion of the antidiuretic hormone vasopressin (VP) from posterior pituitary nerve terminals. VP gene mutations cause adFNDI. Rats expressing an adFNDI VP transgene (Cys67stop) show a neuronal pathology characterized by autophagic structures in the cell body. adFNDI has thus been added to the list of protein aggregation diseases, along with Alzheimer's, Parkinson's and Huntington's, which are associated with autophagy, a bulk process that delivers regions of cytosol to lysosomes for degradation. However, the role of autophagy in these diseases is unclear. To address the relationships between mutant protein accumulation, autophagy, cell survival, and cell death, we have developed a novel and tractable in vitro system. We have constructed adenoviral vectors (Ads) that express structural genes encoding either the Cys67stop mutant protein (Ad-VCAT-Cys67stop) or an epitope-tagged wild-type VP precursor (Ad-VCAT). After infection of mouse neuroblastoma Neuro2a cells, Ad-VCAT encoded material enters neurite processes and accumulates in terminals, while the Cys67stop protein is confined to enlarged vesicles in the cell body. Similar to the intracellular derangements seen in the Cys67stop rats, these structures are of ER origin, and colocalize with markers of autophagy. Neither Ad-VCAT-Cys67stop nor Ad-VCAT expression affected cell viability. However, inhibition of autophagy or lysosomal protein degradation, while having no effect on Ad-VCAT-expressing cells, significantly increased apoptotic cell death following Ad-VCAT-Cys67stop expression. These data suggest that activation of autophagy by the stress of the expression of an adFNDI mutant protein is a prosurvival mechanism.

Cecconi, F. and B. Levine (2008). "The role of autophagy in mammalian development: cell makeover rather than cell death." *Dev Cell* **15**(3): 344-357.

Chen, G., X. Chen, et al. (2011). "Sorption and distribution of copper in unsaturated *Pseudomonas putida* CZ1 biofilms as determined by X-ray fluorescence microscopy." *Appl Environ Microbiol* **77**(14): 4719-4727.

The spatial and temporal distribution of metals in unsaturated *Pseudomonas putida* CZ1 biofilms was determined using synchrotron-based X-ray fluorescence microscopy (XRF). It was found that Fe, Mn, and Ca were mainly distributed near the air-biofilm interface of a biofilm grown on 40 mM citrate, while there were two Fe-, Mn-, and Ca-rich layers within a biofilm grown on 10 mM citrate. The sorption of copper by biofilm grown in medium containing 10 mM citrate was rapid, with copper being found throughout the biofilm after only 1 h of exposure. Copper initially colocalized with Fe and Mn element layers in the biofilm and then precipitated in a 40- μ m-thick layer near the air-biofilm interface when exposed for 12 h. Cu K-edge X-ray absorption near edge structure (XANES) analysis revealed that Cu was primarily bound with citrate within the biofilm, and the precipitate formed in the biofilm exposed to copper for 12 h was most similar to copper phosphate. LIVE/DEAD staining revealed that cells at the biofilm-membrane interface were mostly alive even when the copper concentration reached 80.5 mg copper g(-1) biomass. This suggests that the biofilm matrix provided significant protection for cells in this area. These results significantly improve our understanding of metal acquisition, transportation, and immobilization in unsaturated biofilm systems.

Chen, O. S., R. J. Crisp, et al. (2004). "Transcription of the yeast iron regulon does not respond directly to iron but rather to iron-sulfur cluster biosynthesis." *J Biol Chem* **279**(28): 29513-29518.

Saccharomyces cerevisiae responds to iron deprivation by increased transcription of the iron regulon, including the high affinity cell-surface transport system encoded by FET3 and FTR1. Here we demonstrate that transcription of these genes does not respond directly to cytosolic iron but rather to the mitochondrial utilization of iron for the synthesis of iron-sulfur (Fe-S) clusters. We took advantage of a mutant form of an iron-dependent enzyme in the sterol pathway (Erg25-2p) to assess cytosolic iron levels. We showed that disruption of mitochondrial Fe-S biosynthesis, which results in excessive mitochondrial iron accumulation, leads to transcription of the iron transport system independent of the cytosolic iron level. There is an inverse correlation between the activity of the mitochondrial Fe-S-containing enzyme aconitase and the induction of FET3. Regulation of transcription by Fe-S biosynthesis represents a mechanism by which cellular iron acquisition is integrated with mitochondrial iron metabolism.

Cobine, P. A., L. D. Ojeda, et al. (2004). "Yeast contain a non-proteinaceous pool of copper in the mitochondrial matrix." *J Biol Chem* **279**(14): 14447-14455.

The yeast mitochondrion is shown to contain a pool of copper that is distinct from that associated with the two known mitochondrial cuproenzymes, superoxide dismutase (Sod1) and cytochrome c oxidase (CcO) and the copper-binding CcO assembly proteins Cox11, Cox17, and Sco1. Only a small fraction of mitochondrial copper is associated with these cuproproteins. The bulk of the remainder is localized within the matrix as a soluble, anionic, low molecular weight complex. The identity of the matrix copper ligand is unknown, but the bulk of the matrix copper fraction is not protein-bound. The mitochondrial copper pool is dynamic, responding to changes in the cytosolic copper level. The addition of copper salts to the growth medium leads to an increase in mitochondrial copper, yet the expansion of this matrix pool does not induce any respiration defects. The matrix copper pool is accessible to a

heterologous cuproenzyme. Co-localization of human Sod1 and the metallochaperone CCS within the mitochondrial matrix results in suppression of growth defects of *sod2Delta* cells. However, in the absence of CCS within the matrix, the activation of human Sod1 can be achieved by the addition of copper salts to the growth medium.

Cohen, A., H. Nelson, et al. (2000). "The family of SMF metal ion transporters in yeast cells." J Biol Chem **275**(43): 33388-33394.

Metal ions are vital for all organisms, and metal ion transporters play a crucial role in maintaining their homeostasis. The yeast (*Saccharomyces cerevisiae*) Smf transporters and their homologs in other organisms have a central role in the accumulation of metal ions and their distribution in different tissues and cellular organelles. In this work we generated null mutations in each individual SMF gene in yeast as well as in all combinations of the genes. Each null mutation exhibited sensitivity to metal ion chelators at different concentrations. The combination of null mutants Δ SMF1 + Δ SMF2 and the triple null mutant Δ 3SMF failed to grow on medium buffered at pH 8 and 7.5, respectively. Addition of 5 microm copper or 25 microm manganese alleviated the growth arrest at the high pH or in the presence of the chelating agent. The transport of manganese was analyzed in the triple null mutant and in this mutant expressing each Smf protein. Although overexpression of Smf1p and Smf2p resulted in uptake that was higher than wild type cells, the expression of Smf3p gave no significant uptake above that of the triple mutant Δ 3SMF. Western analysis with antibody against Smf3p indicated that this transporter does not reach the plasma membrane and may function at the Golgi or post-Golgi complexes. The iron uptake resulting from expression of Smf1p and Smf2p was analyzed in a mutant in which its iron transporters FET3 and FET4 were inactivated. Overexpression of Smf1p gave rise to a significant iron uptake that was sensitive to the sodium concentrations in the medium. We conclude that the Smf proteins play a major role in copper and manganese homeostasis and, under certain circumstances, Smf1p may function in iron transport into the cells.

Coonrod, E. M. and T. H. Stevens (2010). "The yeast vps class E mutants: the beginning of the molecular genetic analysis of multivesicular body biogenesis." Mol Biol Cell **21**(23): 4057-4060.

Costanzo, M., A. Baryshnikova, et al. (2010). "The genetic landscape of a cell." Science **327**(5964): 425-431.

A genome-scale genetic interaction map was constructed by examining 5.4 million gene-gene pairs for synthetic genetic interactions, generating quantitative genetic interaction profiles for approximately 75% of all genes in the budding yeast, *Saccharomyces cerevisiae*. A network based on genetic interaction profiles reveals a functional map of the cell in which genes of similar biological processes cluster together in coherent subsets, and highly correlated profiles delineate specific pathways to define gene function. The global network identifies functional cross-connections between all bioprocesses, mapping a cellular wiring diagram of pleiotropy. Genetic interaction degree correlated with a number of different gene attributes, which may be informative about genetic network hubs in other organisms. We also demonstrate that extensive and unbiased mapping of the genetic landscape provides a key for interpretation of chemical-genetic interactions and drug target identification.

Costanzo, M., A. Baryshnikova, et al. (2011). "Charting the genetic interaction map of a cell." Curr Opin Biotechnol **22**(1): 66-74.

Culotta, V. C., S. J. Lin, et al. (1999). "Intracellular pathways of copper trafficking in yeast and humans." Adv Exp Med Biol **448**: 247-254.

Culotta, V. C., M. Yang, et al. (2005). "Manganese transport and trafficking: lessons learned from *Saccharomyces cerevisiae*." Eukaryot Cell **4**(7): 1159-1165.

De Freitas, J., H. Wintz, et al. (2003). "Yeast, a model organism for iron and copper metabolism studies." Biometals **16**(1): 185-197.

Virtually all organisms on earth depend on transition metals for survival. Iron and copper are particularly important because they participate in vital electron transfer reactions, and are thus cofactors of many metabolic enzymes. Their ability to transfer electrons also render them toxic when present in excess. Disturbances of iron and copper steady-state levels can have profound effects on cellular metabolism, growth and development. It is critical to maintain these metals in a narrow range between utility and toxicity. Organisms ranging from bacteria and plants to mammals have developed sophisticated mechanisms to control metal homeostasis. In this review, we will present an overview of the current understanding of iron and copper metabolism in yeast, and the utility of yeast as a model organism to investigate iron and copper metabolism in mammals and plants.

Dimitrov, M. D., M. G. Pesheva, et al. (2013). "New cell-based assay indicates dependence of antioxidant biological activity on the origin of reactive oxygen species." J Agric Food Chem **61**(18): 4344-4351.

The mobility of the Ty1 transposon in *Saccharomyces cerevisiae* was found to vary proportionally with the level of ROS generated in cells, which provides the possibility to determine antioxidant activity by changes in a cellular process instead of using chemical reactions. The study of propolis, royal jelly, and honey with the newly developed Ty1antiROS test reveals an inverse exponential dependence of antioxidant activity on increased concentrations. This dependence can be transformed to proportional by changing the source of ROS: instead of cell-produced to applied as hydrogen peroxide. The different test responses are not due to excess of added hydrogen peroxide, as evidenced by the exponential dependence found by usage of yap1Delta tester cells accumulating cell-generated ROS. Results indicate that the activity of antioxidants to oxidative radicals depends on the origin of ROS, and this activity is elevated for cell-generated ROS compared to ROS added as reagents in the assay.

Dos Santos, S. C., M. C. Teixeira, et al. (2012). "Yeast toxicogenomics: genome-wide responses to chemical stresses with impact in environmental health, pharmacology, and biotechnology." Front Genet **3**: 63.

The emerging transdisciplinary field of Toxicogenomics aims to study the cell response to a given toxicant at the genome, transcriptome, proteome, and metabolome levels. This approach is expected to provide earlier and more sensitive biomarkers of toxicological responses and help in the delineation of regulatory risk assessment. The use of model organisms to gather such genomic information, through the exploitation of Omics and Bioinformatics approaches and tools, together with more focused molecular and cellular biology studies are rapidly increasing our understanding and providing an integrative view on how cells interact with their environment. The use of the model eukaryote *Saccharomyces*

S. cerevisiae in the field of Toxicogenomics is discussed in this review. Despite the limitations intrinsic to the use of such a simple single cell experimental model, *S. cerevisiae* appears to be very useful as a first screening tool, limiting the use of animal models. Moreover, it is also one of the most interesting systems to obtain a truly global understanding of the toxicological response and resistance mechanisms, being in the frontline of systems biology research and developments. The impact of the knowledge gathered in the yeast model, through the use of Toxicogenomics approaches, is highlighted here by its use in prediction of toxicological outcomes of exposure to pesticides and pharmaceutical drugs, but also by its impact in biotechnology, namely in the development of more robust crops and in the improvement of yeast strains as cell factories.

Duennwald, M. L., A. Echeverria, et al. (2012). "Small heat shock proteins potentiate amyloid dissolution by protein disaggregases from yeast and humans." *PLoS Biol* **10**(6): e1001346.

How small heat shock proteins (sHsps) might empower proteostasis networks to control beneficial prions or disassemble pathological amyloid is unknown. Here, we establish that yeast sHsps, Hsp26 and Hsp42, inhibit prionogenesis by the [PSI⁺] prion protein, Sup35, via distinct and synergistic mechanisms. Hsp42 prevents conformational rearrangements within molten oligomers that enable de novo prionogenesis and collaborates with Hsp70 to attenuate self-templating. By contrast, Hsp26 inhibits self-templating upon binding assembled prions. sHsp binding destabilizes Sup35 prions and promotes their disaggregation by Hsp104, Hsp70, and Hsp40. In yeast, Hsp26 or Hsp42 overexpression prevents [PSI⁺] induction, cures [PSI⁺], and potentiates [PSI⁺]-curing by Hsp104 overexpression. In vitro, sHsps enhance Hsp104-catalyzed disaggregation of pathological amyloid forms of alpha-synuclein and polyglutamine. Unexpectedly, in the absence of Hsp104, sHsps promote an unprecedented, gradual depolymerization of Sup35 prions by Hsp110, Hsp70, and Hsp40. This unanticipated amyloid-depolymerase activity is conserved from yeast to humans, which lack Hsp104 orthologues. A human sHsp, HspB5, stimulates depolymerization of alpha-synuclein amyloid by human Hsp110, Hsp70, and Hsp40. Thus, we elucidate a heretofore-unrecognized human amyloid-depolymerase system that could have applications in various neurodegenerative disorders.

Eide, D. J., S. Clark, et al. (2005). "Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in *Saccharomyces cerevisiae*." *Genome Biol* **6**(9): R77.

BACKGROUND: Nutrient minerals are essential yet potentially toxic, and homeostatic mechanisms are required to regulate their intracellular levels. We describe here a genome-wide screen for genes involved in the homeostasis of minerals in *Saccharomyces cerevisiae*. Using inductively coupled plasma-atomic emission spectroscopy (ICP-AES), we assayed 4,385 mutant strains for the accumulation of 13 elements (calcium, cobalt, copper, iron, potassium, magnesium, manganese, nickel, phosphorus, selenium, sodium, sulfur, and zinc). We refer to the resulting accumulation profile as the yeast 'ionome'. **RESULTS:** We identified 212 strains that showed altered ionome profiles when grown on a rich growth medium. Surprisingly few of these mutants (four strains) were affected for only one element. Rather, levels of multiple elements were altered in most mutants. It was also remarkable that only six genes previously shown to be involved in the uptake and utilization of minerals were identified here, indicating that homeostasis is robust under these replete conditions. Many mutants identified affected either mitochondrial or vacuolar function and these groups showed similar effects on the accumulation of many different elements. In addition, intriguing positive and negative correlations among different elements were observed. Finally, ionome

profile data allowed us to correctly predict a function for a previously uncharacterized gene, YDR065W. We show that this gene is required for vacuolar acidification. CONCLUSION: Our results indicate the power of ionomics to identify new aspects of mineral homeostasis and how these data can be used to develop hypotheses regarding the functions of previously uncharacterized genes.

Entian, K. D., T. Schuster, et al. (1999). "Functional analysis of 150 deletion mutants in *Saccharomyces cerevisiae* by a systematic approach." Mol Gen Genet **262**(4-5): 683-702.

In a systematic approach to the study of *Saccharomyces cerevisiae* genes of unknown function, 150 deletion mutants were constructed (1 double, 149 single mutants) and phenotypically analysed. Twenty percent of all genes examined were essential. The viable deletion mutants were subjected to 20 different test systems, ranging from high throughput to highly specific test systems. Phenotypes were obtained for two-thirds of the mutants tested. During the course of this investigation, mutants for 26 of the genes were described by others. For 18 of these the reported data were in accordance with our results. Surprisingly, for seven genes, additional, unexpected phenotypes were found in our tests. This suggests that the type of analysis presented here provides a more complete description of gene function.

Eskelinen, E. L., Y. Tanaka, et al. (2003). "At the acidic edge: emerging functions for lysosomal membrane proteins." Trends Cell Biol **13**(3): 137-145.

Farrugia, G. and R. Balzan (2012). "Oxidative stress and programmed cell death in yeast." Front Oncol **2**: 64.

Yeasts, such as *Saccharomyces cerevisiae*, have long served as useful models for the study of oxidative stress, an event associated with cell death and severe human pathologies. This review will discuss oxidative stress in yeast, in terms of sources of reactive oxygen species (ROS), their molecular targets, and the metabolic responses elicited by cellular ROS accumulation. Responses of yeast to accumulated ROS include upregulation of antioxidants mediated by complex transcriptional changes, activation of pro-survival pathways such as mitophagy, and programmed cell death (PCD) which, apart from apoptosis, includes pathways such as autophagy and necrosis, a form of cell death long considered accidental and uncoordinated. The role of ROS in yeast aging will also be discussed.

Fernandez-Calvino, D., A. Bermudez-Couso, et al. (2012). "Copper release kinetics from a long-term contaminated acid soil using a stirred flow chamber: effect of ionic strength and pH." J Colloid Interface Sci **367**(1): 422-428.

The effect of pH and ionic strength on copper release in a long-term Cu-polluted soil was studied using a stirred flow chamber. The presence of Ca(2+) and Na(+) was also evaluated. More copper was released as the ionic strength increased, and it was significantly higher in the presence of Ca(2+) than in the presence of Na(+). The maximum amount of Cu that could be released under experimental conditions increased logarithmically as the ionic strength increased, and the release rate parameters were not significantly correlated with ionic strength values. The maximum amount of Cu that could be released was similar for solutions with pH values between 5.5 and 8.5. For solutions with a pH value below 4.5, the amount of Cu released increased exponentially as the pH decreased. The release rate parameters and

Cu release pattern were affected by pH, especially for more acidic solutions (pH values of 2.5 and 3.5).

Finnigan, G. C., G. E. Cronan, et al. (2012). "Sorting of the yeast vacuolar-type, proton-translocating ATPase enzyme complex (V-ATPase): identification of a necessary and sufficient Golgi/endosomal retention signal in Stv1p." *J Biol Chem* **287**(23): 19487-19500.

Subunit a of the yeast vacuolar-type, proton-translocating ATPase enzyme complex (V-ATPase) is responsible for both proton translocation and subcellular localization of this highly conserved molecular machine. Inclusion of the Vph1p isoform causes the V-ATPase complex to traffic to the vacuolar membrane, whereas incorporation of Stv1p causes continued cycling between the trans-Golgi and endosome. We previously demonstrated that this targeting information is contained within the cytosolic, N-terminal portion of V-ATPase subunit a (Stv1p). To identify residues responsible for sorting of the Golgi isoform of the V-ATPase, a random mutagenesis was performed on the N terminus of Stv1p. Subsequent characterization of mutant alleles led to the identification of a short peptide sequence, W(83)KY, that is necessary for proper Stv1p localization. Based on three-dimensional homology modeling to the *Meiothermus ruber* subunit I, we propose a structural model of the intact Stv1p-containing V-ATPase demonstrating the accessibility of the W(83)KY sequence to retrograde sorting machinery. Finally, we characterized the sorting signal within the context of a reconstructed Stv1p ancestor (Anc.Stv1). This evolutionary intermediate includes an endogenous W(83)KY sorting motif and is sufficient to compete with sorting of the native yeast Stv1p V-ATPase isoform. These data define a novel sorting signal that is both necessary and sufficient for trafficking of the V-ATPase within the Golgi/endosomal network.

Finnigan, G. C., V. Hanson-Smith, et al. (2011). "The reconstructed ancestral subunit a functions as both V-ATPase isoforms Vph1p and Stv1p in *Saccharomyces cerevisiae*." *Mol Biol Cell* **22**(17): 3176-3191.

The vacuolar-type, proton-translocating ATPase (V-ATPase) is a multisubunit enzyme responsible for organelle acidification in eukaryotic cells. Many organisms have evolved V-ATPase subunit isoforms that allow for increased specialization of this critical enzyme. Differential targeting of the V-ATPase to specific subcellular organelles occurs in eukaryotes from humans to budding yeast. In *Saccharomyces cerevisiae*, the two subunit a isoforms are the only difference between the two V-ATPase populations. Incorporation of Vph1p or Stv1p into the V-ATPase dictates the localization of the V-ATPase to the vacuole or late Golgi/endosome, respectively. A duplication event within fungi gave rise to two subunit a genes. We used ancestral gene reconstruction to generate the most recent common ancestor of Vph1p and Stv1p (Anc.a) and tested its function in yeast. Anc.a localized to both the Golgi/endosomal network and vacuolar membrane and acidified these compartments as part of a hybrid V-ATPase complex. Trafficking of Anc.a did not require retrograde transport from the late endosome to the Golgi that has evolved for retrieval of the Stv1p isoform. Rather, Anc.a localized to both structures through slowed anterograde transport en route to the vacuole. Our results suggest an evolutionary model that describes the differential localization of the two yeast V-ATPase isoforms.

Finnigan, G. C., M. Ryan, et al. (2011). "A genome-wide enhancer screen implicates sphingolipid composition in vacuolar ATPase function in *Saccharomyces cerevisiae*." *Genetics* **187**(3): 771-783.

The function of the vacuolar H(+)-ATPase (V-ATPase) enzyme complex is to acidify organelles; this process is critical for a variety of cellular processes and has implications in human

disease. There are five accessory proteins that assist in assembly of the membrane portion of the complex, the V(0) domain. To identify additional elements that affect V-ATPase assembly, trafficking, or enzyme activity, we performed a genome-wide enhancer screen in the budding yeast *Saccharomyces cerevisiae* with two mutant assembly factor alleles, VMA21 with a dysfunctional ER retrieval motif (vma21QQ) and vma21QQ in combination with voa1Delta, a nonessential assembly factor. These alleles serve as sensitized genetic backgrounds that have reduced V-ATPase enzyme activity. Genes were identified from a variety of cellular pathways including a large number of trafficking-related components; we characterized two redundant gene pairs, HPH1/HPH2 and ORM1/ORM2. Both sets demonstrated synthetic growth defects in combination with the vma21QQ allele. A loss of either the HPH or ORM gene pairs alone did not result in a decrease in vacuolar acidification or defects in V-ATPase assembly. While the Hph proteins are not required for V-ATPase function, Orm1p and Orm2p are required for full V-ATPase enzyme function. Consistent with the documented role of the Orm proteins in sphingolipid regulation, we have found that inhibition of sphingolipid synthesis alleviates Orm-related growth defects.

Fox, J. H., J. A. Kama, et al. (2007). "Mechanisms of copper ion mediated Huntington's disease progression." *PLoS One* **2**(3): e334.

Futai, M., T. Oka, et al. (2000). "Luminal acidification of diverse organelles by V-ATPase in animal cells." *J Exp Biol* **203**(Pt 1): 107-116.

Eukaryotic cells contain organelles bounded by a single membrane in the cytoplasm. These organelles have differentiated to carry out various functions in the pathways of endocytosis and exocytosis. Their lumina are acidic, with pH ranging from 4.5 to 6.5. This article describes recent studies on these animal cell organelles focusing on (1) the primary proton pump (vacuolar-type H(+)-ATPase) and (2) the functions of the organelle luminal acidity. We also discuss similarities and differences between vacuolar-type H(+)-ATPase and F-type ATPase. Our own studies and interests are emphasized.

Gahl, W. A., J. Z. Balog, et al. (2007). "Nephropathic cystinosis in adults: natural history and effects of oral cysteamine therapy." *Ann Intern Med* **147**(4): 242-250.

Gandhi, S. and A. Y. Abramov (2012). "Mechanism of oxidative stress in neurodegeneration." *Oxid Med Cell Longev* **2012**: 428010.

Gao, X. D., J. Wang, et al. (2005). "ERS1 encodes a functional homologue of the human lysosomal cystine transporter." *FEBS J* **272**(10): 2497-2511.

Cystinosis is a lysosomal storage disease caused by an accumulation of insoluble cystine in the lumen of the lysosome. CTNS encodes the lysosomal cystine transporter, mutations in which manifest as a range of disorders and are the most common cause of inherited renal Fanconi syndrome. Cystinosis, the CTNS product, is highly conserved among mammals. Here we show that the yeast Ers1 protein and cystinosis are functional orthologues, despite sharing only limited sequence homology. Ers1 is a vacuolar protein whose loss of function results in growth sensitivity to hygromycin B. This phenotype can be complemented by the human CTNS gene but not by mutant ctns alleles that were previously identified in cystinosis patients. A genetic screen for multicopy suppressors of an ers1Delta yeast strain identified a novel gene, MEH1, which is implicated in regulating Ers1 function. Meh1 localizes to the

vacuolar membrane and loss of MEH1 results in a defect in vacuolar acidification, suggesting that the vacuolar environment is critical for normal ERS1 function. This genetic system has also led us to identify Gtr1 as an Meh1 interacting protein. Like Meh1 and Ers1, Gtr1 associates with vacuolar membranes in an Meh1-dependent manner. These results demonstrate the utility of yeast as a model system for the study of CTNS and vacuolar function.

Goodyer, P. (2011). "The history of cystinosis: lessons for clinical management." Int J Nephrol **2011**: 929456.

Cystinosis is a rare disorder, and, accordingly, progress on the understanding and treatment of this disease has been relatively slow. Although cystinosis was identified over 100 years ago, the history of cystinosis is marked by a few sudden leaps forward in our understanding rather than by a sustained research effort fuelled by the larger research community. Major conceptual break-throughs include (a) its discovery in 1903, (b) recognition of the renal Fanconi syndrome, (c) realization that tissue accumulation of cystine reflects a defective channel in the lysosomal membrane, (d) translation of this discovery to trials of cysteamine, (e) discovery of the CTNS gene, and (f) report of successful stem cell therapy in the cystinotic mouse. This paper focuses on the importance management lessons from these milestones and the potential new therapeutic strategies which may be looming in the near future.

Grazioso, G., L. Moretti, et al. (2005). "Development of a three-dimensional model for the N-methyl-D-aspartate NR2A subunit." J Med Chem **48**(17): 5489-5494.

NR2 subunits of N-methyl-d-aspartic acid (NMDA) receptors are known to bind the neurotransmitter glutamate, competitive agonists, and antagonists. Since crystallographic data of these proteins are not available, we built a homology model of the ligand binding domain of the NR2A subunit. A consensus binding mode of selected AP5-like NMDA antagonists has been ascertained using molecular docking. The present 3D model gives insights for the design of new NMDA subtype selective compounds.

Gu, M., M. T. Gash, et al. (1996). "Mitochondrial defect in Huntington's disease caudate nucleus." Ann Neurol **39**(3): 385-389.

Gunther, J., M. Nguyen, et al. (2005). "Generation and functional in vivo characterization of a lipid kinase defective phosphatidylinositol 3-kinase Vps34p of *Candida albicans*." Microbiology **151**(Pt 1): 81-89.

The phosphatidylinositol (PI) 3-kinase Vps34p of *Candida albicans* has lipid kinase and autophosphorylation activity and is involved in virulence and vesicular protein transport. In order to characterize the roles of lipid kinase activity, a chimeric Vps34 protein was created which lacks lipid kinase but retains autophosphorylation activity. To this end, six amino acids within the putative lipid-binding site of Vps34p were replaced by the homologous region of the PI 3-kinase-like *C. albicans* Tor protein. The resulting chimeric Vps34T protein was recombinantly expressed in *Escherichia coli* and shown to lack lipid kinase activity. The corresponding chimeric VPS34TOR gene was inserted into the genome of *C. albicans*, and this lipid-kinase-defective strain had a distinctive phenotype compared to those of the wild-type strain SC5314 and the vps34 null mutant. The lipid-kinase-defective strain was non-virulent, and showed altered hyphal growth, reduced adherence, as well as defective vacuole morphology and endosomal vesicle transport. These results demonstrate an important role

for the lipid kinase activity of Vps34p in virulence and vesicular protein transport. On the other hand, the lipid-kinase-defective strain and the vps34 null mutant differ in their temperature- and osmotic-stress response. This indicates a possible role for activities different from the lipid kinase function of Vps34p.

Halfdanarson, T. R., N. Kumar, et al. (2008). "Hematological manifestations of copper deficiency: a retrospective review." Eur J Haematol **80**(6): 523-531.

Halliwel, B. (2007). "Dietary polyphenols: good, bad, or indifferent for your health?" Cardiovasc Res **73**(2): 341-347.

Halliwel, B. and C. E. Cross (1994). "Oxygen-derived species: their relation to human disease and environmental stress." Environ Health Perspect **102 Suppl 10**: 5-12.

Halliwel, B. and J. M. C. Gutteridge (2007). Free radicals in biology and medicine. Oxford, Oxford University Press.

Haurie, V., H. Boucherie, et al. (2003). "The Snf1 protein kinase controls the induction of genes of the iron uptake pathway at the diauxic shift in *Saccharomyces cerevisiae*." J Biol Chem **278**(46): 45391-45396.

In *Saccharomyces cerevisiae* the transition between the fermentative and the oxidative metabolism, called the diauxic shift, is associated with major changes in gene expression. In this study, we characterized a novel family of five genes whose expression is induced during the diauxic shift. These genes, FET3, FTR1, TIS11, SIT1, and FIT2, are involved in the iron uptake pathway. We showed that their induction at the diauxic shift is positively controlled by the Snf1/Snf4 kinase pathway. The transcriptional factor Aft1p, which is known to control their induction in response to iron limitation, is also required for their induction during the diauxic shift. The increase of the extracellular iron concentration does not affect this induction, indicating that glucose exhaustion by itself would be the signal. The possibility that the Snf1/Snf4 pathway was also involved in the induction of the same set of genes in response to iron starvation was considered. We demonstrate here that this is not the case. Thus, the two signals, glucose exhaustion and iron starvation, use two independent pathways to activate the same set of genes through the Aft1p transcriptional factor.

Hettema, E. H., C. C. Ruigrok, et al. (1998). "The cytosolic DnaJ-like protein djp1p is involved specifically in peroxisomal protein import." J Cell Biol **142**(2): 421-434.

The *Saccharomyces cerevisiae* DJP1 gene encodes a cytosolic protein homologous to *Escherichia coli* DnaJ. DnaJ homologues act in conjunction with molecular chaperones of the Hsp70 protein family in a variety of cellular processes. Cells with a DJP1 gene deletion are viable and exhibit a novel phenotype among cytosolic J-protein mutants in that they have a specific impairment of only one organelle, the peroxisome. The phenotype was also unique among peroxisome assembly mutants: peroxisomal matrix proteins were mislocalized to the cytoplasm to a varying extent, and peroxisomal structures failed to grow to full size and exhibited a broad range of buoyant densities. Import of marker proteins for the endoplasmic reticulum, nucleus, and mitochondria was normal. Furthermore, the metabolic adaptation to a change in carbon source, a complex multistep process, was unaffected in a DJP1 gene

deletion mutant. We conclude that Djplp is specifically required for peroxisomal protein import.

Hicsonmez, U., F. S. Erees, et al. (2009). "Determination of major and minor elements in the *Malva sylvestris* L. from Turkey using ICP-OES techniques." *Biol Trace Elem Res* **128**(3): 248-257.

In this work, *Malva sylvestris* var. *mauritiana* (L.) leaves were collected from different points in Muradiye region of Manisa-Turkey. The leaves were dissolved by wet digestion method using a mixture of mineral acid. Concentrations of Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, La, Mg, Mn, Na, Ni, Pb, Sn, Sr, Sb, Si, Ti, U, Zn, and Zr in prepared solutions were determined by using inductively coupled plasma optical emission spectrometry (ICP-OES). High Ca (13,848 mg/kg) and Mg (1,936 mg/kg) concentrations were found at the leaves. Obtained values were compared with the internationally permitted (standard) values. The results of elements were analyzed statistically (analysis of variance test). For different leaf sizes, concentration factors were calculated.

Jamieson, D. J. (1998). "Oxidative stress responses of the yeast *Saccharomyces cerevisiae*." *Yeast* **14**(16): 1511-1527.

All aerobically growing organisms suffer exposure to oxidative stress, caused by partially reduced forms of molecular oxygen, known as reactive oxygen species (ROS). These are highly reactive and capable of damaging cellular constituents such as DNA, lipids and proteins. Consequently, cells from many different organisms have evolved mechanisms to protect their components against ROS. This review concentrates on the oxidant defence systems of the budding yeast *Saccharomyces cerevisiae*, which appears to have a number of inducible adaptive stress responses to oxidants, such as H₂O₂, superoxide anion and lipid peroxidation products. The oxidative stress responses appear to be regulated, at least in part, at the level of transcription and there is considerable overlap between them and many diverse stress responses, allowing the yeast cell to integrate its response towards environmental stress.

Jo, W. J., A. Loguinov, et al. (2008). "Identification of genes involved in the toxic response of *Saccharomyces cerevisiae* against iron and copper overload by parallel analysis of deletion mutants." *Toxicol Sci* **101**(1): 140-151.

Iron and copper are essential nutrients for life as they are required for the function of many proteins but can be toxic if present in excess. Accumulation of these metals in the human body as a consequence of overload disorders and/or high environmental exposures has detrimental effects on health. The budding yeast *Saccharomyces cerevisiae* is an accepted cellular model for iron and copper metabolism in humans primarily because of the high degree of conservation between pathways and proteins involved. Here we report a systematic screen using yeast deletion mutants to identify genes involved in the toxic response to growth-inhibitory concentrations of iron and copper sulfate. We aimed to understand the cellular responses to toxic concentrations of these two metals by analyzing the different subnetworks and biological processes significantly enriched with these genes. Our results indicate the presence of two different detoxification pathways for iron and copper that converge toward the vacuole. The product of several of the identified genes in these pathways form molecular complexes that are conserved in mammals and include the retromer, endosomal sorting complex required for transport (ESCRT) and AP-3 complexes, suggesting that the mechanisms involved can be extrapolated to humans. Our data also suggest a disruption in ion homeostasis and, in particular, of iron after copper exposure.

Moreover, the identification of treatment-specific genes associated with biological processes such as DNA double-strand break repair for iron and tryptophan biosynthesis for copper suggests differences in the mechanisms by which these two metals are toxic at high concentrations.

Kalatzis, V., S. Cherqui, et al. (2001). "Cystinosin, the protein defective in cystinosis, is a H(+)-driven lysosomal cystine transporter." EMBO J **20**(21): 5940-5949.

Kaler, S. G. (1996). "Menkes disease mutations and response to early copper histidine treatment." Nat Genet **13**(1): 21-22.

Kaler, S. G. (1998). "Diagnosis and therapy of Menkes syndrome, a genetic form of copper deficiency." Am J Clin Nutr **67**(5 Suppl): 1029S-1034S.

Kanki, T., D. J. Klionsky, et al. (2011). "Mitochondria autophagy in yeast." Antioxid Redox Signal **14**(10): 1989-2001.

The mitochondrion is an organelle that carries out a number of important metabolic processes such as fatty acid oxidation, the citric acid cycle, and oxidative phosphorylation. However, this multitasking organelle also generates reactive oxygen species (ROS), which can cause oxidative stress resulting in self-damage. This type of mitochondrial damage can lead to the further production of ROS and a resulting downward spiral with regard to mitochondrial capability. This is extremely problematic because the accumulation of dysfunctional mitochondria is related to aging, cancer, and neurodegenerative diseases. Accordingly, appropriate quality control of this organelle is important to maintain proper cellular homeostasis. It has been thought that selective mitochondria autophagy (mitophagy) contributes to the maintenance of mitochondrial quality by eliminating damaged or excess mitochondria, although little is known about the mechanism. Recent studies in yeast identified several mitophagy-related proteins, which have been characterized with regard to their function and regulation. In this article, we review recent advances in the physiology and molecular mechanism of mitophagy and discuss the similarities and differences of this degradation process between yeast and mammalian cells.

Kawahata, M., K. Masaki, et al. (2006). "Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p." FEMS Yeast Res **6**(6): 924-936.

Using two types of genome-wide analysis to investigate yeast genes involved in response to lactic acid and acetic acid, we found that the acidic condition affects metal metabolism. The first type is an expression analysis using DNA microarrays to investigate 'acid shock response' as the first step to adapt to an acidic condition, and 'acid adaptation' by maintaining integrity in the acidic condition. The other is a functional screening using the nonessential genes deletion collection of *Saccharomyces cerevisiae*. The expression analysis showed that genes involved in stress response, such as YGP1, TPS1 and HSP150, were induced under the acid shock response. Genes such as FIT2, ARN1 and ARN2, involved in metal metabolism regulated by Aft1p, were induced under the acid adaptation. AFT1 was induced under acid shock response and under acid adaptation with lactic acid. Moreover, green fluorescent protein-fused Aft1p was localized to the nucleus in cells grown in media containing lactic

acid, acetic acid, or hydrochloric acid. Both analyses suggested that the acidic condition affects cell wall architecture. The depletion of cell-wall components encoded by SED1, DSE2, CTS1, EGT2, SCW11, SUN4 and YNL300W and histone acetyltransferase complex proteins encoded by YID21, EAF3, EAF5, EAF6 and YAF9 increased resistance to lactic acid. Depletion of the cell-wall mannoprotein Sed1p provided resistance to lactic acid, although the expression of SED1 was induced by exposure to lactic acid. Depletion of vacuolar membrane H⁺-ATPase and high-osmolarity glycerol mitogen-activated protein kinase proteins caused acid sensitivity. Moreover, our quantitative PCR showed that expression of PDR12 increased under acid shock response with lactic acid and decreased under acid adaptation with hydrochloric acid.

Kawasaki-Nishi, S., T. Nishi, et al. (2001). "Yeast V-ATPase complexes containing different isoforms of the 100-kDa a-subunit differ in coupling efficiency and in vivo dissociation." *J Biol Chem* **276**(21): 17941-17948.

The 100 kDa a-subunit of the yeast vacuolar (H⁺)-ATPase (V-ATPase) is encoded by two genes, VPH1 and STV1. These genes encode unique isoforms of the a-subunit that have previously been shown to reside in different intracellular compartments in yeast. Vph1p localizes to the central vacuole, whereas Stv1p is present in some other compartment, possibly the Golgi or endosomes. To compare the properties of V-ATPases containing Vph1p or Stv1p, Stv1p was expressed at higher than normal levels in a strain disrupted in both genes, under which conditions V-ATPase complexes containing Stv1p appear in the vacuole. Complexes containing Stv1p showed lower assembly with the peripheral V(1) domain than did complexes containing Vph1p. When corrected for this lower degree of assembly, however, V-ATPase complexes containing Vph1p and Stv1p had similar kinetic properties. Both exhibited a K_m for ATP of about 250 microm, and both showed resistance to sodium azide and vanadate and sensitivity to nanomolar concentrations of concanamycin A. Stv1p-containing complexes, however, showed a 4-5-fold lower ratio of proton transport to ATP hydrolysis than Vph1p-containing complexes. We also compared the ability of V-ATPase complexes containing Vph1p or Stv1p to undergo in vivo dissociation in response to glucose depletion. Vph1p-containing complexes present in the vacuole showed dissociation in response to glucose depletion, whereas Stv1p-containing complexes present in their normal intracellular location (Golgi/endosomes) did not. Upon overexpression of Stv1p, Stv1p-containing complexes present in the vacuole showed glucose-dependent dissociation. Blocking delivery of Vph1p-containing complexes to the vacuole in vps21Delta and vps27Delta strains caused partial inhibition of glucose-dependent dissociation. These results suggest that dissociation of the V-ATPase complex in vivo is controlled both by the cellular environment and by the 100-kDa a-subunit isoform present in the complex.

Kiel, J. A., K. B. Rechinger, et al. (1999). "The Hansenula polymorpha PDD1 gene product, essential for the selective degradation of peroxisomes, is a homologue of Saccharomyces cerevisiae Vps34p." *Yeast* **15**(9): 741-754.

Via functional complementation we have isolated the Hansenula polymorpha PDD1 gene essential for selective, macroautophagic peroxisome degradation. HpPDD1 encodes a 116 kDa protein with high similarity (42% identity) to Saccharomyces cerevisiae Vps34p, which has been implicated in vacuolar protein sorting and endocytosis. Western blotting experiments revealed that HpPDD1 is expressed constitutively. In a H. polymorpha pdd1 disruption strain peroxisome degradation is fully impaired. Sequestered peroxisomes, typical for the first stage of peroxisome degradation in H. polymorpha, were never observed, suggesting that HpPdd1p plays a role in the tagging of redundant peroxisomes and/or

sequestration of these organelles from the cytosol. Possibly, HpPdd1p is the functional homologue of ScVps34p, because-like *S. cerevisiae* vps34 mutants-*H. polymorpha* pdd1 mutants are temperature-sensitive for growth and are impaired in the sorting of vacuolar carboxypeptidase Y. Moreover, HpPdd1p is associated to membranes, as was also observed for ScVps34p.

Komaromy-Hiller, G. (1999). "Flame, flameless, and plasma spectroscopy." *Anal Chem* **71**(12): 338R-342R.

Kommuguri, U. N., S. Bodiga, et al. (2012). "Copper deprivation modulates CTR1 and CUP1 expression and enhances cisplatin cytotoxicity in *Saccharomyces cerevisiae*." *J Trace Elem Med Biol* **26**(1): 13-19.

Saccharomyces cerevisiae has been established as a model system for cancer studies, due to the widely conserved family of genes involved in cell cycle progression, proliferation and apoptosis. In the current study, we sought to determine whether copper deprivation modulates sensitivity of yeast to cisplatin. Yeast cultures grown in low copper medium and exposed to bathocuproine disulfate (BCS) resulted in significant reduction of intracellular copper. We report here that low copper medium rendered BY4741 hypersensitive to cisplatin (CDDP). Yeast grown in low copper medium exhibited approximately 2.0 fold enhanced cytotoxicity in survival and colony-forming ability, compared to copper adequate control cells grown in YPD. The effect of copper restriction on CDDP sensitivity appeared to be associated with the up regulation of CTR1, facilitating enhanced uptake and accumulation of CDDP. Also, CDDP further lowered copper deprivation-induced changes in CUP1 metallothionein levels, SOD activity and GSH levels. These changes were associated with increased protein oxidation and lipid peroxidation induced by CDDP. These results thus suggest that cisplatin cytotoxicity is potentiated under low copper conditions due to enhanced uptake and accumulation of cisplatin and also in part due to lowered antioxidant defense and increased oxidative stress imposed by copper deprivation.

Kwon, H. J., J. Y. Heo, et al. (2011). "DJ-1 mediates paraquat-induced dopaminergic neuronal cell death." *Toxicol Lett* **202**(2): 85-92.

There are two causes of Parkinson's disease (PD): environmental insults and genetic mutations of PD-associated genes. Environmental insults and genetic mutations lead to mitochondrial dysfunction, and a combination of mitochondrial dysfunction and increased oxidative stress in dopaminergic neurons is thought to contribute to the pathogenesis of PD. Among the PD-associated genes, DJ-1 acts as a redox sensor for oxidative stress and has been also proposed to maintain mitochondrial complex I activity. To understand molecular functions of DJ-1 in the cell, we have generated DJ-1 null cells from the DJ-1(-/-) mouse embryos. Using these null cells, we investigated the susceptibility to an environmental toxin, paraquat, which is known to inhibit mitochondrial complex I. Interestingly, we found that DJ-1 null cells showed a resistance to paraquat-induced apoptosis, including reduced poly (ADP-ribose) polymerase and procaspase-3. Also DJ-1 null cells generated less superoxide than SN4741 cells by paraquat treatment. Consistent with the reduced paraquat sensitivity, DJ-1 null cells showed reduced complex I activity, which was partially rescued by ectopic DJ-1 expression. In summary, our results suggest that DJ-1 is critical to maintain mitochondrial complex I and complex I could be a key target in interaction of paraquat toxicity and DJ-1 for giving rise to PD.

Lawrence, C. L., C. H. Botting, et al. (2004). "Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress." *Mol Cell Biol* **24**(8): 3307-3323.

Screening the *Saccharomyces cerevisiae* disruptome, profiling transcripts, and determining changes in protein expression have identified an important new role for the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway in the regulation of adaptation to citric acid stress. Deletion of HOG1, SSK1, PBS2, PTC2, PTP2, and PTP3 resulted in sensitivity to citric acid. Furthermore, citric acid resulted in the dual phosphorylation, and thus activation, of Hog1p. Despite minor activation of glycerol biosynthesis, the inhibitory effect of citric acid was not due to an osmotic shock. HOG1 negatively regulated the expression of a number of proteins in response to citric acid stress, including Bmh1p. Evidence suggests that BMH1 is induced by citric acid to counteract the effect of amino acid starvation. In addition, deletion of BMH2 rendered cells sensitive to citric acid. Deletion of the transcription factor MSN4, which is known to be regulated by Bmh1p and Hog1p, had a similar effect. HOG1 was also required for citric acid-induced up-regulation of Ssa1p and Eno2p. To counteract the cation chelating activity of citric acid, the plasma membrane Ca(2+) channel, CCH1, and a functional vacuolar membrane H(+)-ATPase were found to be essential for optimal adaptation. Also, the transcriptional regulator CYC8, which mediates glucose derepression, was required for adaptation to citric acid to allow cells to metabolize excess citrate via the tricarboxylic acid (TCA) cycle. Supporting this, Mdh1p and Idh1p, both TCA cycle enzymes, were up-regulated in response to citric acid.

Leary, S. C., P. A. Cobine, et al. (2007). "The human cytochrome c oxidase assembly factors SCO1 and SCO2 have regulatory roles in the maintenance of cellular copper homeostasis." *Cell Metab* **5**(1): 9-20.

Human SCO1 and SCO2 are metallochaperones that are essential for the assembly of the catalytic core of cytochrome c oxidase (COX). Here we show that they have additional, unexpected roles in cellular copper homeostasis. Mutations in either SCO result in a cellular copper deficiency that is both tissue and allele specific. This phenotype can be dissociated from the defects in COX assembly and is suppressed by overexpression of SCO2, but not SCO1. Overexpression of a SCO1 mutant in control cells in which wild-type SCO1 levels were reduced by shRNA recapitulates the copper-deficiency phenotype in SCO1 patient cells. The copper-deficiency phenotype reflects not a change in high-affinity copper uptake but rather a proportional increase in copper efflux. These results suggest a mitochondrial pathway for the regulation of cellular copper content that involves signaling through SCO1 and SCO2, perhaps by their thiol redox or metal-binding state.

Leary, S. C. and D. R. Winge (2007). "The Janus face of copper: its expanding roles in biology and the pathophysiology of disease. Meeting on Copper and Related Metals in Biology." *EMBO Rep* **8**(3): 224-227.

Lee, J., S. Giordano, et al. (2012). "Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling." *Biochem J* **441**(2): 523-540.

Reactive oxygen and nitrogen species change cellular responses through diverse mechanisms that are now being defined. At low levels, they are signalling molecules, and at high levels, they damage organelles, particularly the mitochondria. Oxidative damage and the associated mitochondrial dysfunction may result in energy depletion, accumulation of cytotoxic mediators and cell death. Understanding the interface between stress adaptation and cell

death then is important for understanding redox biology and disease pathogenesis. Recent studies have found that one major sensor of redox signalling at this switch in cellular responses is autophagy. Autophagic activities are mediated by a complex molecular machinery including more than 30 Atg (AuTophagy-related) proteins and 50 lysosomal hydrolases. Autophagosomes form membrane structures, sequester damaged, oxidized or dysfunctional intracellular components and organelles, and direct them to the lysosomes for degradation. This autophagic process is the sole known mechanism for mitochondrial turnover. It has been speculated that dysfunction of autophagy may result in abnormal mitochondrial function and oxidative or nitrative stress. Emerging investigations have provided new understanding of how autophagy of mitochondria (also known as mitophagy) is controlled, and the impact of autophagic dysfunction on cellular oxidative stress. The present review highlights recent studies on redox signalling in the regulation of autophagy, in the context of the basic mechanisms of mitophagy. Furthermore, we discuss the impact of autophagy on mitochondrial function and accumulation of reactive species. This is particularly relevant to degenerative diseases in which oxidative stress occurs over time, and dysfunction in both the mitochondrial and autophagic pathways play a role.

Marino, M. L., S. Fais, et al. (2010). "Proton pump inhibition induces autophagy as a survival mechanism following oxidative stress in human melanoma cells." *Cell Death Dis* 1: e87.

Proton pump inhibitors (PPI) target tumour acidic pH and have an antineoplastic effect in melanoma. The PPI esomeprazole (ESOM) kills melanoma cells through a caspase-dependent pathway involving cytosolic acidification and alkalization of tumour pH. In this paper, we further investigated the mechanisms of ESOM-induced cell death in melanoma. ESOM rapidly induced accumulation of reactive oxygen species (ROS) through mitochondrial dysfunctions and involvement of NADPH oxidase. The ROS scavenger N-acetyl-L-cysteine (NAC) and inhibition of NADPH oxidase significantly reduced ESOM-induced cell death, consistent with inhibition of cytosolic acidification. Autophagy, a cellular catabolic pathway leading to lysosomal degradation and recycling of proteins and organelles, represents a defence mechanism in cancer cells under metabolic stress. ESOM induced the early accumulation of autophagosomes, at the same time reducing the autophagic flux, as observed by WB analysis of LC3-II accumulation and by fluorescence microscopy. Moreover, ESOM treatment decreased mammalian target of rapamycin signalling, as reduced phosphorylation of p70-S6K and 4-EBP1 was observed. Inhibition of autophagy by knockdown of Atg5 and Beclin-1 expression significantly increased ESOM cytotoxicity, suggesting a protective role for autophagy in ESOM-treated cells. The data presented suggest that autophagy represents an adaptive survival mechanism to overcome drug-induced cellular stress and cytotoxicity, including alteration of pH homeostasis mediated by proton pump inhibition.

Meena, R. C., S. Thakur, et al. (2011). "Regulation of *Saccharomyces cerevisiae* Plasma membrane H(+)-ATPase (Pma1) by Dextrose and Hsp30 during Exposure to Thermal Stress." *Indian J Microbiol* 51(2): 153-158.

Mizushima, N. and M. Komatsu (2011). "Autophagy: renovation of cells and tissues." *Cell* 147(4): 728-741.

Autophagy is the major intracellular degradation system by which cytoplasmic materials are delivered to and degraded in the lysosome. However, the purpose of autophagy is not the simple elimination of materials, but instead, autophagy serves as a dynamic recycling system that produces new building blocks and energy for cellular renovation and homeostasis. Here

we provide a multidisciplinary review of our current understanding of autophagy's role in metabolic adaptation, intracellular quality control, and renovation during development and differentiation. We also explore how recent mouse models in combination with advances in human genetics are providing key insights into how the impairment or activation of autophagy contributes to pathogenesis of diverse diseases, from neurodegenerative diseases such as Parkinson disease to inflammatory disorders such as Crohn disease.

Morikawa, D., H. Nojiri, et al. (2013). "Cytoplasmic reactive oxygen species and SOD1 regulate bone mass during mechanical unloading." *J Bone Miner Res* **28**(11): 2368-2380.

Oxidative stress contributes to the pathogenesis of age-related diseases as well as bone fragility. Our previous study demonstrated that copper/zinc superoxide dismutase (Sod1)-deficient mice exhibit the induction of intracellular reactive oxygen species (ROS) and bone fragility resulting from low-turnover bone loss and impaired collagen cross-linking (Nojiri et al. *J Bone Miner Res*. 2011;26:2682-94). Mechanical stress also plays an important role in the maintenance of homeostasis in bone tissue. However, the molecular links between oxidative and mechanical stresses in bone tissue have not been fully elucidated. We herein report that mechanical unloading significantly increased intracellular ROS production and the specific upregulation of Sod1 in bone tissue in a tail-suspension experiment. We also reveal that Sod1 loss exacerbated bone loss via reduced osteoblastic abilities during mechanical unloading. Interestingly, we found that the administration of an antioxidant, vitamin C, significantly attenuated bone loss during unloading. These results indicate that mechanical unloading, in part, regulates bone mass via intracellular ROS generation and the Sod1 expression, suggesting that activating Sod1 may be a preventive strategy for ameliorating mechanical unloading-induced bone loss.

Murphy, M. P., A. Holmgren, et al. (2011). "Unraveling the biological roles of reactive oxygen species." *Cell Metab* **13**(4): 361-366.

Reactive oxygen species are not only harmful agents that cause oxidative damage in pathologies, they also have important roles as regulatory agents in a range of biological phenomena. The relatively recent development of this more nuanced view presents a challenge to the biomedical research community on how best to assess the significance of reactive oxygen species and oxidative damage in biological systems. Considerable progress is being made in addressing these issues, and here we survey some recent developments for those contemplating research in this area.

Nagano, T., T. Toyoda, et al. (2005). "Clinical features of hematological disorders caused by copper deficiency during long-term enteral nutrition." *Intern Med* **44**(6): 554-559.

Naik, E. and V. M. Dixit (2011). "Mitochondrial reactive oxygen species drive proinflammatory cytokine production." *J Exp Med* **208**(3): 417-420.

Nelson, N., N. Perzov, et al. (2000). "The cellular biology of proton-motive force generation by V-ATPases." *J Exp Biol* **203**(Pt 1): 89-95.

The vacuolar H(+)-ATPase (V-ATPase) is one of the most fundamental enzymes in nature. It functions in almost every eukaryotic cell and energizes a wide variety of organelles and membranes. In contrast to F-ATPases, whose primary function in eukaryotic cells is to form

ATP at the expense of the proton-motive force, V-ATPases function exclusively as ATP-dependent proton pumps. The proton-motive force generated by V-ATPases in organelles and across plasma membranes of eukaryotic cells is utilized as a driving force for numerous secondary transport processes. The enzyme is also vital for the proper functioning of endosomes and the Golgi apparatus. In contrast to yeast vacuoles, which maintain an internal pH of approximately 5.5, it is believed that the vacuoles of lemon fruit may have a pH as low as 2. Similarly, some brown and red algae maintain an internal pH as low as 1 in their vacuoles. It was yeast genetics that allowed the identification of the special properties of individual subunits and the discovery of the factors that are involved in V-ATPase biogenesis and assembly. Null mutations in genes encoding V-ATPase subunits of *Saccharomyces cerevisiae* result in a phenotype that is unable to grow at high pH and is sensitive to high and low metal-ion concentrations. Treatment of these null mutants with ethyl methanesulphonate causes mutations that suppress the V-ATPase null phenotype, and these cells are able to grow at pH 7.5. The suppressor mutants were denoted as *svf* (Suppressor of V-ATPase Function). The *svf* mutations are recessive: crossing the *svf* mutants with their corresponding V-ATPase null mutants resulted in diploid strains that were not able to grow at pH 7.5. A novel gene family in which null mutations cause pleiotropic effects on metal-ion resistance or on the sensitivity and distribution of membrane proteins in different targets was discovered. We termed this gene family VTC (Vacuolar Transporter Chaperon) and discovered four genes in *S. cerevisiae* that belong to the family. Inactivation of one of them, VTC1, in the background of V-ATPase null mutations resulted in an *svf* phenotype that was able to grow at pH 7.5. Apparently, Vtc1p is one of a few membrane organizers that determine the relative amounts of different membrane proteins in the various cellular membranes. We utilize the numerous yeast mutants generated in our laboratory to identify the specific organelle whose acidification is vital. The interaction between V-ATPase and the secretory pathway is investigated.

Norambuena, L., J. Zouhar, et al. (2008). "Identification of cellular pathways affected by Sortin2, a synthetic compound that affects protein targeting to the vacuole in *Saccharomyces cerevisiae*." BMC Chem Biol 8: 1.

Obara, K., T. Sekito, et al. (2006). "Assortment of phosphatidylinositol 3-kinase complexes--Atg14p directs association of complex I to the pre-autophagosomal structure in *Saccharomyces cerevisiae*." Mol Biol Cell 17(4): 1527-1539.

In the yeast *Saccharomyces cerevisiae*, two similar phosphatidylinositol 3-kinase complexes (complexes I and II) function in distinct biological processes, complex I in autophagy and complex II in the vacuolar protein sorting via endosomes. Atg14p is only integrated into complex I, likely facilitating the function of complex I in autophagy. Deletion analysis of Atg14p revealed that N-terminal region containing the coiled-coil structures was essential and sufficient for autophagy. Atg14p localized to pre-autophagosomal structure (PAS) and vacuolar membranes, whereas Vps38p, a component specific to complex II, localized to endosomes and vacuolar membranes. Vps34p and Vps30p, components shared by the two complexes, localized to the PAS, vacuolar membranes, and several punctate structures that included endosomes. The localization of these components to the PAS was Atg14p dependent but not dependent on Vps38p. Conversely, localization of these proteins to endosomes required Vps38p but not Atg14p. Vps15p, regulatory subunit of the Vps34p complexes, localized to the PAS, vacuolar membranes, and punctate structures independent of both Atg14p and Vps38p. Together, these results indicate that complexes I and II function in distinct biological processes by localizing to specific compartments in a manner mediated by specific components of each complex, Atg14p and Vps38p, respectively.

Okorokov, L. A., T. V. Kulakovskaya, et al. (1985). "H⁺/ion antiport as the principal mechanism of transport systems in the vacuolar membrane of the yeast *Saccharomyces carlsbergensis*." *FEBS Lett* **192**(2): 303-306.

The secondary transport systems of the yeast vacuolar membrane have been investigated by the method of radioactive isotopes [(¹⁴C]arginine); activation of H⁺-ATPase by cations (Cat⁺), when the enzyme is under H⁺ control and measurement of changes in the proton gradient (Δ pH) and membrane potential (E_m) due to the supposed substrates of the transporters. The main mechanism of cation transport across the yeast tonoplast is probably H⁺/Cat⁺ antiport. The apparent K_m of antiporters for Ca²⁺, Mg²⁺, Mn²⁺, Zn²⁺ and Pi are 0.06, 0.3, 0.8, 0.055-0.17 and 1.5 mM, respectively.

Oot, R. A. and S. Wilkens (2010). "Domain characterization and interaction of the yeast vacuolar ATPase subunit C with the peripheral stator stalk subunits E and G." *J Biol Chem* **285**(32): 24654-24664.

The proton pumping activity of the eukaryotic vacuolar ATPase (V-ATPase) is regulated by a unique mechanism that involves reversible enzyme dissociation. In yeast, under conditions of nutrient depletion, the soluble catalytic V(1) sector disengages from the membrane integral V(o), and at the same time, both functional units are silenced. Notably, during enzyme dissociation, a single V(1) subunit, C, is released into the cytosol. The affinities of the other V(1) and V(o) subunits for subunit C are therefore of particular interest. The C subunit crystal structure shows that the subunit is elongated and dumbbell-shaped with two globular domains (C(head) and C(foot)) separated by a flexible helical neck region (Drory, O., Frolow, F., and Nelson, N. (2004) *EMBO Rep.* **5**, 1148-1152). We have recently shown that subunit C is bound in the V(1)-V(o) interface where the subunit is in contact with two of the three peripheral stators (subunit EG heterodimers): one via C(head) and one via C(foot) (Zhang, Z., Zheng, Y., Mazon, H., Milgrom, E., Kitagawa, N., Kish-Trier, E., Heck, A. J., Kane, P. M., and Wilkens, S. (2008) *J. Biol. Chem.* **283**, 35983-35995). In vitro, however, subunit C binds only one EG heterodimer (Fethiere, J., Venzke, D., Madden, D. R., and Bottcher, B. (2005) *Biochemistry* **44**, 15906-15914), implying that EG has different affinities for the two domains of the C subunit. To determine which subunit C domain binds EG with high affinity, we have generated C(head) and C(foot) and characterized their interaction with subunit EG heterodimer. Our findings indicate that the high affinity site for EGC interaction is C(head). In addition, we provide evidence that the EGC(head) interaction greatly stabilizes EG heterodimer.

Pagani, A., L. Villarreal, et al. (2007). "The *Saccharomyces cerevisiae* Crs5 Metallothionein metal-binding abilities and its role in the response to zinc overload." *Mol Microbiol* **63**(1): 256-269.

Crs5 is a *Saccharomyces cerevisiae* Metallothionein (MT), non-homologous to the paradigmatic Cu-thionein Cup1. Although considered a secondary copper-resistance agent, we show here that it determines survival under zinc overload in a CUP1-null background. Its overexpression prevents the deleterious effects exhibited by CUP1-CRS5-null cells when exposed to combined Zn/Cu, as it does the mouse MT1 Zn-thionein, but not Cup1. The detailed characterization of Crs5 in vivo and in vitro Zn(II)-, Cd(II)- and Cu(I)-binding abilities fully supports its resemblance to mammalian MTs. Hence, Crs5 exhibits a good divalent metal-binding ability, yielding homometallic, highly chiral and stable Zn and Cd complexes when expressed in media enriched with these metal ions. In Cu-supplemented cultures, heterometallic Zn,Cu complexes are recovered, unless aeration is kept to a minimum. These

features define a Crs5 dual metal-binding behaviour that is significantly closer to Zn-thioneins than to Cu-thioneins. Protein sequence similarities fully support these findings. Overall, a Crs5 function in global metal cell homeostasis, based on its Zn-binding features, is glimpsed. The comparative evaluation of Crs5 in the framework of MT functional differentiation and evolution allows its consideration as a representative of the primeval eukaryotic forms that progressively evolved to give rise to the Zn-thionein lineage.

Pal, P. K., A. Samii, et al. (1999). "Manganese neurotoxicity: a review of clinical features, imaging and pathology." Neurotoxicology 20(2-3): 227-238.

Manganese intoxication can result in a syndrome of parkinsonism and dystonia. If these extrapyramidal findings are present, they are likely to be irreversible and even progress after termination of the exposure to manganese. Clinical features are usually sufficient to distinguish these patients from those with Parkinson's disease. The neurological syndrome does not respond to levodopa. Imaging of the brain may reveal MRI signal changes in the globus pallidus, striatum, and midbrain. Positron emission tomography reveals normal presynaptic and postsynaptic nigrostriatal dopaminergic function. The primary site of neurological damage has been shown by pathological studies to be the globus pallidus. The mechanism of toxicity is not clear.

Pena-Castillo, L. and T. R. Hughes (2007). "Why are there still over 1000 uncharacterized yeast genes?" Genetics 176(1): 7-14.

Pena, M. M., J. Lee, et al. (1999). "A delicate balance: homeostatic control of copper uptake and distribution." J Nutr 129(7): 1251-1260.

The cellular uptake and intracellular distribution of the essential but highly toxic nutrient, copper, is a precisely orchestrated process. Copper homeostasis is coordinated by several proteins to ensure that it is delivered to specific subcellular compartments and copper-requiring proteins without releasing free copper ions that will cause damage to cellular components. Genetic studies in prokaryotic organisms and yeast have identified membrane-associated proteins that mediate the uptake or export of copper from cells. Within cells, small cytosolic proteins, called copper chaperones, have been identified that bind copper ions and deliver them to specific compartments and copper-requiring proteins. The identification of mammalian homologues of these proteins reveal a remarkable structural and functional conservation of copper metabolism between bacteria, yeast and humans. Furthermore, studies on the function and localization of the products of the Menkes and Wilson's disease genes, which are defective in patients afflicted with these diseases, have provided valuable insight into the mechanisms of copper balance and their role in maintaining appropriate copper distribution in mammals.

Perzov, N., V. Padler-Karavani, et al. (2002). "Characterization of yeast V-ATPase mutants lacking Vph1p or Stv1p and the effect on endocytosis." J Exp Biol 205(Pt 9): 1209-1219.

Subunit a of V-ATPase in the yeast *Saccharomyces cerevisiae*, in contrast to its other subunits, is encoded by two genes VPH1 and STV1. While disruption of any other gene encoding the V-ATPase subunits results in growth arrest at pH 7.5, null mutants of Vph1p or Stv1p can grow at this pH. We used a polyclonal antibody to yeast Stv1p and a commercially available monoclonal antibody to Vph1p for analysis of yeast membranes by sucrose gradient fractionation, and two different vital dyes to characterize the phenotype of vph1 triangle up

and *stv1* triangle up mutants as compared to the double mutant and the wild-type cells. Immunological assays of sucrose gradient fractions revealed that the amount of Stv1p was elevated in the *vph1* triangle up strain, and that vacuoles purified by this method with no detectable endosomal contamination contain an assembled V-ATPase complex, but with much lower activity than the wild type. These results suggest that Stv1p compensates for the loss of Vph1p in the *vph1* triangle up strain. LysoSensor Green DND-189 was used as a pH sensor to demonstrate unexpected changes in vacuolar acidification in *stv1* triangle up as the Vph1p-containing V-ATPase complex is commonly considered to acidify the vacuoles. In the *vph1* triangle up strain, the dye revealed slight but definite acidification of the vacuole as well. The lipophilic dye FM4-64 was used as an endocytic marker. We show that the null V-ATPase mutants, as well as the *vph1* triangle up one, markedly slow down endocytosis of the dye.

Petroi, D., B. Popova, et al. (2012). "Aggregate clearance of alpha-synuclein in *Saccharomyces cerevisiae* depends more on autophagosome and vacuole function than on the proteasome." *J Biol Chem* **287**(33): 27567-27579.

Parkinson disease is the second most common neurodegenerative disease. The molecular hallmark is the accumulation of proteinaceous inclusions termed Lewy bodies containing misfolded and aggregated alpha-synuclein. The molecular mechanism of clearance of alpha-synuclein aggregates was addressed using the bakers' yeast *Saccharomyces cerevisiae* as the model. Overexpression of wild type alpha-synuclein or the genetic variant A53T integrated into one genomic locus resulted in a gene copy-dependent manner in cytoplasmic proteinaceous inclusions reminiscent of the pathogenesis of the disease. In contrast, overexpression of the genetic variant A30P resulted only in transient aggregation, whereas the designer mutant A30P/A36P/A76P neither caused aggregation nor impaired yeast growth. The alpha-synuclein accumulation can be cleared after promoter shut-off by a combination of autophagy and vacuolar protein degradation. Whereas the proteasomal inhibitor MG-132 did not significantly inhibit aggregate clearance, treatment with phenylmethylsulfonyl fluoride, an inhibitor of vacuolar proteases, resulted in significant reduction in clearance. Consistently, a *cim3-1* yeast mutant restricted in the 19 S proteasome regulatory subunit was unaffected in clearance, whereas an *Deltaatg1* yeast mutant deficient in autophagy showed a delayed aggregate clearance response. A *cim3-1Deltaatg1* double mutant was still able to clear aggregates, suggesting additional cellular mechanisms for alpha-synuclein clearance. Our data provide insight into the mechanisms yeast cells use for clearing different species of alpha-synuclein and demonstrate a higher contribution of the autophagy/vacuole than the proteasome system. This contributes to the understanding of how cells can cope with toxic and/or aggregated proteins and may ultimately enable the development of novel strategies for therapeutic intervention.

Petti, A. A., C. A. Crutchfield, et al. (2011). "Survival of starving yeast is correlated with oxidative stress response and nonrespiratory mitochondrial function." *Proc Natl Acad Sci U S A* **108**(45): E1089-1098.

Survival of yeast during starvation has been shown to depend on the nature of the missing nutrient(s). In general, starvation for "natural" nutrients such as sources of carbon, phosphate, nitrogen, or sulfate results in low death rates, whereas starvation for amino acids or other metabolites in auxotrophic mutants results in rapid loss of viability. Here we characterized phenotype, gene expression, and metabolite abundance during starvation for methionine. Some methionine auxotrophs (those with blocks in the biosynthetic pathway) respond to methionine starvation like yeast starving for natural nutrients such as phosphate

or sulfate: they undergo a uniform cell cycle arrest, conserve glucose, and survive. In contrast, methionine auxotrophs with defects in the transcription factors Met31p and Met32p respond poorly, like other auxotrophs. We combined physiological and gene expression data from a variety of nutrient starvations (in both respiratory competent and incompetent cells) to show that successful starvation response is correlated with expression of genes encoding oxidative stress response and nonrespiratory mitochondrial functions, but not respiration per se.

Phadnis, N. and E. Ayres Sia (2004). "Role of the putative structural protein Sed1p in mitochondrial genome maintenance." *J Mol Biol* **342**(4): 1115-1129.

The nuclear gene MIP1 encodes the mitochondrial DNA polymerase responsible for replicating the mitochondrial genome in *Saccharomyces cerevisiae*. A number of other factors involved in replicating and segregating the mitochondrial genome are yet to be identified. Here, we report that a bacterial two-hybrid screen using the mitochondrial polymerase, Mip1p, as bait identified the yeast protein Sed1p. Sed1p is a cell surface protein highly expressed in the stationary phase. We find that several modified forms of Sed1p are expressed and the largest of these forms interacts with the mitochondrial polymerase in vitro. Deletion of SED1 causes a 3.5-fold increase in the rate of mitochondrial DNA point mutations as well as a 4.3-fold increase in the rate of loss of respiration. In contrast, we see no change in the rate of nuclear point mutations indicating the specific role of Sed1p function in mitochondrial genome stability. Indirect immunofluorescence analysis of Sed1p localization shows that Sed1p is targeted to the mitochondria. Moreover, Sed1p is detected in purified mitochondrial fractions and the localization to the mitochondria of the largest modified form is insensitive to the action of proteinase K. Deletion of the sed1 gene results in a reduction in the quantity of Mip1p and also affects the levels of a mitochondrially-expressed protein, Cox3p. Our results point towards a role for Sed1p in mitochondrial genome maintenance.

Pimentel, C., L. Batista-Nascimento, et al. (2012). "Oxidative stress in Alzheimer's and Parkinson's diseases: insights from the yeast *Saccharomyces cerevisiae*." *Oxid Med Cell Longev* **2012**: 132146.

Alzheimer's (AD) and Parkinson's (PD) diseases are the two most common causes of dementia in aged population. Both are protein-misfolding diseases characterized by the presence of protein deposits in the brain. Despite growing evidence suggesting that oxidative stress is critical to neuronal death, its precise role in disease etiology and progression has not yet been fully understood. Budding yeast *Saccharomyces cerevisiae* shares conserved biological processes with all eukaryotic cells, including neurons. This fact together with the possibility of simple and quick genetic manipulation highlights this organism as a valuable tool to unravel complex and fundamental mechanisms underlying neurodegeneration. In this paper, we summarize the latest knowledge on the role of oxidative stress in neurodegenerative disorders, with emphasis on AD and PD. Additionally, we provide an overview of the work undertaken to study AD and PD in yeast, focusing the use of this model to understand the effect of oxidative stress in both diseases.

Pittman, Y. R., L. Valente, et al. (2006). "Mg²⁺ and a key lysine modulate exchange activity of eukaryotic translation elongation factor 1B alpha." *J Biol Chem* **281**(28): 19457-19468.

To sustain efficient translation, eukaryotic elongation factor B alpha (eEF1B alpha) functions as the guanine nucleotide exchange factor for eEF1A. Stopped-flow kinetics using 2'-(or 3')-O-N-methylanthraniloyl (mant)-GDP showed spontaneous release of nucleotide from eEF1A

is extremely slow and accelerated 700-fold by eEF1B alpha. The eEF1B alpha-stimulated reaction was inhibited by Mg²⁺ with a K(1/2) of 3.8 mM. Previous structural studies predicted the Lys-205 residue of eEF1B alpha plays an important role in promoting nucleotide exchange by disrupting the Mg²⁺ binding site. Co-crystal structures of the lethal K205A mutant in the catalytic C terminus of eEF1B alpha with eEF1A and eEF1A.GDP established that the lethality was not due to a structural defect. Instead, the K205A mutant drastically reduced the nucleotide exchange activity even at very low concentrations of Mg²⁺. A K205R eEF1B alpha mutant on the other hand was functional in vivo and showed nearly wild-type nucleotide dissociation rates but almost no sensitivity to Mg²⁺. These results indicate the significant role of Mg²⁺ in the nucleotide exchange reaction by eEF1B alpha and establish the catalytic function of Lys-205 in displacing Mg²⁺ from its binding site.

Prohaska, C., K. Pomazal, et al. (2000). "Determination of Ca, Mg, Fe, Cu, and Zn in blood fractions and whole blood of humans by ICP-OES." Fresenius J Anal Chem **367**(5): 479-484.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) was applied to the determination of the elements Ca, Mg, Fe, Cu, and Zn in blood plasma, erythrocytes, lymphocytes, and whole blood to obtain reliable data on their distribution in blood fractions. The samples were carefully collected to avoid contamination. Two different nebulizers (Babington and Meinhard) were tested and optimized for this analytical problem. Line selections for all elements of interest were performed (LODs were 0.8 microg/L for Ca, 1.7 microg/L for Cu, 3.0 microg/L for Fe, 1.1 microg/L for Mg, and 4.2 microg/L for Zn). Recoveries were determined as approx. 100%, and standard reference material was analyzed to obtain reliable data on element distribution. The optimized method was applied to the determination of Ca, Mg, Fe, Cu, and Zn in the course of a clinical study on blood and blood fractions of two groups of humans of differing health. The concentrations measured in blood fractions were verified by balancing with the values found in whole blood.

Puig, S., E. Askeland, et al. (2005). "Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation." Cell **120**(1): 99-110.

Iron (Fe) is an essential micronutrient for virtually all organisms and serves as a cofactor for a wide variety of vital cellular processes. Although Fe deficiency is the primary nutritional disorder in the world, cellular responses to Fe deprivation are poorly understood. We have discovered a posttranscriptional regulatory process controlled by Fe deficiency, which coordinately drives widespread metabolic reprogramming. We demonstrate that, in response to Fe deficiency, the *Saccharomyces cerevisiae* Cth2 protein specifically downregulates mRNAs encoding proteins that participate in many Fe-dependent processes. mRNA turnover requires the binding of Cth2, an RNA binding protein conserved in plants and mammals, to specific AU-rich elements in the 3' untranslated region of mRNAs targeted for degradation. These studies elucidate coordinated global metabolic reprogramming in response to Fe deficiency and identify a mechanism for achieving this by targeting specific mRNA molecules for degradation, thereby facilitating the utilization of limited cellular Fe levels.

Puig, S. and D. J. Thiele (2002). "Molecular mechanisms of copper uptake and distribution." Curr Opin Chem Biol **6**(2): 171-180.

Pulkes, T. and M. G. Hanna (2001). "Human mitochondrial DNA diseases." Adv Drug Deliv Rev **49**(1-2): 27-43.

The mitochondrial encephalomyopathies are a genetically heterogeneous group of disorders associated with impaired oxidative phosphorylation. Patients may exhibit a wide range of clinical symptoms and experience significant morbidity and mortality. There is currently no curative treatment. At present the majority of genetically defined mitochondrial encephalomyopathies are caused by mutations in mitochondrial DNA. The underlying molecular mechanisms and the complex relationship between genotype and phenotype in these mitochondrial DNA diseases remain only partially understood. We describe the key features of mitochondrial DNA genetics and outline some of the common disease phenotypes associated with mtDNA defects. A classification of pathogenic mitochondrial DNA point mutations which may have therapeutic implications is outlined.

Qiu, X. B., Y. M. Shao, et al. (2006). "The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones." *Cell Mol Life Sci* **63**(22): 2560-2570.

DnaJ/Hsp40 (heat shock protein 40) proteins have been preserved throughout evolution and are important for protein translation, folding, unfolding, translocation, and degradation, primarily by stimulating the ATPase activity of chaperone proteins, Hsp70s. Because the ATP hydrolysis is essential for the activity of Hsp70s, DnaJ/Hsp40 proteins actually determine the activity of Hsp70s by stabilizing their interaction with substrate proteins. DnaJ/Hsp40 proteins all contain the J domain through which they bind to Hsp70s and can be categorized into three groups, depending on the presence of other domains. Six DnaJ homologs have been identified in *Escherichia coli* and 22 in *Saccharomyces cerevisiae*. Genome-wide analysis has revealed 41 DnaJ/Hsp40 family members (or putative members) in humans. While 34 contain the typical J domains, 7 bear partially conserved J-like domains, but are still suggested to function as DnaJ/Hsp40 proteins. DnaJA2b, DnaJB1b, DnaJC2, DnaJC20, and DnaJC21 are named for the first time in this review; all other human DnaJ proteins were dubbed according to their gene names, e.g. DnaJA1 is the human protein named after its gene DNAJA1. This review highlights the progress in studying the domains in DnaJ/Hsp40 proteins, introduces the mechanisms by which they interact with Hsp70s, and stresses their functional diversity.

Ramsay, L. M. and G. M. Gadd (1997). "Mutants of *Saccharomyces cerevisiae* defective in vacuolar function confirm a role for the vacuole in toxic metal ion detoxification." *FEMS Microbiol Lett* **152**(2): 293-298.

Reddi, A. R. and V. C. Culotta (2011). "Regulation of manganese antioxidants by nutrient sensing pathways in *Saccharomyces cerevisiae*." *Genetics* **189**(4): 1261-1270.

In aerobic organisms, protection from oxidative damage involves the combined action of enzymatic and nonproteinaceous cellular factors that collectively remove harmful reactive oxygen species. One class of nonproteinaceous antioxidants includes small molecule complexes of manganese (Mn) that can scavenge superoxide anion radicals and provide a backup for superoxide dismutase enzymes. Such Mn antioxidants have been identified in diverse organisms; however, nothing regarding their physiology in the context of cellular adaptation to stress was known. Using a molecular genetic approach in Baker's yeast, *Saccharomyces cerevisiae*, we report that the Mn antioxidants can fall under control of the same pathways used for nutrient sensing and stress responses. Specifically, a serine/threonine PAS-kinase, Rim15p, that is known to integrate phosphate, nitrogen, and carbon sensing, can also control Mn antioxidant activity in yeast. Rim15p is negatively regulated by the phosphate-sensing kinase complex Pho80p/Pho85p and by the nitrogen-

sensing Akt/S6 kinase homolog, Sch9p. We observed that loss of either of these upstream kinase sensors dramatically inhibited the potency of Mn as an antioxidant. Downstream of Rim15p are transcription factors Gis1p and the redundant Msn2/Msn4p pair that typically respond to nutrient and stress signals. Both transcription factors were found to modulate the potency of the Mn antioxidant but in opposing fashions: loss of Gis1p was seen to enhance Mn antioxidant activity whereas loss of Msn2/4p greatly suppressed it. Our observed roles for nutrient and stress response kinases and transcription factors in regulating the Mn antioxidant underscore its physiological importance in aerobic fitness.

Reddy, J. V. and M. N. Seaman (2001). "Vps26p, a component of retromer, directs the interactions of Vps35p in endosome-to-Golgi retrieval." *Mol Biol Cell* **12**(10): 3242-3256.

Endosome-to-Golgi retrieval of the carboxypeptidase Y receptor Vps10p is mediated by a recently discovered membrane coat complex termed retromer. Retromer comprises five conserved proteins: Vps35p, Vps29p, Vps5p, Vps17p, and Vps26p. Vps35p recognizes cargo molecules such as Vps10p and interacts strongly with Vps29p. Vps5p forms a subcomplex with Vps17p and has been proposed to play a structural role by self-assembling into large multimeric structures. The function of Vps26p is currently unknown. We have investigated the role that Vps26p plays in retromer-mediated endosome-to-Golgi transport by analyzing dominant negative alleles of Vps26p. These mutants have identified a crucial region of Vps26p that plays an important role in its function. Functional domains of Vps26p have been investigated by the creation of yeast-mouse hybrid molecules in which domains of Vps26p have been replaced by the similar domain in the protein encoded by the mouse VPS26 gene, Hbeta58. These domain swap experiments have shown that Vps26p promotes the interactions between the cargo-selective component Vps35p and the structural components Vps5p/Vps17p.

Rees, E. M., J. Lee, et al. (2004). "Mobilization of intracellular copper stores by the ctr2 vacuolar copper transporter." *J Biol Chem* **279**(52): 54221-54229.

Rios, G., M. Cabedo, et al. (2013). "Role of the yeast multidrug transporter Qdr2 in cation homeostasis and the oxidative stress response." *FEMS Yeast Res* **13**(1): 97-106.

We have identified QDR2 in a screening for genes able to confer tolerance to sodium and/or lithium stress upon overexpression. Qdr2 is a multidrug transporter of the major facilitator superfamily, originally described for its ability to transport the antimalarial drug quinidine and the herbicide barban. To identify its physiological substrate, we have screened for phenotypes dependent on QDR2 and found that Qdr2 is able to transport monovalent and divalent cations with poor selectivity, as shown by growth tests and the determination of internal cation content. Moreover, strains overexpressing or lacking QDR2 also exhibit phenotypes when reactive oxygen species- producing agents, such as hydrogen peroxide or menadione were added to the growth medium. We have also found that the presence of copper and hydrogen peroxide repress the expression of QDR2. In addition, the copper uptake of a qdr2 mutant strain is similar to a wild type, but the extrusion is clearly impaired. Based on our results, we propose that free divalent copper is the main physiological substrate of Qdr2. As copper is a substrate for several redox reactions that occur within the cytoplasm, its function in copper homeostasis explains its role in the oxidative stress response.

Rivera-Mancia, S., I. Perez-Neri, et al. (2010). "The transition metals copper and iron in neurodegenerative diseases." *Chem Biol Interact* **186**(2): 184-199.

Roberts, E. A. and D. W. Cox (1998). "Wilson disease." *Baillieres Clin Gastroenterol* **12**(2): 237-256.

Wilson disease is a recessively inherited disorder of copper transport. Clinical features are highly variable, with any combination of neurological, hepatic or psychiatric illness. The age of onset varies from 3 to 50 years of age. Diagnosis is challenging because no specific combination of clinical or biochemical features is necessarily definitive. The genetic defect is due to a variety of abnormalities in a copper-transporting membrane ATPase. Most of the more than 80 mutations are present at a low frequency, and mutations differ between ethnic groups. At least two mutations are sufficiently common to aid in rapid diagnosis, in European and Asian populations respectively. Molecular analysis can provide a definitive diagnosis for asymptomatic sibs. Treatment, using chelating agents or zinc, is most effective when started before permanent tissue damage occurs.

Roberts, R. A., R. A. Smith, et al. (2010). "Toxicological and pathophysiological roles of reactive oxygen and nitrogen species." *Toxicology* **276**(2): 85-94.

'Oxidative and Nitritative Stress in Toxicology and Disease' was the subject of a symposium held at the EUROTOX meeting in Dresden 15th September 2009. Reactive oxygen (ROS) and reactive nitrogen species (RNS) produced during tissue pathogenesis and in response to viral or chemical toxicants, induce a complex series of downstream adaptive and reparative events driven by the associated oxidative and nitritative stress. As highlighted by all the speakers, ROS and RNS can promote diverse biological responses associated with a spectrum of disorders including neurodegenerative/neuropsychiatric and cardiovascular diseases. Similar pathways are implicated during the process of liver and skin carcinogenesis. Mechanistically, reactive oxygen and nitrogen species drive sustained cell proliferation, cell death including both apoptosis and necrosis, formation of nuclear and mitochondrial DNA mutations, and in some cases stimulation of a pro-angiogenic environment. Here we illustrate the pivotal role played by oxidative and nitritative stress in cell death, inflammation and pain and its consequences for toxicology and disease pathogenesis. Examples are presented from five different perspectives ranging from in vitro model systems through to in vivo animal model systems and clinical outcomes.

Rodriguez-Rocha, H., A. Garcia-Garcia, et al. (2013). "Compartmentalized oxidative stress in dopaminergic cell death induced by pesticides and complex I inhibitors: Distinct roles of superoxide anion and superoxide dismutases." *Free Radic Biol Med* **61C**: 370-383.

The loss of dopaminergic neurons induced by the parkinsonian toxins paraquat, rotenone, and 1-methyl-4-phenylpyridinium (MPP+) is associated with oxidative stress. However, controversial reports exist regarding the source/compartmentalization of reactive oxygen species (ROS) generation and its exact role in cell death. We aimed to determine in detail the role of superoxide anion ($O_2^{\bullet-}$), oxidative stress, and their subcellular compartmentalization in dopaminergic cell death induced by parkinsonian toxins. Oxidative stress and ROS formation were determined in the cytosol, intermembrane (IMS), and mitochondrial matrix compartments, using dihydroethidine derivatives and the redox sensor roGFP, as well as electron paramagnetic resonance spectroscopy. Paraquat induced an increase in ROS and oxidative stress in both the cytosol and the mitochondrial matrix prior to cell death. MPP+ and rotenone primarily induced an increase in ROS and oxidative stress in the mitochondrial matrix. No oxidative stress was detected at the level of the IMS. In contrast to previous

studies, overexpression of manganese superoxide dismutase (MnSOD) or copper/zinc SOD (CuZnSOD) had no effect on alterations in ROS steady-state levels, lipid peroxidation, loss of mitochondrial membrane potential ($\Delta\psi$), and dopaminergic cell death induced by MPP⁺ or rotenone. In contrast, paraquat-induced oxidative stress and cell death were selectively reduced by MnSOD overexpression, but not by CuZnSOD or manganese-porphyrins. However, MnSOD also failed to prevent $\Delta\psi$ loss. Finally, paraquat, but not MPP⁺ or rotenone, induced the transcriptional activation of the redox-sensitive antioxidant response elements (ARE) and nuclear factor kappa-B (NF- κ B). These results demonstrate a selective role of mitochondrial O₂^{•-} in dopaminergic cell death induced by paraquat, and show that toxicity induced by the complex I inhibitors rotenone and MPP⁺ does not depend directly on mitochondrial O₂^{•-} formation.

Rothman, J. H., I. Howald, et al. (1989). "Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae*." *EMBO J* 8(7): 2057-2065.

To further our studies of protein sorting and biogenesis of the lysosome-like vacuole in yeast, we have isolated spontaneous mutations in 11 new VPL complementation groups, as well as additional alleles of the eight previously described VPL genes. These mutants were identified by selecting for cells that mislocalize vacuolar proteins to the cell surface. Morphological examination of the vpl mutants indicated that most contain vacuoles of normal appearance; however, some of the mutants generally lack a large vacuole, and instead accumulate smaller organelles. Of the 19 VPL complementation groups, 12 were found to be identical to 12 of 33 VPT complementation groups identified in a separate study. Moreover, the end1 mutant and all of the previously reported pep mutants, with the exception of pep4, were found to exhibit a profound vacuolar protein sorting defect, and complementation tests between the PEP, VPL VPT and END1 groups demonstrated that there are extensive overlaps between these groups. Collectively, mutants in these four collections define 49 complementation groups required to deliver or retain soluble vacuolar enzymes, including carboxypeptidase Y (CPY) and proteinase A. We have also isolated 462 new mutants that lack normal levels of vacuolar CPY activity. Among these latter mutants, only pep4 mutants were found to be specifically defective in vacuolar zymogen activation. We conclude that there is a large number of gene products required for sorting or retention of vacuolar proteins in yeast, and only a single gene, PEP4, that is essential for activation of CPY and other vacuolar zymogens.

Salmon, T. B., B. A. Evert, et al. (2004). "Biological consequences of oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*." *Nucleic Acids Res* 32(12): 3712-3723.

Reactive oxygen species (ROS), generated by endogenous and exogenous sources, cause significant damage to macromolecules, including DNA. To determine the cellular effects of induced, oxidative DNA damage, we established a relationship between specific oxidative DNA damage levels and biological consequences produced by acute H₂O₂ exposures in yeast strains defective in one or two DNA damage-handling pathways. We observed that unrepaired, spontaneous DNA damage interferes with the normal cellular response to exogenous oxidative stress. In addition, when base excision repair (BER) is compromised, there is a preference for using recombination (REC) over translesion synthesis (TLS) for handling H₂O₂-induced DNA damage. The global genome transcriptional response of these strains to exogenous H₂O₂ exposure allowed for the identification of genes responding specifically to induced, oxidative DNA damage. We also found that the presence of DNA damage alone was sufficient to cause an increase in intracellular ROS levels. These results, linking DNA damage and intracellular ROS production, may provide insight into the role of DNA damage in tumor progression and aging. To our knowledge, this is the first report

establishing a relationship between H₂O₂-induced biological endpoints and specific oxidative DNA damage levels present in the genome.

Samimi, G., K. Katano, et al. (2004). "Modulation of the cellular pharmacology of cisplatin and its analogs by the copper exporters ATP7A and ATP7B." *Mol Pharmacol* **66**(1): 25-32.

The copper efflux transporters ATP7A and ATP7B sequester intracellular copper into the vesicular secretory pathway for export from the cell. The influence of these transporters on the pharmacodynamics of cisplatin, carboplatin, and oxaliplatin was investigated using human Menkes' disease fibroblasts (Me32a) that do not express either transporter and sublines molecularly engineered to express either ATP7A (MeMNK) or ATP7B (MeWND). Cellular copper levels were significantly higher in the Me32a cells than in the MeMNK and MeWND sublines. These transporter-proficient sublines were resistant to the cytotoxic effect of copper, cisplatin, and carboplatin but were hypersensitive to oxaliplatin. Whole-cell accumulation of platinum after a 24-h exposure was significantly increased in the MeMNK and MeWND cells for all three platinum drugs, but this was accompanied by an increase in the amount of platinum reaching the DNA only for oxaliplatin. Vesicles isolated from MeMNK cells contained more platinum after exposure to cisplatin and carboplatin, whereas the platinum content of vesicles from MeWND cells was increased after exposure to all three drugs. Although copper triggered relocalization of ATP7A from the perinuclear region to more peripheral locations, the platinum drugs did not. These results demonstrate that both ATP7A and ATP7B modulate the pharmacodynamics of all three clinically used platinum drugs. The data are consistent with the hypothesis that these copper exporters sequester the platinum drugs into subcellular compartments, limiting their cytotoxicity, similar to their effect on copper. However, in this model system, although copper is readily exported after vesicular sequestration, the platinum drugs are not.

Sansanwal, P. and M. M. Sarwal (2010). "Abnormal mitochondrial autophagy in nephropathic cystinosis." *Autophagy* **6**(7): 971-973.

Cystinosis, which is characterized by lysosomal accumulation of cystine in many tissues, was the first known storage disorder caused by defective metabolite export from the lysosome. The molecular and cellular mechanisms underlying nephropathic cystinosis, the most severe form, which exhibits generalized proximal tubular dysfunction and progressive renal failure, remain largely unknown. We used renal proximal tubular epithelial (RPTE) cells and fibroblasts from patients with three clinical variants of cystinosis: nephropathic, intermediate and ocular to explore the specific injury mechanism in nephropathic cystinosis. We demonstrate enhanced autophagy of mitochondria, increase in apoptosis and mitochondrial dysfunction in the nephropathic cystinosis phenotype. Furthermore, specific inhibition of autophagy results in significant attenuation of cell death in nephropathic cystinosis. This study provides ultrastructural and functional evidence of abnormal mitochondrial autophagy in nephropathic cystinosis, which may contribute to renal Fanconi syndrome and progressive renal injury.

Sansanwal, P., B. Yen, et al. (2010). "Mitochondrial autophagy promotes cellular injury in nephropathic cystinosis." *J Am Soc Nephrol* **21**(2): 272-283.

The molecular and cellular mechanisms underlying nephropathic cystinosis, which exhibits generalized proximal tubular dysfunction and progressive renal failure, remain largely unknown. Renal biopsies from patients with this disorder can reveal abnormally large mitochondria, but the relevance of this and other ultrastructural abnormalities is unclear. We

studied the ultrastructure of fibroblasts and renal proximal tubular epithelial cells from patients with three clinical variants of cystinosis: Nephropathic, intermediate, and ocular. Electron microscopy revealed the presence of morphologically abnormal mitochondria and abnormal patterns of mitochondrial autophagy (mitophagy) with a high number of autophagic vacuoles and fewer mitochondria ($P < 0.02$) in nephropathic cystinosis. In addition, we observed increased apoptosis in renal proximal tubular epithelial cells, greater expression of LC3-II/LC3-I (microtubule-associated protein 1 light chain 3), and significantly more autophagosomes in the nephropathic variant. The autophagy inhibitor 3-methyl adenine rescued cell death in cystinotic cells. Cystinotic cells had increased levels of beclin-1 and aberrant mitochondrial function with a significant decrease in ATP generation and an increase in reactive oxygen species. This study provides ultrastructural and functional evidence of abnormal mitophagy in nephropathic cystinosis, which may contribute to the renal Fanconi syndrome and progressive renal injury.

Schapira, A. H. (1996). "Oxidative stress and mitochondrial dysfunction in neurodegeneration." Curr Opin Neurol 9(4): 260-264.

Scherz-Shouval, R. and Z. Elazar (2011). "Regulation of autophagy by ROS: physiology and pathology." Trends Biochem Sci 36(1): 30-38.

Reactive oxygen species (ROS) are small and highly reactive molecules that can oxidize proteins, lipids and DNA. When tightly controlled, ROS serve as signaling molecules by modulating the activity of the oxidized targets. Accumulating data point to an essential role for ROS in the activation of autophagy. Be the outcome of autophagy survival or death and the initiation conditions starvation, pathogens or death receptors, ROS are invariably involved. The nature of this involvement, however, remains unclear. Moreover, although connections between ROS and autophagy are observed in diverse pathological conditions, the mode of activation of autophagy and its potential protective role remain incompletely understood. Notably, recent advances in the field of redox regulation of autophagy focus on the role of mitochondria as a source of ROS and on mitophagy as a means for clearance of ROS.

Schilsky, M. L., R. J. Stockert, et al. (1998). "Copper resistant human hepatoblastoma mutant cell lines without metallothionein induction overexpress ATP7B." Hepatology 28(5): 1347-1356.

Mutant human hepatoblastoma cell lines resistant to copper toxicity were isolated from mutagenized HuH7. Two copper resistant cell lines (CuR), CuR 23 and CuR 27, had reduced basal expression of metallothionein (MT) messenger RNA (mRNA) and exhibited minimal or no increase in resistance to cadmium or zinc toxicity. Copper uptake, efflux of newly transported copper, glutathione content, and efflux rate were comparable with HuH7, whereas holoceruloplasmin synthesis and secretion were slightly decreased. Subcellular distribution of copper at steady-state showed an increase in organelle and membrane fractions with a reduction in cytosol. Expression of ATP7B mRNA was fivefold increased, and ATP7B protein approximately threefold increased in both CuR 23 and 27. Another cell line, CuR 41, showed increased basal expression of MT and ATP7B mRNA but not ATP7B protein, and resistance to cadmium and zinc toxicity. Copper uptake in CuR 41 was comparable with HuH7, but initial rates of efflux of copper and glutathione were reduced. The synthesis of holoceruloplasmin but not ceruloplasmin peptide was markedly diminished in CuR 41. Subcellular distribution of copper showed an increase in cytosolic and decreased organelle and membrane-associated copper. These data suggest that cellular resistance to copper

toxicity was achieved in two independent cell lines without MT induction and that the induction of ATP7B may lead to the enhanced intracellular sequestration of copper by organelles.

Schuller, A., G. Auffermann, et al. (2013). "Overexpression of ctr1Delta300, a high-affinity copper transporter with deletion of the cytosolic C-terminus in *Saccharomyces cerevisiae* under excess copper, leads to disruption of transition metal homeostasis and transcriptional remodelling of cellular processes." *Yeast* **30**(5): 201-218.

In an approach to generating *Saccharomyces cerevisiae* strains with increased intracellular copper amounts for technical applications, we overexpressed the copper transporter CTR1 and a variant of CTR1 with a truncation in the C-terminus after the 300th amino acid (ctr1Delta300). We determined the copper sensitivity of the generated strains and used inductively coupled plasma spectrometry analysis (ICP-OES and ICP-MS) to investigate the effects of overexpression of both constructs under excess copper on the cellular content of different elements in *S. cerevisiae*. In addition, we performed DNA microarray analysis to obtain the gene expression profile under the changed element contents. Overexpression of CTR1 increased the copper content in the cells to 160% and 78 genes were differentially regulated. Overexpression of the truncated ctr1Delta300 resulted in an increased copper, iron and zinc content of > 200% and 980 genes showed differential expression. We found that transition metal ion homeostasis was disrupted in ctr1Delta300-overexpressing strains under excess copper and that this was combined with a transcriptional remodelling of cellular processes.

Seaman, M. N., J. M. McCaffery, et al. (1998). "A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast." *J Cell Biol* **142**(3): 665-681.

We have recently characterized three yeast gene products (Vps35p, Vps29p, and Vps30p) as candidate components of the sorting machinery required for the endosome-to-Golgi retrieval of the vacuolar protein sorting receptor Vps10p (Seaman, M.N.J., E.G. Marcusson, J.-L. Cereghino, and S.D. Emr. 1997. *J. Cell Biol.* 137:79-92). By genetic and biochemical means we now show that Vps35p and Vps29p interact and form part of a multimeric membrane-associated complex that also contains Vps26p, Vps17p, and Vps5p. This complex, designated here as the retromer complex, assembles from two distinct subcomplexes comprising (a) Vps35p, Vps29p, and Vps26p; and (b) Vps5p and Vps17p. Density gradient fractionation of Golgi/endosomal/vesicular membranes reveals that Vps35p cofractionates with Vps5p/Vps17p in a vesicle-enriched dense membrane fraction. Furthermore, gel filtration analysis indicates that Vps35p and Vps5p are present on a population of vesicles and tubules slightly larger than COPI/coatomer-coated vesicles. We also show by immunogold EM that Vps5p is localized to discrete regions at the rims of the prevacuolar endosome where vesicles appear to be budding. Size fractionation of cytosolic and recombinant Vps5p reveals that Vps5p can self-assemble in vitro, suggesting that Vps5p may provide the mechanical impetus to drive vesicle formation. Based on these findings we propose a model in which Vps35p/Vps29p/Vps26p function to select cargo for retrieval, and Vps5p/Vps17p assemble onto the membrane to promote vesicle formation. Conservation of the yeast retromer complex components in higher eukaryotes suggests an important general role for this complex in endosome-to-Golgi retrieval.

Serrano, R., D. Bernal, et al. (2004). "Copper and iron are the limiting factors for growth of the yeast *Saccharomyces cerevisiae* in an alkaline environment." *J Biol Chem* **279**(19): 19698-19704.

Exposure of the yeast *Saccharomyces cerevisiae* to an alkaline environment represents a stress situation that negatively affects growth and results in an adaptive transcriptional response. We screened a collection of 4825 haploid deletion mutants for their ability to grow at mild alkaline pH, and we identified 118 genes, involved in numerous cellular functions, whose absence results in reduced growth. The list includes several key genes in copper and iron homeostasis, such as CCC2, RCS1, FET3, LYS7, and CTR1. In contrast, a screen of high-copy number plasmid libraries for clones able to increase tolerance to alkaline pH revealed only two genes: FET4 (encoding a low affinity transporter for copper, iron, and zinc) and CTR1 (encoding a high affinity copper transporter). The beneficial effect of overexpression of CTR1 requires a functional high affinity iron transport system, as it was abolished by deletion of FET3, a component of the high affinity transport system, or CCC2, which is required for assembly of the transport system. The growth-promoting effect of FET4 was not modified in these mutants. These results suggest that the observed tolerance to alkaline pH is because of improved iron uptake and indicate that both iron and copper are limiting factors for growth under alkaline pH conditions. Addition to the medium of micromolar concentrations of copper or iron ions drastically improved growth at high pH. Supplementation with iron improved somewhat the tolerance of a *fet3* strain but was ineffective in a *ctr1* mutant, suggesting the existence of additional copper-requiring functions important for tolerance to an alkaline environment.

Singh, A., N. Kaur, et al. (2007). "The metalloredoxase Fre6p in Fe-efflux from the yeast vacuole." *J Biol Chem* **282**(39): 28619-28626.

The yeast vacuole is the storage depot for cellular iron. In this report we quantify the import-export balance in the vacuole because of the import of iron by Ccc1p and to export by the combined activity of Smf3p and the ferroxidase, permease pair of proteins, Fet5p and Fth1p. Our data indicate that the two efflux pathways are equally efficient in trafficking iron out of the vacuole. A major focus of this work was to identify the ferrereductase(s) that supplies the Fe(II) for efflux whether by Smf3p or the Fet5p-Fth1p complex. Using a combination of flameless atomic absorption spectrophotometry to quantify vacuolar and whole cell iron content and a reporter assay for cytoplasmic iron we demonstrate that Fre6p supplies Fe(II) to both efflux systems, while Fre7p plays no role in Fe-efflux from the vacuole. Enzymatic assay shows the two fusions to have similar reductase activity, however. Confocal fluorescence microscopy demonstrates that Fre6:GFP localizes to the vacuolar membrane; in contrast, Fre7:GFP fusions exhibit a variable and diffuse cellular distribution. Demonstrating a role for a vacuolar metalloredoxase in Fe-efflux supports the model that iron is stored in the vacuole in the ferric state.

Stack, J. H., D. B. DeWald, et al. (1995). "Vesicle-mediated protein transport: regulatory interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeast." *J Cell Biol* **129**(2): 321-334.

Stack, J. H. and S. D. Emr (1994). "Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities." *J Biol Chem* **269**(50): 31552-31562.

The Vps15 protein kinase and the Vps34 phosphatidylinositol 3-kinase have been shown to function as a membrane-associated complex which facilitates the delivery of proteins to the vacuole in yeast. Biochemical characterization of the autophosphorylation reaction catalyzed by Vps15p demonstrates that it is a functional serine/threonine protein kinase. In addition,

we show that the Vps34 phosphatidylinositol 3-kinase undergoes an autophosphorylation event both in vivo and in vitro, indicating that it represents a novel multiple specificity kinase capable of phosphorylating both protein and lipid substrates. Vps34p is phosphorylated predominately on serine in vivo and is able to phosphorylate serine, threonine, and tyrosine residues in vitro. Mutant Vps34 proteins containing alterations in conserved amino acids in the lipid kinase domain are severely defective for both PI 3-kinase activity and autophosphorylation. Characterization of the PI 3-kinase activity of Vps34p demonstrates that it, unlike the mammalian p110 PI 3-kinase, is highly resistant to the PI 3-kinase inhibitors wortmannin and LY294002. We also find that Vps34p is a phosphatidylinositol-specific 3-kinase, as it is able to utilize phosphatidylinositol (PtdIns) but not PtdIns(4)P or PtdIns(4,5)P₂ as substrates in an in vitro PI kinase reaction. The substrate specificity, wortmannin resistance, and other biochemical characteristics of its PtdIns 3-kinase activity suggest that Vps34p is quite similar to a PtdIns-specific 3-kinase activity recently characterized from mammalian cells. These data indicate the existence of a family of PI 3-kinases composed of p110-like PI 3-kinases and Vps34p-like PtdIns-specific 3-kinases. On the basis of the role for Vps34p in vacuolar protein sorting, we propose that the production of a specific phosphoinositide, PtdIns(3)P, is involved in regulating intracellular protein sorting reactions in eukaryotic cells.

Stafford, S. L., N. J. Bokil, et al. (2013). "Metal ions in macrophage antimicrobial pathways: emerging roles for zinc and copper." *Biosci Rep* **33**(4).

The immunomodulatory and antimicrobial properties of zinc and copper have long been appreciated. In addition, these metal ions are also essential for microbial growth and survival. This presents opportunities for the host to either harness their antimicrobial properties or limit their availability as defence strategies. Recent studies have shed some light on mechanisms by which copper and zinc regulation contribute to host defence, but there remain many unanswered questions at the cellular and molecular levels. Here we review the roles of these two metal ions in providing protection against infectious diseases in vivo, and in regulating innate immune responses. In particular, we focus on studies implicating zinc and copper in macrophage antimicrobial pathways, as well as the specific host genes encoding zinc transporters (SLC30A, SLC39A family members) and CTRs (copper transporters, ATP7 family members) that may contribute to pathogen control by these cells.

Steinmetz, L. M., C. Scharfe, et al. (2002). "Systematic screen for human disease genes in yeast." *Nat Genet* **31**(4): 400-404.

High similarity between yeast and human mitochondria allows functional genomic study of *Saccharomyces cerevisiae* to be used to identify human genes involved in disease. So far, 102 heritable disorders have been attributed to defects in a quarter of the known nuclear-encoded mitochondrial proteins in humans. Many mitochondrial diseases remain unexplained, however, in part because only 40-60% of the presumed 700-1,000 proteins involved in mitochondrial function and biogenesis have been identified. Here we apply a systematic functional screen using the pre-existing whole-genome pool of yeast deletion mutants to identify mitochondrial proteins. Three million measurements of strain fitness identified 466 genes whose deletions impaired mitochondrial respiration, of which 265 were new. Our approach gave higher selection than other systematic approaches, including fivefold greater selection than gene expression analysis. To apply these advantages to human disorders involving mitochondria, human orthologs were identified and linked to heritable diseases using genomic map positions.

Takeshige, K., M. Baba, et al. (1992). "Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction." *J Cell Biol* **119**(2): 301-311.

For determination of the physiological role and mechanism of vacuolar proteolysis in the yeast *Saccharomyces cerevisiae*, mutant cells lacking proteinase A, B, and carboxypeptidase Y were transferred from a nutrient medium to a synthetic medium devoid of various nutrients and morphological changes of their vacuoles were investigated. After incubation for 1 h in nutrient-deficient media, a few spherical bodies appeared in the vacuoles and moved actively by Brownian movement. These bodies gradually increased in number and after 3 h they filled the vacuoles almost completely. During their accumulation, the volume of the vacuolar compartment also increased. Electron microscopic examination showed that these bodies were surrounded by a unit membrane which appeared thinner than any other intracellular membrane. The contents of the bodies were morphologically indistinguishable from the cytosol; these bodies contained cytoplasmic ribosomes, RER, mitochondria, lipid granules and glycogen granules, and the density of the cytoplasmic ribosomes in the bodies was almost the same as that of ribosomes in the cytosol. The diameter of the bodies ranged from 400 to 900 nm. Vacuoles that had accumulated these bodies were prepared by a modification of the method of Ohsumi and Anraku (Ohsumi, Y., and Y. Anraku. 1981. *J. Biol. Chem.* 256:2079-2082). The isolated vacuoles contained ribosomes and showed latent activity of the cytosolic enzyme glucose-6-phosphate dehydrogenase. These results suggest that these bodies sequestered the cytosol in the vacuoles. We named these spherical bodies "autophagic bodies." Accumulation of autophagic bodies in the vacuoles was induced not only by nitrogen starvation, but also by depletion of nutrients such as carbon and single amino acids that caused cessation of the cell cycle. Genetic analysis revealed that the accumulation of autophagic bodies in the vacuoles was the result of lack of the PRB1 product proteinase B, and disruption of the PRB1 gene confirmed this result. In the presence of PMSF, wild-type cells accumulated autophagic bodies in the vacuoles under nutrient-deficient conditions in the same manner as did multiple protease-deficient mutants or cells with a disrupted PRB1 gene. As the autophagic bodies disappeared rapidly after removal of PMSF from cultures of normal cells, they must be an intermediate in the normal autophagic process. This is the first report that nutrient-deficient conditions induce extensive autophagic degradation of cytosolic components in the vacuoles of yeast cells.

Tarsio, M., H. Zheng, et al. (2011). "Consequences of loss of Vph1 protein-containing vacuolar ATPases (V-ATPases) for overall cellular pH homeostasis." *J Biol Chem* **286**(32): 28089-28096.

In yeast cells, subunit a of the vacuolar proton pump (V-ATPase) is encoded by two organelle-specific isoforms, VPH1 and STV1. V-ATPases containing Vph1 and Stv1 localize predominantly to the vacuole and the Golgi apparatus/endosomes, respectively. Ratiometric measurements of vacuolar pH confirm that loss of STV1 has little effect on vacuolar pH. Loss of VPH1 results in vacuolar alkalinization that is even more rapid and pronounced than in *vma* mutants, which lack all V-ATPase activity. Cytosolic pH responses to glucose addition in the *vph1Delta* mutant are similar to those in *vma* mutants. The extended cytosolic acidification in these mutants arises from reduced activity of the plasma membrane proton pump, Pma1p. Pma1p is mislocalized in *vma* mutants but remains at the plasma membrane in both *vph1Delta* and *stv1Delta* mutants, suggesting multiple mechanisms for limiting Pma1 activity when organelle acidification is compromised. pH measurements in early prevacuolar compartments via a pHluorin fusion to the Golgi protein Gef1 demonstrate that pH responses of these compartments parallel cytosolic pH changes. Surprisingly, these compartments remain acidic even in the absence of V-ATPase function, possibly as a result of

cytosolic acidification. These results emphasize that loss of a single subunit isoform may have effects far beyond the organelle where it resides.

Town, M., G. Jean, et al. (1998). "A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis." *Nat Genet* **18**(4): 319-324.

Uauy, R., M. Olivares, et al. (1998). "Essentiality of copper in humans." *Am J Clin Nutr* **67**(5 Suppl): 952S-959S.

van der Vaart, A., J. Griffith, et al. (2010). "Exit from the Golgi is required for the expansion of the autophagosomal phagophore in yeast *Saccharomyces cerevisiae*." *Mol Biol Cell* **21**(13): 2270-2284.

The delivery of proteins and organelles to the vacuole by autophagy involves membrane rearrangements that result in the formation of large vesicles called autophagosomes. The mechanism underlying autophagosome biogenesis and the origin of the membranes composing these vesicles remains largely unclear. We have investigated the role of the Golgi complex in autophagy and have determined that in yeast, activation of ADP-ribosylation factor (Arf)1 and Arf2 GTPases by Sec7, Gea1, and Gea2 is essential for this catabolic process. The two main events catalyzed by these components, the biogenesis of COPI- and clathrin-coated vesicles, do not play a critical role in autophagy. Analysis of the *sec7* strain under starvation conditions revealed that the autophagy machinery is correctly assembled and the precursor membrane cisterna of autophagosomes, the phagophore, is normally formed. However, the expansion of the phagophore into an autophagosome is severely impaired. Our data show that the Golgi complex plays a crucial role in supplying the lipid bilayers necessary for the biogenesis of double-membrane vesicles possibly through a new class of transport carriers or a new mechanism.

Vitvitsky, V. M., S. K. Garg, et al. (2012). "Na⁺ and K⁺ ion imbalances in Alzheimer's disease." *Biochim Biophys Acta* **1822**(11): 1671-1681.

Alzheimer's disease (AD) is associated with impaired glutamate clearance and depressed Na⁽⁺⁾/K⁽⁺⁾ ATPase levels in AD brain that might lead to a cellular ion imbalance. To test this hypothesis, [Na⁽⁺⁾] and [K⁽⁺⁾] were analyzed in postmortem brain samples of 12 normal and 16 AD individuals, and in cerebrospinal fluid (CSF) from AD patients and matched controls. Statistically significant increases in [Na⁽⁺⁾] in frontal (25%) and parietal cortex (20%) and in cerebellar [K⁽⁺⁾] (15%) were observed in AD samples compared to controls. CSF from AD patients and matched controls exhibited no differences, suggesting that tissue ion imbalances reflected changes in the intracellular compartment. Differences in cation concentrations between normal and AD brain samples were modeled by a 2-fold increase in intracellular [Na⁽⁺⁾] and an 8-15% increase in intracellular [K⁽⁺⁾]. Since amyloid beta peptide (Abeta) is an important contributor to AD brain pathology, we assessed how Abeta affects ion homeostasis in primary murine astrocytes, the most abundant cells in brain tissue. We demonstrate that treatment of astrocytes with the Abeta 25-35 peptide increases intracellular levels of Na⁽⁺⁾ (~2-3-fold) and K⁽⁺⁾ (~1.5-fold), which were associated with reduced levels of Na⁽⁺⁾/K⁽⁺⁾ ATPase and the Na⁽⁺⁾-dependent glutamate transporters, GLAST and GLT-1. Similar increases in astrocytic Na⁽⁺⁾ and K⁽⁺⁾ levels were also caused by Abeta 1-40, but not by Abeta 1-42 treatment. Our study suggests a previously unrecognized impairment in AD brain cell ion homeostasis that might be triggered by Abeta and could significantly affect electrophysiological activity of brain cells, contributing to the pathophysiology of AD.

Walsh, P., D. Bursac, et al. (2004). "The J-protein family: modulating protein assembly, disassembly and translocation." *EMBO Rep* 5(6): 567-571.

Wang, X., O. Okonkwo, et al. (2013). "Physiological basis of copper tolerance of *Saccharomyces cerevisiae* nonsense-mediated mRNA decay mutants." *Yeast* 30(5): 179-190.

The eukaryotic nonsense-mediated mRNA decay pathway (NMD) is a specialized pathway that contributes to the recognition and rapid degradation of mRNA with premature termination codons. In addition to mRNAs containing premature termination codons, NMD degrades non-nonsense-containing, natural mRNAs. Approximately 5-10% of the total *Saccharomyces cerevisiae* transcriptome is affected when NMD is inactivated. The regulation of natural mRNAs by NMD has physiological consequences. However, the physiological outcomes associated with the degradation of specific natural mRNAs by NMD are not fully understood. Here, we examined the physiological consequences resulting from the NMD-mediated regulation of an mRNA involved in copper homeostasis, in an attempt to understand why *nmd* mutant strains are more tolerant of toxic copper levels than wild-type yeast strains. We found that wild-type (UPF1) and *upf1*Δ mutants accumulate similar amounts of total copper when grown in medium containing elevated levels of copper; however, the copper levels in the cytoplasm of wild-type yeast cells were higher than in the *upf1*Δ mutant. Copper tolerance by the *upf1*Δ mutant is dependent on the presence of CTR2. Deletion of CTR2 resulted in similar cytoplasmic copper levels in wild-type and *upf1*Δ mutant strains, regardless of the environmental copper levels. This suggests that CTR2 plays a role in regulating the level of copper in the cytoplasm. We also found that the *upf1*Δ mutant contained elevated copper levels in the vacuole relative to wild-type yeast cells, after both strains were exposed to elevated copper levels.

Wu, J., M. Ricker, et al. (2006). "Copper deficiency as cause of unexplained hematologic and neurologic deficits in patient with prior gastrointestinal surgery." *J Am Board Fam Med* 19(2): 191-194.

Wu, M. J., P. J. O'Doherty, et al. (2011). "Different Reactive Oxygen Species Lead to Distinct Changes of Cellular Metal Ions in the Eukaryotic Model Organism *Saccharomyces cerevisiae*." *Int J Mol Sci* 12(11): 8119-8132.

Elemental uptake and export of the cell are tightly regulated thereby maintaining the ionic homeostasis. This equilibrium can be disrupted upon exposure to exogenous reactive oxygen species (ROS), leading to reduction or elevation of the intracellular metal ions. In this study, the ionic composition in the eukaryotic model organism *Saccharomyces cerevisiae* was profiled using the inductively-coupled plasma optical emission spectrometer (ICP-OES) following the treatment with individual ROS, including hydrogen peroxide, cumen hydroperoxide, linoleic acid hydroperoxide (LAH), the superoxide-generating agent menadione, the thiol-oxidising agent diamide [diazine-dicarboxylic acid-bis(dimethylamide)], dimedone and peroxyxynitrite. The findings demonstrated that different ROS resulted in distinct changes in cellular metal ions. Aluminium (Al³⁺) level rose up to 50-fold after the diamide treatment. Cellular potassium (K⁺) in LAH-treated cells was 26-fold less compared to the non-treated controls. The diamide-induced Al³⁺ accumulation was further validated by the enhanced Al³⁺ uptake along the time course and diamide doses. Pre-incubation of yeast with individual elements including iron, copper, manganese and magnesium failed to block diamide-induced Al³⁺ uptake, suggesting Al³⁺-specific

transporters could be involved in Al(3+) uptake. Furthermore, LAH-induced potassium depletion was validated by a rescue experiment in which addition of potassium increased yeast growth in LAH-containing media by 26% compared to LAH alone. Taken together, the data, for the first time, demonstrated the linkage between ionic profiles and individual oxidative conditions.

Yang, F., F. Ding, et al. (2013). "ROS generation and proline metabolism in calli of halophyte *Nitraria tangutorum* Bobr. to sodium nitroprusside treatment." Protoplasma.

Nitric oxide (NO) is a stress factor or a signal molecule involved in various plant physiological and developmental processes. In the present study, the generation of reactive oxygen species and the metabolism of proline due to different sodium nitroprusside (SNP, an NO donor) concentrations were investigated in callus from halophyte *Nitraria tangutorum* Bobr. Treatment with SNP led to significant increases of hydrogen peroxide (H_2O_2) content and cell viability but notable reductions in hydrogen radical level and lipid peroxidation degree, and superoxide anion (O_2^-) content also enhanced in 100 μM SNP-treated calli. Using a chemical inhibitor for plasma membrane (PM) NADPH oxidase diphenylene iodonium (DPI), we found low O_2^- generation in untreated and 25 μM SNP-treated calli, whereas in those treated with 100 μM SNP O_2^- level exhibited a very little alteration, comparable to the absence of DPI. These suggest a high activity of PM NADPH oxidase in untreated calli. H_2O_2 scavenging enzymes (catalase, peroxidase [POD] and ascorbate peroxidase) and H_2O_2 forming enzymes (superoxide dismutase [SOD], cell wall-POD and diamine oxidase [DAO]) stimulated significantly in calli treated with different SNP concentrations while glutathione reductase activity decreased. In addition, a reduction in proline content was observed in SNP-treated calli. Moreover, different SNP concentrations stimulated proline dehydrogenase (PDH) and ornithine delta-aminotransferase but inhibited γ -glutamyl kinase (GK). In conclusion, our results suggest that the increasing H_2O_2 generation was associated with the stimulation of SOD, cell wall-POD and DAO, and that the reduction of proline content might be the consequence of increased PDH activity and decreased GK activity in *N. tangutorum* Bobr. calli under SNP treatment.

Yang, J., S. Dong, et al. (2013). "Changes in expression of manganese superoxide dismutase, copper and zinc superoxide dismutase and catalase in *Brachionus calyciflorus* during the aging process." PLoS One 8(2): e57186.

Rotifers are useful model organisms for aging research, owing to their small body size (0.1-1 mm), short lifespan (6-14 days) and the relative ease in which aging and senescence phenotypes can be measured. Recent studies have shown that antioxidants can extend the lifespan of rotifers. In this paper, we analyzed changes in the mRNA expression level of genes encoding the antioxidants manganese superoxide dismutase (MnSOD), copper and zinc SOD (CuZnSOD) and catalase (CAT) during rotifer aging to clarify the function of these enzymes in this process. We also investigated the effects of common life-prolonging methods [dietary restriction (DR) and resveratrol] on the mRNA expression level of these genes. The results showed that the mRNA expression level of MnSOD decreased with aging, whereas that of CuZnSOD increased. The mRNA expression of CAT did not change significantly. This suggests that the ability to eliminate reactive oxygen species (ROS) in the mitochondria reduces with aging, thus aggravating the damaging effect of ROS on the mitochondria. DR significantly increased the mRNA expression level of MnSOD, CuZnSOD and CAT, which might explain why DR is able to extend rotifer lifespan. Although resveratrol also increased the mRNA expression level of MnSOD, it had significant inhibitory effects on the mRNA expression of

CuZnSOD and CAT. In short, mRNA expression levels of CAT, MnSOD and CuZnSOD are likely to reflect the ability of mitochondria to eliminate ROS and delay the aging process.

Yang, Q., G. Siganos, et al. (2006). "Evolution versus "intelligent design": comparing the topology of protein-protein interaction networks to the Internet." Comput Syst Bioinformatics Conf: 299-310.

Recent research efforts have made available genome-wide, high-throughput protein-protein interaction (PPI) maps for several model organisms. This has enabled the systematic analysis of PPI networks, which has become one of the primary challenges for the system biology community. In this study, we attempt to understand better the topological structure of PPI networks by comparing them against man-made communication networks, and more specifically, the Internet. Our comparative study is based on a comprehensive set of graph metrics. Our results exhibit an interesting dichotomy. On the one hand, both networks share several macroscopic properties such as scale-free and small-world properties. On the other hand, the two networks exhibit significant topological differences, such as the cliqueishness of the highest degree nodes. We attribute these differences to the distinct design principles and constraints that both networks are assumed to satisfy. We speculate that the evolutionary constraints that favor the survivability and diversification are behind the building process of PPI networks, whereas the leading force in shaping the Internet topology is a decentralized optimization process geared towards efficient node communication.

Yasokawa, D., S. Murata, et al. (2008). "Mechanisms of copper toxicity in *Saccharomyces cerevisiae* determined by microarray analysis." Environ Toxicol **23**(5): 599-606.