STUDY OF THE ROLE OF Pap1 AS A SENSOR OF H_2O_2 AND AS A TRANSCRIPTIONAL ACTIVATOR OF STRESS RESPONSES IN *Schizosaccharomyces pombe*

Isabel A. Calvo

Tesi Doctoral, Barcelona, Octubre 2012

Memòria presentada per optar al títol de Doctora

per la Universitat Pompeu Fabra

Treball dirigit per la Dra. Elena Hidalgo Hernando
Oxidative Stress and Cell Cycle Group

Departament de Ciències Experimentals i de la Salut

Programa de Doctorat en Ciències de la Salut i la Vida

de la Universitat Pompeu Fabra

A mi madre, padre y hermano,

AGRADECIMIENTOS

Bueno, llegó la hora...parecía que nunca iba a pasar; y por fin después de seis años llenos de experiencias inolvidables, estoy aquí, a punto de conseguir el título más duro a la vez que más enriquecedor de toda mi vida. No sé por donde empezar, ha sido tanto y tan bueno que es imposible resumir en unas líneas todo lo que he vivido durante este tiempo en Barcelona.

En primer lugar, quiero agradecer esta tesis a José y Elena. Gracias por darme la oportunidad de formar parte de la historia de este lab, estaré eternamente agradecida; además de apoyarme y ayudarme en momentos difíciles. Sólo deciros que espero poder seguir aprendiendo de vosotros estando fuera.

A Elena, por enseñarme tanto en cada reunión en ese despacho (que no han sido pocas, jeje) endulzadas con esos caramelos que tanto nos gustan a las dos. Gracias Elena! Gracias por estar siempre disponible cuando llamaba a esa puerta, por ayudarme a crecer como persona y sobre todo a querer llegar a ser una buena científica.

A José, todavía recuerdo el día que me dijiste que conocías Tudela y cocorico...☺ por resolvernos cada día un millón de dudas en el lab y amenizarnos con tu buena música; Gracias por esos consejos científicos y no tan científicos que me has dado, han sido clave!!

Gracias al lab, a todos mis compañeros pasados que me recibieron con los brazos abiertos y que vivimos tanto juntos. Mención especial a Ana por enseñarme tanto de Pap1 en esas escaleras de emergencia con el "piti" en mano, nunca dejaré de ser tu estudiantil!. Os deseo lo mejor en esta vida; Iván gracias por esos momenticos!. Gracias también a Alice, siempre pendiente de todos, que nos cuidaba tanto con los mejores tiramisús. Suerte pastelera!!

A mis grandes amigos del lab que ya no están, que sin vuestra ayuda no seguiría aquí. A Albertito, bueno mejor, Dr. Moldón, por enseñarme a amar la ciencia como tú sabes y no dejarme rendir por nada ni por nadie, gracias por darme tan buenos momentos dentro y fuera del lab, por estar siempre ahí, por no fallarme nunca en lo bueno y no tan bueno que vivimos, porfa, no cambies nunca!!. A "bellotita" por ser tan dulce, tan especial y tan fuerte!. Por ser la mejor amiga que cualquiera desearía tener. Ni te imaginas lo que significas para mi, que gran amiga!. A la "Birri", esta gran científica y sobre todo tan buenísima persona. Gracias por enseñarme cada día en el lab y sobre todo por vivir y disfrutar tanto juntas; y lo que nos queda por vivir. ¡¡Os quiero chicos!!

A gente que pasó por el labo poco o mucho tiempo y que dejaron huella. A Iván, el granaíno, porque conseguiste lo imposible en esas grandes noches. A Mónica, "la mami" por enseñarme esos "in vitros" y darme la oportunidad de formar parte de la historia de Tpx1. A Deib, por esas charlas filosóficas y grandes momentos; y sobre todo a Itzel, por traernos esa sonrisa al lab cada día y dejarnos compartir esas magníficas comidas mexicanas…¡Te espero en mi tesis!

Y ahora, gracias a la gente del lab con la que sigo compartiéndolo todo cada día. A Nati, la argentina, la hiperactiva que casi la tesis acaba con ella! Compañera de poyata y de screening, gracias por estos años, por superar juntas tan duros momentos!. A Sarela, esa gallega que calladita está siempre ahí; gracias por enseñarme tanto! Ya queda poco, un último esfuerzo a Ahmed también!. A Tsveti, por ser mi compañera deportista y amiga durante todos estos años y por escucharme mientras corríamos juntas por la playa. Te deseo lo mejor baby! Y a ti también José, un andaluz tan salado y tan gran persona!

A los postdocs del lab, Jorge, Ángel, Susana y Patricia. A Isabel, "la portuguesa", por esos grandes desayunos juntas y apoyarme siempre, y por aguantar esas discusiones de futbol. A mi estudiantita Alba, que ya es una predoc bien entrenada! Gracias por ayudarme estos últimos meses…mucha suerte!!

Mil gracias a MERCE. la mejor técnico, lab manager, predoc… q existe, una doctora sin título. Por aguantarnos tanto y conseguir un labo tan organizado; por llegar a tiempo a todo y mantener una sonrisa siempre! Esa Navarrica adoptada! Y sobre todo por ser compañera de grandes momentos fuera! Gracias de todo corazón a Jordi y a tí, ¡viva San Fermín!

Y por supuesto, gracias especialmente a Esther-Iva. Sólo pensar estar en un labo sin vosotras se me hace imposible. A la meva nena Esther, una catalana de pura casta, gràcies per fer que aquests anys hagin estat tan bons, per compartir tant, compres, viatges, festa, Sant Fermines, lab… ¡un diamant en brut!. A Iva, por llegar al labo cuando más la necesitábamos, q grande!! Por esa pijamada y momentos chicas…cuidame a mi peque porfa. Os espero fuera, ¡q nos queda mucho!

Y no m puedo olvidar del lab de al lado, los "posas" los que están y ya no están: Nuria, Gloria, Alex, Sergi, Jonathan, Micky, Laia...por esas gran fiestas o desmadres, jiji, como a Claudia, Cris, Mariona, Carme...y a grandes amigas que he hecho. Alba, llegaras muy lejos; y a Isabel, una persona muy especial que durante todo este tiempo me ha ayudado mucho en tantas cosas, y a Matt, por acogerme en Boston y cuidarme tanto. ¡¡Nos vemos en Boston!! Y no podías faltar tú, Gemma, GRACIAS por ayudarme tanto en estos dos meses, por organizarlo todo y no dejar que se me pase nada, has hecho que sean menos duros estos meses; tranquila que nos vemos fuera y más relajadas.

A no+3sis por esos partidos! Y sobre todo a todo el equipo de cool blue por permitirme vivir un sueño este último año! que grandes sois!! Al maritim, Sergi, Maite, Juan Carlos y Anna (mi fisio) por hacernos los días menos duros. Y a Alex, te deseo lo mejor guapa, ¡¡mucha suerte!! Y en esta tesis tiene que haber un espacio especialmente dedicado a ellos, gracias a su trabajo nosotras podemos currar cada día tan duro; Rafa de mantenimiento y las chicas de cocina, y a Jacinta por supuesto.

A Diego, gran amigo y el mejor bioinformático de la historia, ¡gracias por ayudarme tanto boludo!

Buf, esto no se acaba nunca. Pero no me puedo olvidar de mis grandes amigos fuera del lab; Víctor y José! gracias majicos! Pepón, gracias por ser tan buen confidente y estar siempre cuando te necesito. A la mejor profe de ioga y por supuesto buenísima amiga Raquel. A Ale, Eva, Darko, y a todos los gallegos por todas esas risas en esas grandes noches!

A Fon, por ser como es, por hacerme vivir momentos inolvidables y tan especiales durante tantos años, por madurar juntos y ayudarme siempre a todo lo q lo he necesitado. Un gran chef y mejor persona. ¡¡Siempre serás especial!!! Y a toda tu familia por seguir estando ahí! gracias Carol.

Y mención especial, a mi gran compañera de piso y GRAN amiga VALENTINA. Una española ya adoptada! te irá genial! Gracias por todo este tiempo juntas y esas noches hablando y hablando…¡por estos seis años de amistad y muchos más! Imposible de agradecer tanto…¡¡Ven pronto!! Y a esa mami tan dulce, gracias Neus por esas comidicas juntas y dejarme formar parte de esta nueva etapa, mil besicos a Julia! Agradezco de todo corazón a las mejores compis de piso,

que me han aguantado cada noche, María del Mar y Jenny. Habéis participado en esta tesis casi tanto como yo! Demasiadas cosas buenas! MMar cuanto hemos reído y llorado en la biblio-salón! Gracias chicas!. A mis ex compis de piso, Alex y Nico, que trajeron el equilibrio a un piso de tres locas.

Y no podía faltar sobre todo Tudela, gracias a mi gente de siempre, a mis amigas del alma, que aún estando lejos no me han fallado nunca, que sin ellas nose si seguiría aquí y sería tan feliz. ¡¡¡GRACIAS!!! A mis amores Juan luis y Diego q son los mejores! Y a mi queridísimo Iñigo por estar siempre ahí, por sus consejos y broncas.

Por último, y no por ello menos importante, a mi familia. Con especial cariño a mi abuelica, por esa paga y jamoncico que me metía en la maleta. A la mejor cuñada del mundo, por esos findes debajo del sol en Salou, por cuidar de mi hermano.
Y ahora, sí, sois los mejores, ¡¡sin vosotros esto no hubiera sido posible!! A mi madre, por darme a luz y permitirme disfrutar de este bonito viaje que es la vida, por esa comprensión y por hacer de mi una mujer hecha y derecha. ¡¡Por esos tupes durante tantos años!! Eres la mejor persona que he conocido nunca!. A mi padre, por intentar entenderme aunque sin éxito que hago aquí, por darme verdurica limpia cada vez que iba. Y para terminar, a mi hermanico, Oscar, no tengo palabras, ¡¡MUCHAS GRACIAS POR EXISTIR!! ¡Va a ser muy duro estar sin vosotros!

Volveré pronto, lo prometo!

Isabel , Agosto 2012

SUMMARY

"La perfección es una pulida colección de errores"

Mario Benedetti

Schizosaccharomyces pombe (S. pombe) has specific pathways to respond to extracellular hydrogen peroxide (H_2O_2), the main one being the Pap1 (pombe AP-1-like) pathway. Activation of Pap1 (oxidized state) occurs mainly at low H_2O_2 concentrations in a Tpx1-dependent manner. Previous genetic analysis seemed to indicate that an intramolecular disulfide bond is formed in Pap1 upon oxidative stress, which triggers the nuclear accumulation of the transcription factor and Pap1-dependent gene induction.

In this work we have characterized Pap1 as a sensor of H_2O_2 and as a transcription factor. Regarding the role of Pap1 as a sensor of H_2O_2, we have now performed a detailed mutagenesis analysis of all cysteine residues in Pap1 and have identified new cysteine residues essential for its activation. Furthermore, we have confirmed this data regarding with a proteomic characterization of active and inactive Pap1. We have deciphered the role of different components of the thioredoxin system in the regulation of Pap1, the participation of some proteins such as Trx1 and Trr1 in the control of Pap1 redox state; and we have seen that the loss of Trx1 prolongs H_2O_2-induced Pap1-dependent gene expression.

With regard to the study of Pap1 as a transcription factor, we have demonstrated here that the expression of some Pap1-induced genes such as *ctt1, trr1, srx1, obr1, SPCC663.08c* and *caf5* have different requirements regarding Pap1 activity/subcellular localization/oxidation state. The drug resistance genes only require nuclear Pap1 for activation, whereas another subset of genes, those coding for the antioxidants catalase, sulfiredoxin or thioredoxin reductase, do need oxidized Pap1 to form a heterodimer with the constitutively nuclear transcription factor Prr1. The ability of Pap1 to bind and activate drug tolerance

promoters is independent on Prr1, whereas its ability to bind to the antioxidant promoters is significantly enhanced upon association with Prr1.

En el laboratorio trabajamos en el estudio de la respuesta a estrés oxidativo, utilizando como sistema modelo la levadura *Schizosaccaromyces pombe. S. pombe* responde específicamente a estrés oxidativo a través del factor de transcripción Pap1. Cuando aplicamos a la célula bajas dosis de H_2O_2, Pap1 se encuentra reducido y en el citosol, sufre un cambio conformacional debido a la formación de puentes disulfuro (forma oxidada) y se acumula en el núcleo dónde se une a los diferentes promotores para inducir la respuesta transcripcional.

Los resultados presentados en este trabajo se centran en el estudio del mecanismo molecular de activación de Pap1 y concretamente en la caracterización de Pap1 como sensor de H_2O_2 y como factor de transcripción. Respecto a su papel como sensor, caracterizamos a Pap1 bioquímica y genéticamente y pudimos identificar nuevos residuos aminoacídicos que son esenciales para la transmisión de la señal. Estos resultados se pudieron confirmar con la caracterización proteómica de la proteína Pap1 activa e inactiva.

Además, intentamos averiguar cuál es el papel de los diferentes componentes del sistema tiorredoxina, descifrar el mecanismo por el cual proteínas como tiorredoxina o tiorredoxina reductasa participan en la regulación (oxidación-reducción) de Pap1.

En relación al papel de Pap1 como factor de transcripción, hemos observado que la expresión de genes Pap1 dependientes tiene diferentes requerimientos dependiendo de la localización/estado redox de la proteína, dando lugar a dos grupos de genes: tolerancia a drogas, como *obr1* o *caf5* que sólo necesitan a Pap1

nuclear para su activación. O antioxidantes, como *ctt1, srx1 o trr1* que requieren no sólo a Pap1 nuclear sino también activado (oxidado), además de la presencia de Prr1 (proteína nuclear). Ambas proteínas necesitan de la presencia de la otra para llevar a cabo correctamente su función y poder unirse a los diferentes promotores e inducir la transcripción.

PROLOGUE

Reactive oxygen species (ROS) are a variety of molecules derived from molecular oxygen during different metabolic processes. ROS can react with a wide variety of cellular components resulting in lipid peroxidation, protein oxidation and DNA damage. ROS-induced damage has been implicated in many chronic diseases and in ageing. Oxidative stress is associated with increased production of oxidizing species or a significant decrease in the capability of antioxidant defenses. Cells can counteract oxidative damage by inducing the expression of a large number of genes many of which encode proteins that decrease the levels of ROS and repair the accumulated damage.

Schizosaccharomyces pombe (*S. pombe*) is an ideal model to study stress responses due to the diversity of mechanisms that uses to respond to environmental stresses. *S. pombe* has specific sensors for oxidative stress such as the transcription factor Pap1 (pombe AP-1-like). At the beginning of this project, it was known that the activation of the transcription factor Pap1 occurs mainly at low hydrogen peroxide (H_2O_2) concentrations in a thioredoxin peroxidase (Tpx1)-dependent manner. In this work we have characterized Pap1 as a sensor of H_2O_2 as well as its activation in a Tpx1, Trx1 and Trr1-dependent manner and the role of Pap1 as a transcription factor. This signaling pathway seems to be essential for cell survival under oxidative stress conditions.

INDEX

"Life is like riding a bicycle. To keep your balance you must keep moving"

Albert Einstein

SUMMARY XII

PROLOGUE XXI

CONTENTS XXV

CHAPTER 1: INTRODUCTION

1. REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

1.1. ROS 3

1.1.1. The superoxide anion 3

1.1.2. Hydrogen peroxide 4

1.1.3. The hydroxyl radical 4

1.2. Oxidative stress: breaking an equilibrium 5

1.3. Cellular processes in which ros are generated 6

1.4. ROS associated toxicity 6

1.4.1. DNA damage 7

1.4.2. Lipid damage 8

1.4.3. Protein damage 9

1.4.3.1. Cysteine oxidation

2. DETOXIFYING SYSTEMS AGAINST CELLULAR ROS

2.1. Molecules acting as antioxidants 13

2.1.1. GSH 13

2.1.2. Thioredoxin (Trx) 15

2.1.2.1. Thioredoxin structure and mechanism

2.1.2.2. Roles of thioredoxin in diverse organisms

2.1.2.3. Thioredoxin reductase (Trr1)

2.1.3. Glutaredoxins (Grxs) 22

2.1.3.1. Classification and mechanism of Grxs

2.2. Enzymatic ros detoxification 26

2.2.1. Catalases 26

2.2.2. Glutathione Peroxidases (Gpxs) 28

2.2.3. Peroxiredoxins (Prxs) 29

2.2.3.1. Classification and mechanism of Prxs

2.2.3.2. Reversible inactivation and reactivation of 2-Cys Prx enzymes

2.2.3.3. Other functions of Prxs

2.2.4. Superoxide dismutases (SODs) 35

2.2.5. Glutathione transferasas (GSTs) 36

3. CYSTEINE HOMEOSTASIS

3.1. Interplay between the thioredoxin and glutaredoxin systems 39

3.2. Cytosol: a reducing compartment 41

3.3. Oxidizing compartments 42

3.3.1. Bacterial periplasm 43

3.3.2. Eukaryotic cells 45

3.3.2.1. The endoplasmic reticulum (ER)

3.3.2.2. The intermembrane mitochondrial space (IMS)

4. HYDROGEN PEROXIDE SENSING AND SIGNALING

4.1. Prokaryotic cells*: E. coli* 46

4.1.1. OxyR 47

4.1.2. SoxR 48

4.2. Eukaryotic cells 50

4.2.1. *Saccharomyces cerevisiae* *50*

4.2.1.1. YAP1
4.2.1.2. SKN7
4.2.1.3. Association of SKN7 and YAP1

***5.* OXIDATIVE STRESS *RESPONSES* IN *Schizosaccharomyces* pombe**
5.1. *Schizosaccharomyces* pombe as a model organism in the study of stress responses 56
5.2. General response against stress: map kinase sty1 pathway 58
5.3. The response regulator Prr1 61
5.4. Fission yeast specific sensors 63
5.4.1. Pap1 and Tpx1 63

OBJETIVES
73

CHAPTER 2: RESULTS

1. GENOME WIDE SCREEN OF *S. pombe* GENES ESSENTIAL FOR ROS HOMEOSTASIS AND FOR WILD-TYPE TOLERANCE TO CAFFEINE

1.1. Description of the screening performed with the deletion collection 79
1.2. Identification of the *s. pombe* genes required for wild-type tolerance to extracellular peroxides 80
1.3. Identification of genes required for growth on both h_2o_2-containing plates and on respiratory-prone conditions 83

1.3.1. Mitochondrial Dysfunction Increases Oxidative Stress and Decreases Chronological Life Span in fission yeast

1.4. Genome wide screen of genes required for caffeine tolerance in fission yeast

1.4.1. Genome-wide screen of caffeine-sensitive mutants 85

1.4.2. Involvement of the Pap1 pathway in cellular tolerance to caffeine 86

2. GENETIC AND PROTEOMIC CHARACTERIZATION OF ACTIVE Pap1

2.1. Genetic analysis and identification of new cys residues essential for pap1 activation 89

2.1.1. Proteomic characterization of active and inactive Pap1 90

3. ROLE OF Tpx1 AND THE THIOREDOXIN SYSTEM IN Pap1 ACTIVATION

3.1. Tpx1 is the main activator of Pap1 115

3.2. Trx1 can be regulating the h_2o_2-induced activation and inactivation of pap1 116

4. CHARACTERIZATION OF Pap1 AS A TRANSCRIPTION FACTOR IN RESPONSE TO H_2O_2

4.1. **Characterization of different mutant strains expressing constitutively nuclear pap1** 154
4.2. **Constitutively nuclear reduced pap1 can bind and activate only a subset of pap1-dependent genes** 155
4.3. **The role of prr1 in the pap1-dependent antioxidant response** 156

CHAPTER 3: GLOBAL DISCUSSION

1. **The pap1 and sty1 pathways are essential for wild-type tolerance to oxidative stress and to caffeine** 171

2. **Identification of four cysteines residues in pap1 involved in the response to h_2o_2** 173

3. **The study of thioredoxin system in pap1 activation** 174

4. **Characterization of pap1-dependent gene expression program** 175

CONCLUSIONS 181

APPENDICES 185

A.1 Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast 187
A.2 Genome-wide screen of genes required for caffeine tolerance in fission yeast 197
A.3. The peroxiredoxin Tpx1 is essential as a H_2O_2 scavenger during aerobic growth in fission yeast 209

A.4. Yox1 links MBF-dependent transcription to completion of DNA synthesis. 219

REFERENCES

CHAPTER 1: INTRODUCTION

"The only source of knowledge is experience"

Albert Einstein

1. REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

All organisms are exposed to reactive oxygen species (ROS) during the course of normal aerobic metabolism, or following exposure to radical-generating compounds (Halliwell 2006). Molecular oxygen is relatively unreactive and harmless in its ground state, but can undergo partial reduction to form a number of ROS including the superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) which can further react to produce the highly reactive hydroxyl radical (Fig. 1).

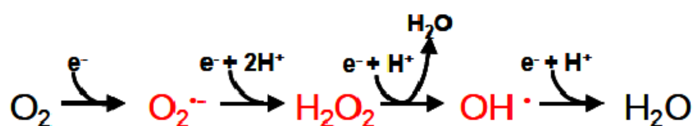


Figure 1. Reactive oxygen species (ROS), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) are generated from the partial reduction of molecular oxygen (O_2).

1.1. Reactive oxygen species (ROS)

1.1.1. The superoxide anion ($O_2^{\cdot -}$)

$O_2^{\cdot -}$ is generated by one electron reduction of O_2, which occurs widely in nature (see Fig. 1). $O_2^{\cdot -}$ is the major ROS product resulting from electron leakage from the mitochondrial electron transport chain (Halliwell 2006). The $O_2^{\cdot -}$ can readily be generated in cells using redox-cycling drugs such as menadione and paraquat, which transfer electrons to molecular oxygen.

1.1.2. Hydrogen peroxide (H_2O_2)

H_2O_2 is the simplest peroxide (a compound with an oxygen-oxygen single bond) and an oxidizer as a result of the ganancy of two electrons by O_2 (see Fig. 1). H_2O_2 is a ubiquitous molecule formed as a by-product of aerobic respiration and following exposure to diverse biological and

environmental factors. The stability of its link O-O limits its reactivity, however it can be easily spread through the cell compartments. Due to its easy use, including water solubility and relative stability, H_2O_2 is widely used to trigger oxidative stress in cell cultures.

H_2O_2 must be removed from cells to avoid Fenton and Haber - Weiss reactions leading to the formation of highly reactive hydroxyl radical ($OH^•$) (see Fig. 1). Organisms also naturally produce H_2O_2 as a by-product of oxidative metabolism. Consequently, nearly all-living things (specifically, aerobic organisms) possess enzymes known as catalase peroxidases, which harmlessly and catalytically decompose low concentrations of H_2O_2 to water and O_2.

1.1.3. The hydroxyl radical ($OH^•$)

$OH^•$ is the neutral form of the hydroxide ion ($OH^–$). $OH^•$ is highly reactive and consequently short-lived. It bears a single negative charge largely residing on the more electronegative oxygen. The $OH^•$ is generated from H_2O_2 via Fenton reaction, which requires iron (or another divalent metal ion, such as copper) and a source of reducing equivalents (possibly NADH) to regenerate the metal. In short, the radical $OH^•$ is generated when a ferrous iron (II) atom transfers an electron to H_2O_2 leading to ferric iron (III), an $OH^•$ and an $OH^–$.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

The Fenton reaction was suggested by Haber and Weiss in the 1930s and previously the Fenton's reagent was developed in the 1890s by Henry John Horstman Fenton as an analytical reagent (Fenton H.J.H. et al., 1894).

The $OH^•$ can damage virtually all types of macromolecules such as carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation) and amino acids (e.g. conversion of Phenylalanine to m-Tyrosine and o-Tyrosine) (Novogrodsky, Ravid et al. 1982). Unlike $O_2^{\cdot-}$ which can be detoxified by superoxide dismutase (SOD), the $OH^•$ cannot be eliminated by an enzymatic reaction.

1.2. Oxidative stress: breaking an equilibrium

Oxidative stress represents an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Some ROS can even act as messengers through a phenomenon called redox signaling.

Fluctuations in the external environment, genetic alterations or metabolic disturbances can result in a variety of cellular perturbations that disrupt this internal equilibrium and generate oxidative stress which can turn into pathologies, among them neurodegenerative diseases such as Alzheimer and Parkinson. They are known to be accelerated by the production of ROS causing DNA damage (Halliwell and Gutteridge 1988).

1.3. Sources of ROS generation

Mitochondrial respiration is thought to provide the main source of ROS in cells via the process of oxidative phosphorylation (Murphy and Porter 2009). Similarly, the use of O_2 as a terminal electron acceptor during oxidative protein folding means that the endoplasmic reticulum (ER) is also a significant source of ROS (see Fig. 2) (Tu and Weissman 2004). Also, other metabolic processes can potentially generate endogenous ROS in yeast depending on the growth conditions, including peroxisomal fatty acid degradation in the β-oxidation pathway (Hiltunen, Mursula et al. 2003) and oxidative deamination of amino acids by D-amino acid oxidases during growth on D-amino acids as carbon sources (Pollegioni, Piubelli et al. 2007). Besides the ROS generated in cellular processes, cells can receive insults from exogenous sources, like xenobiotics, exposition to UV and ionizing radiation or carcinogens (Halliwell 2006).

1.4. ROS targets

Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals, which damage all components of the cell including proteins, lipids, and DNA (Fig. 2).

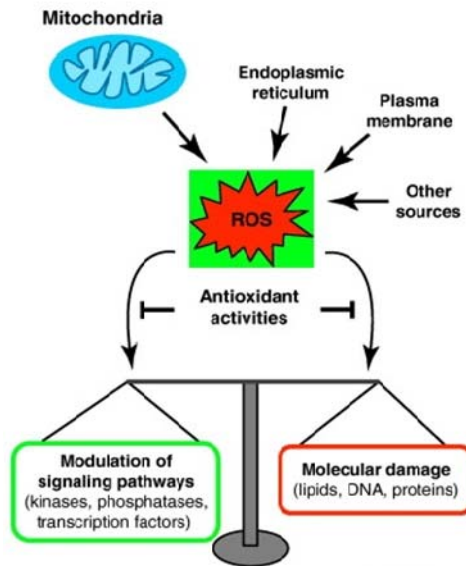


Figure 2. Sources and targets of ROS (Hekimi, Lapointe et al. 2011).

1.4.1. DNA damage

ROS can lead to strand breaks formation, base and nucleotide modifications, particularly in sequences with high guanosine content (Burney, Niles et al. 1999). Oxidative modification induces a robust repair response, characterized by excision of modified bases and nucleotides. Moreover, double-stranded DNA breaks also activate DNA repair enzymes such as mutated in ataxia telangiectasia (ATM) or ATM-related kinase (ATR). Both ATM and ATR directly phosphorylate and activate

specific checkpoint kinases, such as *chk2* and hCDS1, with subsequent phosphorylation of the tumor suppressor gene *p53*.
The best known DNA lesion is the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an oxidative modification of DNA produced by hydroxylation in the C-8 position of deoxyguanosine residues by $OH^{\bullet}$ (Floyd, West et al. 1990).

1.4.2. Lipid damage

ROS such as $OH^{\bullet}$ and hydroperoxyl radical (HO_2) combine with a hydrogen atom to make water and a fatty acid radical. The oxidative degradation of lipids is the lipid peroxidation in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. The most highly susceptible to oxidation by ROS are the polyunsaturated fatty acids present in phospholipids of biological membranes.

The fatty acid radical is not a very stable molecule, so it reacts readily with $O_2^{\cdot -}$, thereby creating a peroxyl-fatty acid radical. It is also an unstable species that reacts with another free fatty acid, producing a different fatty acid radical and lipid peroxide, or cyclic peroxide if it had reacted with itself. This reaction stops when two radicals react and produce a non-radical species and only happens when the concentration of radical species is high enough for there to be a high probability of collision of two radicals. Living organisms have evolved different molecules for its protection, such as vitamin E. Other antioxidants made within the body include the enzymes superoxide dismutase, catalase, and peroxidase (Halliwell and Gutteridge 1986; Coyle and Puttfarcken 1993).

In short, malondialdehyde (MDA), was used for the estimation of damage by ROS, is a major reactive aldehyde resulting from the peroxidation of biological membranes (Vaca, Wilhelm et al. 1988). MDA and 4-hydroxy-2-noneal (HNE) are the two most prominent lipid peroxidative products (Esterbauer, Cheeseman et al. 1982; Esterbauer and Cheeseman 1990).

1.4.3. Protein damage

There is a whole array of protein modifications, which can lead to protein loss of catalytic activity such as amino acid modifications, carbonyl group formation, formation of protein-protein cross-links or formation of disulfide bonds (S–S) among others. These modifications can impair protective functions through an increase in acidity, decrease in thermal stability, change in viscosity, change in fluorescence, fragmentation and increased susceptibility to proteolysis.

Protein oxidation is defined as a covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary products of oxidative stress (Shacter 2000). These oxidative modifications can be generated by prooxidant agents or *in vivo* during aging or some diseases. The amino acid side chains can be oxidatively modified leading to functional changes and, consequently, the protein structure is altered disturbing cellular metabolism.

Among amino acids, the most prone to oxidative attack are cysteine (Cys) and methionine (Met) residues, both of which contain susceptible sulfur atoms (Roos and Messens 2011). In the case of Cys, oxidation leads to the formation of S–S, mixed disulfides (e.g., with glutathione), and thiyl radicals (Hu 1994). For Met residues, the major product under biological conditions is Met sulfoxide (MetSO), which is associated with loss of function (Vogt 1995). Most biological systems contain disulfide reductases and MetSO reductases that can convert the oxidized forms of Cys and Met residues back to their unmodified forms. These are the only oxidative modifications of proteins that can be repaired (Berlett and Stadtman 1997). In the case of an oxidized protein cannot be repaired, they can be removed by proteolytic degradation and then be regenerated by de novo synthesis. For instance, proteins which are irreversibly inactivated by formation of Met sulfones, sulfinic (SO_2H) or sulfonic (SO_3H) acids and carbonyl derivatives cannot be repaired and have to be recognized and degraded by cellular proteolytic processes. It is worth mentioning that SO_2H in peroxiredoxins (Prxs) can be reversed to the thiol by sulfiredoxines (Biteau, Labarre et al. 2003; Wood, Poole et al. 2003).

The major form of irreversible protein oxidation is carbonylation. Therefore, the presence of carbonyl groups in proteins has been used as a marker of ROS-mediated protein oxidation (Berlett and Stadtman 1997). Carbonyl derivatives are a consequence of direct oxidation of lysine, arginine, proline, and threonine residues and may be introduced into proteins by reactions with aldehydes HNE, MDA produced during lipid peroxidation (Winkler, Lindner et al. 1984; Uchida and Stadtman 1993) or with ketoamines, ketoaldehydes or deoxyosones generated during the reaction of reducing sugars (Kristal and Yu 1992; Levine, Williams et al. 1994) (glycation and glycoxidation reactions).

Finally, indirect oxidative modification of protein amino acyl side chains occurs through the formation of adducts with products of oxidatively modified lipids, amino acids, sugars, and glutathione (GSH).

1.4.3.1. Cys oxidation

The oxidation-reduction (redox) reactions of Cys in a cell are often tightly regulated and play essential physiological roles (Held and Gibson 2012).

Cys is the major H_2O_2 target of different ROS such as peroxynitrite or superoxide (Radi, Beckman et al. 1991). There are a lot of Cys modifications such as sulfenic (-SOH), and SO_2H acids, thiol radicals (-SH), sulfenyl-amides and thiosulfinates (see Fig. 3), which come together to enable redox sensing, activation, catalysis, switching and cellular signaling. The Cys oxidation may result in various changes to the protein structure and function. These changes can be beneficial in the context of the folding, oligomerization and associated redox activation of proteins able to counteract oxidative stress (Gruber, Cemazar et al. 2006).

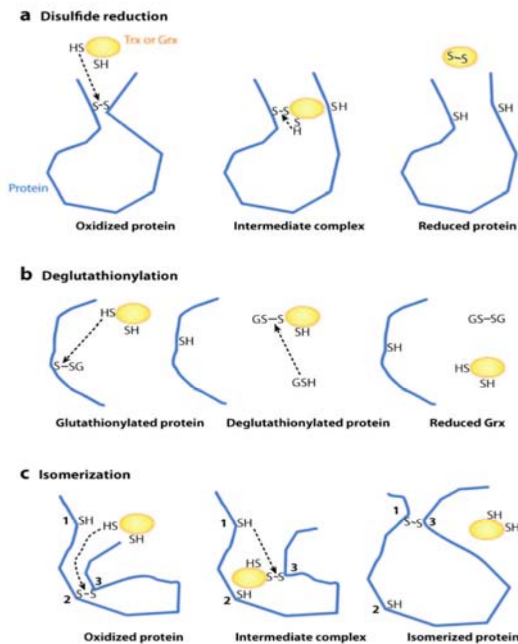


Figure 3. Reversible and irreversible modifications of cysteine residues (from Dansen T lab webpage).

The oxidation state of a free sulfhydryl is -2 and Cys can form up to ten different oxidation states *in vivo* (Giles, Watts et al. 2003). The diverse array of Cys oxoforms differs widely in their reactivity, origin, stability and reducibility. The Cys residue exists *in vivo* in the fully reduced free thiol form (-SH or $-S^{-}$) and in different oxidation forms, the thiyl radical ($-S^{\cdot}$), the -SOH, the disulfide bond (Cys-S-S-Cys), SO_2H, and SO_3H acid forms, and the S-nitrosylated form (-S-NO) (Poole, Karplus et al. 2004). Disulfide bonds are relatively unstable, reversing to the reduced state by thiol-disulfide exchange with kinetics depending on the protein context and the redox nature of the milieu. However, constitutive disulfide bonds, typically found in non-reducing environments such as ER or extracellular domains, are highly stable and generally not redox-regulated. On the other hand, $-S^{\cdot}$ and -SOH acid are very unstable because of their highly reactive nature and thus cannot be easily identified biochemically.

Reversibility is an essential property of any regulatory signaling network, but also allows Cys modifications such as glutathionylation to act

as a temporary buffer to oxidative stress, which can in turn be removed enzymatically.

In contrast, SO_2H and SO_3H acid are irreversible forms of Cys oxidation; these oxoforms are hallmarks of oxidative stress and in most cases are terminal, 'over-oxidized' modifications that are irreversible by chemical or enzymatic means. In a unique instance, the SO_2H- modified Prx family members can be enzymatically reduced by sulfiredoxins (Biteau, Labarre et al. 2003; Wood, Poole et al. 2003).

Finally, it was recently determined that in the mitochondrial matrix the concentration of solvent exposed Cys is higher than the Cys-containing tripeptide GSH. This confirms the role of exposed proteins thiols as the primary cellular antioxidant (Requejo, Hurd et al. 2010).

2. DETOXIFYING SYSTEMS AGAINST CELLULAR ROS

Antioxidant defenses include a huge number of protective enzymes, which are up regulated in response to ROS exposure. Non-enzymatic defenses typically consist of small molecules, which can act as free radical scavengers. Up to now only ascorbic acid and GSH have been extensively characterized in yeast.

2.1. Molecules acting as antioxidants

2.1.1. GSH

γ-glutamyl-cysteinyl-glycine (GSH) is the best-known example of non-enzymatic defense system. GSHis typically found as the most abundant low-molecular-weight sulfhydryl compound (2-10 mM intracellular concentration) in most organisms. GSH, an essential metabolite in yeast, was proposed to be required as a reductant during normal growth conditions (Grant, MacIver et al. 1996). However, it has recently been described as essential only for iron sulfur cluster assembly (Kumar, Igbaria et al. 2011). GSH acts as a radical scavenger with the redox-active sulfydryl group reacting with oxidants to produce GSSG, its oxidized form.

The levels of reduced GSH are maintained in balance between its rate of synthesis and reduction (see Fig. 4). Biosynthesis enzymes (Gsh1, γ-glutamylcysteine synthetase and Gsh2, glutathione synthetase) and enzymes for the reduction of GSSG to GSH such asGlr1 and glutathione reductase which is considered essential antioxidant are pivotal to maintain the reduced form of GSH.

GSH is synthesized via two ATP-dependent steps. During the first one , Gsh1 catalyzes the formation of dipeptide γ-Glu-Cys from glutamate and Cys. The second step is catalyzed by Gsh2 and ligates γ-Glu-Cys with glycine to yield GSH (Grant 2001). Gsh1 enzyme activity is feedback inhibited mechanism by GSH (Meister 1988) and *gsh1* gene expression is regulated by the cellular concentrations of GSH in parallel with sulfur amino acid biosynthesis (Wheeler, Quinn et al. 2002; Wheeler, Trotter et al. 2003). In addition, Glr1 is an NADPH-dependent oxido reductase which converts GSSG to GSH using reducing power generated by the pentose phosphate pathway (Llobell, Lopez-Ruiz et al. 1988).

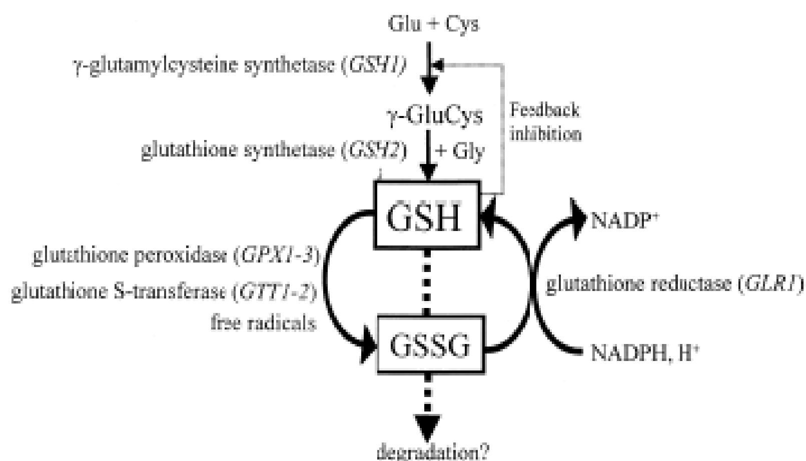


Figure 4. The yeast glutathione cycle (Grant 2001).

GSH is an important antioxidant molecule in yeast.It has been demonstrated that cells without GSH are very sensitive to oxidative stress induced by peroxides,the $O_2^{\cdot}$ and other compounds such as cadmium and methylglyoxyl (Izawa, Inoue et al. 1995; Grant, Collinson et al. 1996; Grant, MacIver et al. 1996). The requirement for GSH for oxidative stress

protection in bacteria is analogous to that occurs in higher eukaryotes. However, in bacteria where GSH is dispensable for the growth during both normal and oxidative stress conditions. Under normal aerobic conditions, the relation between reduced and oxidized form of GSH is 10-15:1 (Grant, Collinson et al. 1996; Muller 1996; Stephen and Jamieson 1996) indicating that the reduced GSH form is predominant in yeast and other eukaryotes due to the constitutive action of Glr1.

Most of the total cell GSH is unbound. The 58% is intracellular, and the 39% is in extracellular form. Exposure to H_2O_2 leads to an increase of GSSG, protein-bound (GSSP) and extracellular GSH indicating a shift in the redox balance. This increase is consistent with the role of GSH as both a free radical scavenger and a cofactor for various antioxidant enzymes, including glutathione peroxidases (Gpxs), glutathione S-transferases (Gsts) and glutaredoxins (Grxs) (Grant, Perrone et al. 1998).

2.1.2. Thioredoxin

Thioredoxin (Trx) was first described in 1964 by Laurent et al (Laurent, Moore et al. 1964) in *Escherichia coli (E. coli)* as a small redox protein. In contrast to bacteria, the yeast and particularly plants have multiple Trxs. However, only one cytoplasmic Trx has been found in human cells so far (Powis and Montfort 2001).

2.1.2.1. Thioredoxin structure and mechanism

Trxs are a family of proteins with a highly conserved amino acid sequence, the thioredoxin fold, Trp-Cys-Gly-Pro-Cys-Lys, at its active site (CxxC: in single-letter amino acid code and where X denotes any amino acid). These two Cys residues are oxidized and form a disulfide through the transfer of reducing equivalents from the catalytic site residues to a disulfide substrate. The oxidized Trx is then reduced back to the –SH form by a NADPH-dependent enzyme, the Trx reductase (Trr). This reaction is reversible and Trx may perform it depending on the redox potential of its substrate.

The mechanism of reduction of disulfide bonds in Trx substrates starts with the attack of the N-terminal SH of the Trx -CxxC motif to the disulfide bond of the target, reducing it and forming a transient mixed disulfide

bond. Secondly, the C-terminal SH of the CxxC motif breaks this disulfide bond leading to an oxidized Trx and the reduced target protein (Brandes, Larimer et al. 1993). The overall difference between the oxidized and reduced forms is subtle and involves a local conformational change in and around the redox-active disulfide.

The N-terminal active site Cys residue has a low pKa value and is the attacking nucleophile in disulfide reduction of proteins. The low redox potential of Trx (*E. coli* Trx1 = -270 mV) ensures that reduced Trx is the major dithiol reductant in the cytosol or an advanced equivalent to dithiothreitol of cells (Holmgren 1995). Apart from Trx, all organisms contain a lot of proteins which contain a Trx fold. Some of them also share a functional similarity with Trxs, such as Grxs, DsbA and protein disulfide isomerases (see section 3.3).

2.1.2.2. Roles of Trxs in diverse organisms

Trx plays multiple roles in cellular processes like proliferation or apoptosis, among others. Theseare critical for redox regulation of protein function and signaling via SH redox control (Fig. 5) (Holmgren 2000).

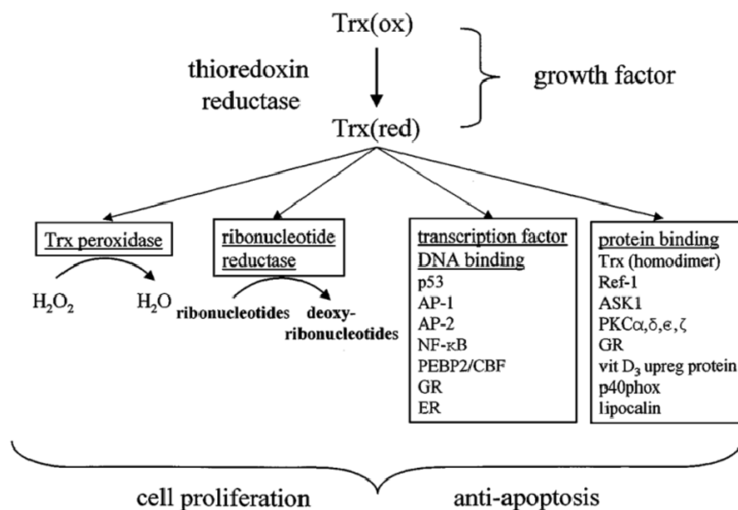


Figure 5. Actions of Trx in the cell (Powis and Montfort 2001).

Among cellular functions, the first one, Trx acts as a hydrogen donor for enzymes such as ribonucleotide reductase (RNR). Trx is required for maintaining dNTP pools for DNA synthesis. Also it participates in ROS

detoxification via Prxs and also it is involved in the reparation of protein due to MetSO reductases which use Trx as electron donors (Holmgren 1984). Briefly, Trx acts as the major RNR reducing system and its deletion leads to accumulation of RNR in its oxidized form (Muller, Wright et al. 1991).

Secondly, Trx is a key player for maintaining intracellular protein disulfides generally reduced with Grx (see section 3.1), both catalyzing thiol-disulfide oxidoreductions and protects cells from oxidative stress (Holmgren 1995).

In addition to its overall antioxidant properties, Trx participates also in the regulation of specific signaling pathways. For example, Trx regulates the affinity of many transcription factors for binding to DNA, due to its capacity for reduction of SH:p53 (Ueno, Masutani et al. 1999), NFkB (Matthews, Wakasugi et al. 1992) and AP-1 (see Fig. 5), among others (Hirota, Matsui et al. 1997). These proteins contain Cys residues critical for DNA-binding, which are sensitive to oxidation, thus Trx maintains them in their reduced state and functionally active (Yamawaki, Haendeler et al. 2003).

The exposure of cells to H_2O_2 generates an increased oxidizing environment that results in oxidation of Trx1.This accumulation of oxidized Trxs could be dangerous for the cell and can induce a S-S formation in other proteins. Therefore, the cell responds to this challenge by activation of the regulatory protein OxyR, which increases the expression of some components of the reducing machinery (Zheng, Aslund et al. 1998).

In plants, Trx regulates the activity of chloroplast photosynthetic enzymes by light via ferredoxin (Buchanan 1991).

In mammals, Trx has many functions, inside the cell to stimulate cell growth and inhibit apoptosis. An increased level of Trx characterizes the majority of human tumor cells. Trx seems to be an atypical growth factor because it has no specific receptor (Kalinina, Chernov et al. 2008). The reduced form of Trx interacts directly with another key signaling molecule, the apoptosis signal regulating kinase 1 (ASK1), preventing downstream signaling for apoptosis (Saitoh, Nishitoh et al. 1998). There are two Trx forms in mammals, one cytosolic Trx (Trx1), one mitochondrial Trx

precursor (Trx2). Also two Trr, in the cytosol (Trr1) and in the mitochondria (Trr2) (see below) (Gasdaska, Berggren et al. 1999).

In bacteria, Trx is an efficient reductant for 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase, the enzyme that converts PAPS to sulfite. Trx mutants are defective in sulphate assimilation (Gan 1991; Muller, Wright et al. 1991). *E. coli* genome contains three very effective cytoplasmic disulfide-reducing proteins and a Trr1.Trr1 isthe product of the *trxB* gene, which maintain Trx in reduced form. Trx1 isthe product of the *trxA* gene, Trx2 is the product of the *trxC* gene, and Grx1 is the product of the *grxA* gene whose physiological role is to act as reductants. However, Trx may act as an oxidant under certain conditions, such as whena *trxB* mutant (Stewart, Aslund et al. 1998) or when it is exported to the oxidizing environment of the *E. coli* periplasm (see section 3.3.1) (Debarbieux and Beckwith 1998). The expression of any one of these is sufficient to support aerobic growth. Moreover, Trx2 is the Trx that contributes to the important reducing processes normally taking place in the cytoplasm. It is due to that *trxBgshA* double mutant (GSH is the product of *gshA)* is inviable. However the double mutant *trxAgshA* is viable.

Saccharomyces cerevisiae (S. cerevisiae) contains a cytoplasmic Trx system with two Trx (TRX1, TRX2), a TRR1, and a mitochondrial comprising TRX3 and TRR2 (see below), which are redundant and independent in protection against oxidative stress (Miranda-Vizuete, Damdimopoulos et al. 1999). The cells can survive in the absence of both systems (Sun, Kirnarsky et al. 2001). Only the expression of *TRX2* is strongly up regulated in response to oxidative stress conditions, in a Yap1-dependent manner, (Kuge and Jones 1994) and *TRX2* mutants are very sensitive to H_2O_2 (Rundlof, Carlsten et al. 2000). Moreover, the double *TRX1TRX2* mutant exhibits an elongated S phase and a shortened G1 interval with an increase in the level of oxidized glutathione (GSSG) assimilation (Muller, Wright et al. 1991).

S. pombe contains two genes encoding cytoplasmic Trxs (Trx1 and Trx3), which are dispensable under normal growth conditions and one mitochondrial Trx (Trx2) (Gan 1991; Muller, Wright et al. 1991). Trx1 and Trx3 expression are enhanced upon H_2O_2 in a Pap1-dependent manner

(Song and Roe 2008); (Kim, Jung et al. 2007). In addition, Trx1, but not Trx3, is necessary to reduce Pap1 leading to a negative feed back loop (se section 5.4.1) (Song and Roe 2008). The ∆*trx1* mutant was found to be sensitive to diverse stresses such as various oxidants, heat and salt. They also exhibited Cys auxotrophy, which can be restored by the addition of Cys to the cells. However, ∆*trx2* mutant was not sensitive (except to paraquat, a superoxide generator). At a more general level, the Trx system participates in modulating the redox state of cell protein sulfidryls and consequently in protein folding (Rietsch and Beckwith 1998; Garcia-Santamarina, Boronat et al. 2011). Similarly, several proteins sensitive to SH oxidation, including Trx1 substrates such as Cdc22, Met16 or Sam1, were identified (Garcia-Santamarina, Boronat et al. 2011).

2.1.2.3. Thioredoxin reductase (Trr)

In 1996, Casso and Beach isolated the Trx reductase-coding gene (*trr1*) as a multicopy suppressor of the *p53*-induced growth inhibition of *S. pombe*. They demonstrated a sensitivity of the deletion strain to oxidant agents (Casso and Beach 1996) and an increase of total GSH (Muller 1996).

Trr is a dimeric enzyme with two Cys residues in its redox active center. It reduces oxidized Trx to the active SH form using NADPH as a cofactor (Holmgren 1989); (Arner and Holmgren 2000; Mustacich and Powis 2000).

Three mammalian Trr isoforms have been found: cytoplasmic (Trr1), mitochondrial (Trr2), and the one which catalyzes the reduction of both Trx and GSSG,called thioredoxin glutathione reductase (Tgr) (Miranda-Vizuete, Damdimopoulos et al. 1999; Sun, Kirnarsky et al. 2001); (Rundlof, Carlsten et al. 2000). Mammals Trr has a higher molecular weight than *E.* coli and yeast enzymes (Holmgren 1985);(Luthman and Holmgren 1982). The latter ones carry the same domain and particularly selenocysteine in the C-terminal active center (Glycine–Cys–SelenoCys–Glycine– COOH). This residue is very important for the catalytic activity of Trr, because its impaired function leads to inactivation (Gorlatov and Stadtman 1998; Zhong, Arner et al. 1998). Moreover, Trr reduces not only Trx from different species but also many non-disulfide substrates, such as selenite (Kumar, Bjornstedt et al. 1992), lipid hydroperoxides (Bjornstedt, Hamberg et al. 1995), and H_2O_2 (Zhong and Holmgren 2000).

A Δ*trr1* strain was also isolated in the Sipiczki´s laboratory due to its implication in multidrug resistance; *caf4 (trr1)-83*, can trigger accumulation or constitutive nuclear localization of Pap1 (see below) (Benko, Sipiczki et al. 1998; Benko, Fenyvesvolgyi et al. 2004; Vivancos, Castillo et al. 2004).

S. cerevisiae has cytoplasmic TRR1 and mitochondrial TRR2. This system can prevent the accumulation of GSSG in cells lacking GLR1 (Tan, Greetham et al. 2010). *TRR1* expression is up regulated by YAP1 and SKN7 (see section 4.2.1). TRR2 contains specific N-terminal domain with signals characteristics for transport into mitochondria (Pedrajas, Kosmidou et al. 1999); and oxidized Prx1, mitochondria contain a single 1-Cys PRX1, is glutathionylated and reduced by TRR2. The *TRR2* mutants are sensitive to H_2O_2. However, a mitochondrial *TRX3* mutant shows no increase in the sensitivity to H_2O_2 (Rundlof, Carlsten et al. 2000). On the contrary, only one Trr has been identified in *S. pombe*; it shows 69% homology to TRR1 and 73% homology to TRR2 from the budding yeast (Pedrajas, Kosmidou et al. 1999). Trr1 is necessary for Pap1 reduction and it is induced by H_2O_2 in a Pap1 and Prr1- dependent manner (see section 5.4.1). Furthermore, Trr1 is crucial in tumor cells as a potential molecular target for enhancing the cytotoxicity of anticancer agents that induce oxidative stress (Smart, Ortiz et al. 2004).

2.1.3. Glutaredoxins (Grx)

The formation of mixed disulfides from protein thiols and GSH is a key event in regulation of cell response to oxidative stress (Fig. 6). Grx is the main enzyme catalyzing both production and reduction of mixed disulfides (Gravina and Mieyal 1993).

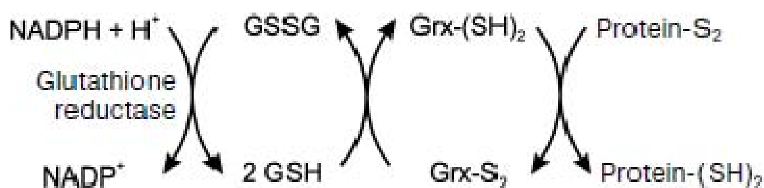


Figure 6. Scheme of reactions catalyzed by the Grx-dependent system (Kalinina, Chernov et al. 2008).

2.1.3.1. Classification and mechanism of Grx

Grx are small heat-stable oxidoreductases, with low molecular masses (9-14 KDa).Thesewere firstly discovered in *E. coli* as GSH-dependent hydrogen donors for ribonucleotide reductase (Holmgren 1989). They are found in virtually all taxonomic groups including prokaryotes and eukaryotes (from yeasts to plants and humans) (Fernandes and Holmgren 2004). Their structurebelong to Grx superfamily, and are responsible for reduction of protein disulfides or GSH protein mixed disulfides, indicating that Grx play an important role in cellular redox dependent processes (Collinson, Wheeler et al. 2002). They also have multiple roles in many cellular processes including protein folding and regulation, reduction of dehydroascorbate, protection against ROS and sulfur metabolism (Holmgren 1989).

Typical cellular Grxs contain a conserved dithiol active site (Cys-Proline-Tyrosine-Cys), a certain surface hydrophobic area and a binding site for GSH (Nordstrand, slund et al. 1999). In the Grx-dependent system, the electron transfer occurs from NADPH-dependent Glr1 to the GSSG to form GSH, which, in turn, reduces the oxidized Grx (Bushweller, Billeter et al. 1994). In the dithiol Grx, the N-terminal Cys residue and the C-terminal of the active center act in the reduction of the substrate.

However, only in the monothiol Grx promotes the reduction of mixed disulfides and glutathionylated proteins and this process is called deglutathionylation. As a consequence, Grx only uses the N-terminal Cys residue (Nordstrand, Aslund et al. 1999). The absence of the second Cys in their redox-active site results in the inability of monocysteinic proteins to perform complete reduction of a disulfide group (Fig. 7).

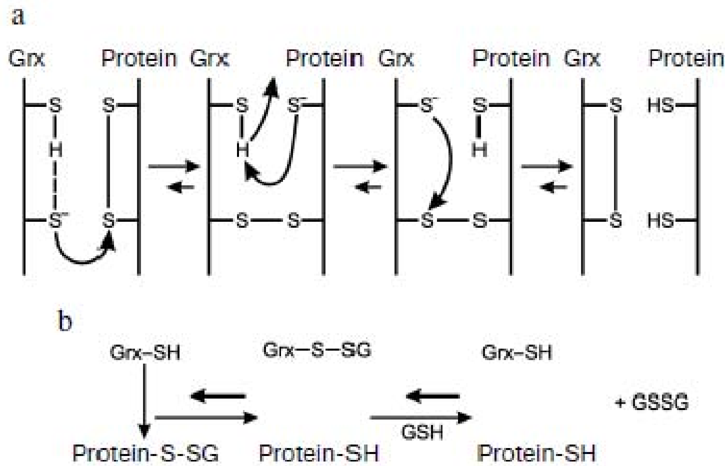


Figure 7. Dithiol (a) and monothiol (b) catalytic mechanisms of Grx (Kalinina, Chernov et al. 2008).

Three Grx isoforms have been found in mammals, including humans: cytosolic Grx1,containing the Cys-Pro-Tyr-Cys motif in its active center and two mitochondrial, Grx2 and Grx5, whose active centers contain the sequences Cys-Ser-Tyr-Cys and Cys-Gly-Phe-Ser respectively (Lundberg, Johansson et al. 2001); (Wingert, Galloway et al. 2005). Grx1 and Grx2 are dithiol isoforms and contain two Cys residues in their active centers, whereas Grx5 has only one Cys residue.

Two yeast genes encode typical dithiol Grxs in *S. cerevisiae*, designated *GRX1* and *GRX2*, which share identity and similarity with those from bacterial and mammalian species (Luikenhuis, Perrone et al. 1998). While both GRX1 and GRX2 are present in the cytosol, a fraction of GRX2 is also located at mitochondria. This happends because ofalternative translation initiation from two in frame ATG sites (Porras, Padilla et al. 2006). Studies concerning the respective mutants have shown that GRX1 confers protection against the $O_2^{.-}$ and hydroperoxides, while GRX2 is specialized in protection against hydroperoxides, indicating that their functions are only partially overlapping. This difference in oxidant sensitivity may reflect differences in the substrate proteins regulated by

GRX1 and GRX2, or in their ability to detoxify ROS-mediated damage (Luikenhuis, Perrone et al. 1998; Grant, Luikenhuis et al. 2000).

Differences in the expression of *GRX1* and *GRX2* have also been further described indicating that the two GRX isoforms may play distinct roles during normal growth and stress conditions (Grant, Luikenhuis et al. 2000). In addition, six related GRX have also been identified (GRX3-8) which are conserved throughout evolution from bacterial to mammalian species (Rodriguez-Manzaneque, Ros et al. 1999). They have been found in different subcellular compartments including nuclear ones (GRX3-4). They play an essential role in intracellular iron trafficking (Muhlenhoff, Molik et al. 2010) and in the mitochondrial matrix (GRX5) which is required for mitochondrial [4Fe-4S] cluster assembly (Rodriguez-Manzaneque, Tamarit et al. 2002). These GRX-like proteins differ from classical GRX because they contain a single Cys residue at their putative active sites. Also, GRX6-7 are located in the endoplasmic reticulum and Golgi, which are thought to function in sulfhydryl regulation in the early secretory pathway during stress conditions (Izquierdo, Casas et al. 2008; Mesecke, Mittler et al. 2008; Eckers, Bien et al. 2009). GRX8 is a dithiol GRX but does not play a role in the oxidative stress response (Eckers, Bien et al. 2009). GRX3, GRX4 and GRX5 is linked to Fe assimilation and FeS cluster formation, overall, these monothiol GRXs of *S. cerevisiae* play an important role in iron homeostasis (Toledano, Kumar et al. 2007); the function of.

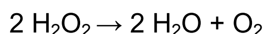
The *S. pombe* genome code for two dithiol Grxs: Grx1 in the cytosol and Grx2 in mitochondria (Chung, Kim et al. 2004).Three putative monothiol Grxs: Grx3 (nucleus and ER), Grx4 (nucleus and essential its deletion) and Grx5 (mitochondria) (Chung, Kim et al. 2005). Grx1 and Grx2 perform distinct roles mediating GSH-dependent redox homeostasis. *grx1* gene expression is induced dramatically by various stressful conditions including oxidants, salt, heat, and stationary growth and also its deletion is sensitive to H_2O_2. Meanwhile the *grx2* expression is constitutive and cells lacking it are sensitive to paraquat and superoxide generator (Chung, Kim et al. 2004). One of the three monothiol Grxs, the Grx3, response to different stresses (Moon, Lim et al. 2005). In addition, Grx4 is critically required for aerobic growth in *S. pombe* and plays an essential role in inhibiting Fep1 function under conditions of iron deficiency (Grx4 is a

binding partner of Fep1) (Jbel, Mercier et al. 2011). *S. pombe* Grx5 may play a crucial role in responses to nitrosative and osmotic stresses in a Pap1 dependent manner (Kim, Park et al. 2005). It also plays a role in the Fe/S assembly process through interaction with Fe/S scaffold proteins Isa1 and Isa2 in mitochondria (Chung, Kim et al. 2005). The *grx2 grx5* double mutant is not lethal, in contrast to the synthetic lethal phenotype of the same mutant in *S. cerevisiae.*

2.2. Enzymatic ROS detoxification

2.2.1. Catalases

Catalases are ubiquitous heme-containing enzymes that catalyze the dismutation of H_2O_2 into H_2O and O_2:



$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

It has been recently published an increase in ctt1 expression in an invertase mutant which is defective in glucose signaling pathway (*ird11*). This could reflect a possible link between oxidative stress response and glucose signaling, in a manner different than that caused by glucose deprivation (Palabiyik, Kig et al. 2012).

S. cerevisiae has two catalases; catalase A encoded by *CTA1*, which localizes at peroxisomes, and catalase T encoded by *CTT1*, which is cytosolic. CTA1 is supposedly involved in the detoxification of H_2O_2 generated by acyl-CoA oxidase during fatty acid beta-oxidation at peroxisomes (Hiltunen, Mursula et al. 2003). On the other hand, CTT1 is thought to play a more general role as an antioxidant during exposure to oxidative stress, since *CTT1* expression is induced by various stress conditions including heat, osmotic, starvation and H_2O_2 stress (Martinez-Pastor, Marchler et al. 1996). Surprisingly, yeast mutants lacking both catalases are unaffected by H_2O_2 tolerance during the exponential phase of growth (Izawa, Inoue et al. 1996).

S. pombe has just one catalase called Ctt1, which is cytosolic and H_2O_2 dependent expression by Pap1 and Prr1 (see below) (Ohmiya, Kato et al. 1999) and also the Sty1/Atf1 pathways (Degols and Russell 1997; Nakagawa, Yamada et al. 2000; Soto, Beltran et al. 2002). The deletion

strain has an increased susceptibility to H_2O_2 but it shows no growth defect in medium without H_2O_2. This suggests that the level of H_2O_2 generated, *in vivo,* in aerobically growing cells, is low and does not affect the growth or the mutation rate of cells lacking this H_2O_2 detoxification enzyme. Ctt1 is not able to detoxify low levels of H_2O_2 due to its low Km. Moreover, its catalytic cycle requires the interaction of two molecules of H_2O_2 within its active center (Gutteridge and Halliwell 2000). *S. pombe* cells overexpressing catalase activity showed increased resistance to H_2O_2 similar to the wild type strain (Mutoh, Nakagawa et al. 1999)

2.2.2. GSH peroxidases (Gpx)

Gpx reduces H_2O_2 and organic peroxides and require GSH as an electron donor. Then, GSSG is reduced by GSH reductase using nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing power (Fig. 8).

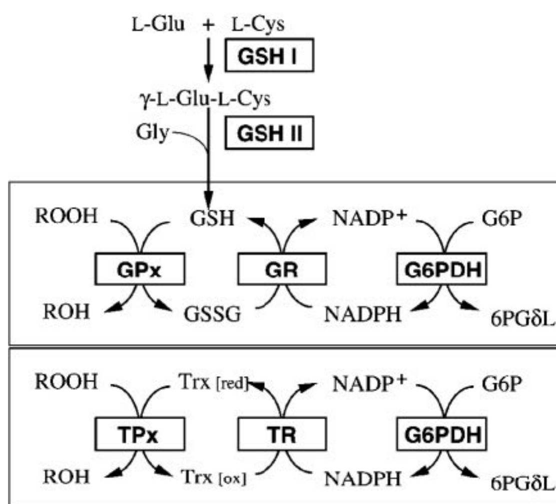


Figure 8. Catalytic cycle of GPx and TPx reaction (Inoue, Matsuda et al. 1999).

The Gpx of yeast has significant homology (36% identity) to mammalian GSH peroxidases (Inoue, Matsuda et al. 1999). The greatest sequence homology to them is found in the region around their active sites (Kho, Lee et al. 2008).

In mammals, there are two Gpx isotypes, with either selenocysteine or Cys as an essential residue in their active site. In contrast, *S. cerevisiae* contains only Cys.

S. cerevisiae contains three, GPX1, GPX2 and GPX3, which use reduced TRX rather than GSH. Certain peroxidases can serve as a primary oxidant receptor to convey oxidation to secondary oxidant target proteins; the best example is the interaction between GPX3 and YAP1. The *GPX3* mutant is hypersensitive to peroxides, whereas null mutants of *GPX1* and *GPX2* did not show any obvious phenotypes (Inoue, Matsuda et al. 1999).

In *S. pombe,* one Gpx (SPBC32F12.03c) has been identified. Gpx acted as a Trx-dependent peroxidase, especially in stationary phase. It was crucial to the long-term survival of the fission yeast (Lee, Song et al. 2008) and it eliminates hydrogen peroxide using reduced Trx. In addition, Gpx possesses a weak molecular chaperone activity.

2.2.3. Peroxiredoxins (Prx)

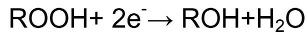
Prx are a ubiquitous family of antioxidant enzymes that have been identified in eubacteria, archaea, yeast, algae, higher plants and animals (Dietz 2003; Wood, Schroder et al. 2003). They were identified as a group of peroxidase enzymes.Thesecatalyze the reduction of H_2O_2, alkyl hydroperoxides and peroxy nitrite into water, the corresponding alcohol or nitrite, respectively with the presence of Trx, Trr1 and NADPH (Nogoceke, Gommel et al. 1997; Bryk, Griffin et al. 2000).

Prxs are localized in the cytosol, mitochondria, chloroplasts, peroxisomes, nucleus and membrane. The Prx Ahpc of *E. coli* is one of the ten most abundant proteins in this cell type. Mammalian PrxII is the third most abundant protein in erythrocytes (Low, Hampton et al. 2008) ranging from 0.1 to 0.8% of soluble protein in mammals (Chae, Kim et al. 1999).In addition to its abundance, Prx have high affinity for the substrate, although moderate catalytic efficiency (Hofmann, Hecht et al. 2002).

Prx have multiple roles in stress protection or antioxidant function, molecular chaperones and regulating H_2O_2-mediated signal transduction (Wood, Poole et al. 2003).

2.2.3.1. Classification and mechanism of Prx

Peroxidases share the same mechanism; an N-terminal activated Cys (the peroxidative Cys, C_P) is oxidized to a SOH by the peroxide substrate (H_2O_2, peroxynitrite, organic hydroperoxides), reducing to the alcohol or water (in the case of H_2O_2). Then, it can be reduced and regenerated by different electron donors, such as Trx, glutaredoxin, GSH, cyclophilin or tryparedoxin (Ellis and Poole 1997);(Ellis and Poole 1997).



$$ROOH + 2e^- \rightarrow ROH + H_2O$$

According to the recycling step there are three types of Prxs.

The first one, typical 2-Cys, these Prxs are homodimeric and contain two redox active Cys residues required for enzyme activity (Chae, Uhm et al. 1994). The C_P of one subunit is oxidized to a SOH, which condenses with the resolving Cys (C_R) from the other subunit to form an intermolecular disulfide between them during the catalytic cycle. This disulfide is then reduced by Trx (Fig. 9) (Chae and Rhee 1994). However, AhpF reduces AhpC. For example, AhpC from *S. typhimurium*, TSA1 of S*. cerevisiae*, Tpx1 of *S. pombe* and human PrxI-IV (Hofmann, Hecht et al. 2002).

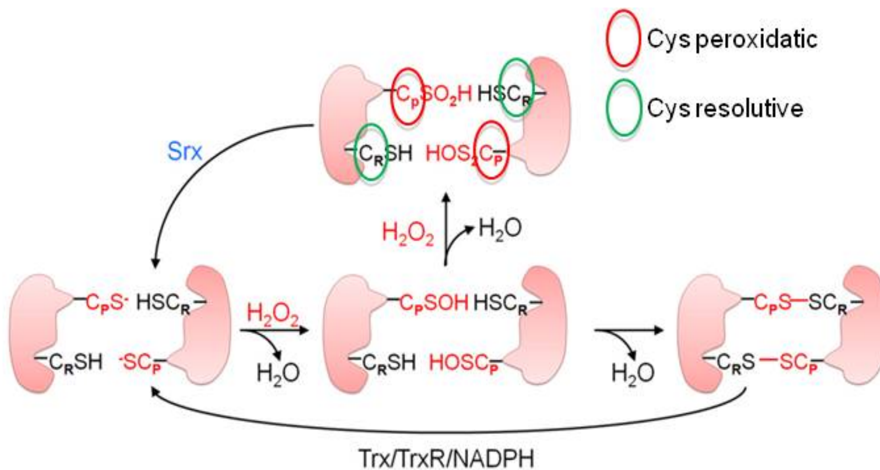


Figure 9. The reaction mechanism of ttypical 2-Cys Prx (Luthman and Holmgren 1982).

Prxs of the second one class, atypical 2-Cys which also require two Cys residues for catalysis, although only the N-terminal one is conserved, and an intra-molecular disulfide between both residues of the same monomer is formed during substrate reduction (Fig. 10). The human PrxV belongs to this group (Seo, Kang et al. 2000).

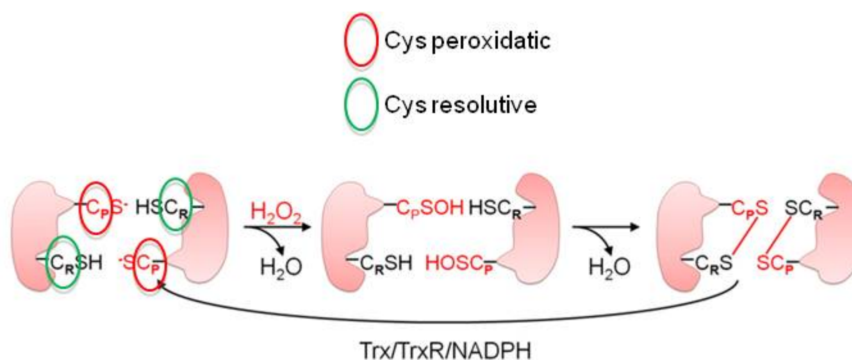


Figure 10. Attypical 2-Cys Prx (Luthman and Holmgren 1982).

And with 1-Cys PRXs, such as the PrxVI of humans that is monomeric and employs a single active site Cys (Fig. 11). H_2O_2 leads to a SOH form which needs to be recycled by GSH. These Prxs cannot form a disulfide bond with other Cys residue because there has only one (Kang *et al.,* 1998).

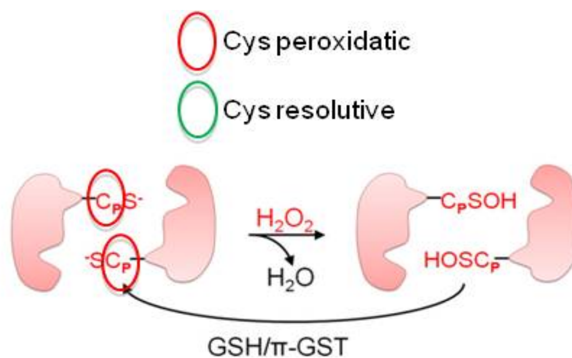


Figure 11. 1-Cys Prx (Luthman and Holmgren 1982).

2.2.3.2. Reversible inactivation and reactivation of 2-Cys PRx enzymes

There are studies with 2-Cys Prxs, mainly PrxI, that indicate that C_P-SOH intermediate is occasionally further oxidized to SO_2H before it is able to form a disulfide with C_R-SH. The SO_2H form appears at high levels of H_2O_2 resulting in inactivation of Prx (Chae, Kim et al. 1993; Yang, Kang et al. 2002). SH oxidation to SO_2H is an irreversible step, with the exception of 2-Cys Prx which is reversible (Woo, Chae et al. 2003; Woo, Kang et al. 2003; Woo, Jeong et al. 2005);(Chevallet, Wagner et al. 2003). Toledano's laboratory identified the enzyme responsible for the reduction of sulfinylated Prx, called designated sulfiredoxin (Srx) (Woo, Jeong et al. 2005)Woo, Jeong et al. 2005). This process requires ATP for hydrolysis, Mg2+, and a SH as an electron donor (Biteau, Labarre et al. 2003). The enzyme sulfiredoxin (Srx) was initially identified because the deletion causes a decrease of tolerance to H_2O_2 and it has a huge expression by H_2O_2.

Srx defines a conserved protein family in lower and higher eukaryotes. All of them share a conserved Cys domain. Srx specifically binds to and reduces the SO_2H forms of 2-Cys Prx isoforms but not those of other proteins exemplified by atypical 2-Cys Prx, 1-Cys Prx, and GAPDH. This occurs because they do not contain the C-terminal GGLG tail of their eukaryotic counterparts and also because prokaryotic cells do not contain Srx (Biteau, Labarre et al. 2003).

Furthermore, the Prx are classified as ‘‘robust’’ Prx or ‘‘sensitive’’ Prx, based on their tendency to be inactivated under high H_2O_2 treatment. This sensitivity to inactivation by H_2O_2 correlates with two amino acid sequence motifs from eukaryotic organisms (Hall, Nelson et al. 2011). Sensitive Prxs, such as Tpx1, have two sensitivity-related motifs: GGLG motif and YF motif, which allow the formation of the SO_2H, form (Wood, Poole et al. 2003). They are thought to act as a molecular switch for stress-related signaling that is influenced by a change in peroxide level (Yuan, Meng et al. 2011). In support of the role of the C-terminal tail, *S. pombe* 2-Cys Prx enzymes show no sensitivity to over oxidation when its C-terminal tail is truncated (Koo, Lee et al. 2002).

2.2.3.3. Other functions of Prxs

In addition to scavenging peroxides, some Prxs also function as regulators of redox-mediated signal transduction in some eukaryotes (Horling, Lamkemeyer et al. 2003; Veal, Findlay et al. 2004). It has also been suggested that Prxs can act as molecular chaperones in a higher-order complex after heat shock (Jang, Lee et al. 2004);(Chuang, Wu et al. 2006).

The redox state of the catalytic Cys is a critical factor that determines their dimer-decamer equilibrium. The reduced enzymes favor decameric forms and the disulfide enzymes are mainly in dimeric forms (Wood, Poole et al. 2002). Decameric forms exhibit higher peroxidase activity than dimeric forms (Chauhan and Mande 2001). Thus, the redox-dependent oligomerization can be a mechanism for the regulation of 2-Cys Prx enzymes as has been previously suggested (Wood, Poole et al. 2003).

2.2.4. Superoxide dismutases (SODs)

SODs are ubiquitous antioxidants, which differ in their intracellular location and metal cofactor requirements in different organisms. They have an antioxidant function by catalyzing the disproportionation of $O_2^{\cdot -}$ to H_2O_2, which can then be reduced to water by catalases or peroxidases.

The SOD-catalyzed dismutation of superoxide may be written with the following half-reactions:

- $M^{(n+1)+}\text{-SOD} + O_2^{-} \rightarrow M^{n+}\text{-SOD} + O_2$
- $M^{n+}\text{-SOD} + O_2^{-} + 2H^{+} \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2$.

where M = Cu (n=1) ; Mn (n=2) ; Fe (n=2) ; Ni (n=2).
In this reaction the oxidation state of the metal cation oscillates between n and n+1.

The enzyme activity is dependent on redox cycling of the bound metal cofactor. This means that SOD1 activity needs an intermolecular disulfide bond. The absence of the intermolecular disulfide bond in SOD1 facilitates protein aggregation (Sturtz, Diekert et al. 2001). The maturation of SOD1

requires the copper chaperone Ccs1, which also participates in copper delivery to the SOD1 apoprotein (Furukawa, Torres et al. 2004).

S. pombe and *S. cerevisiae* cells, in common with other eukaryotes, contain two intracellular SODs, a cytoplasmic Cu, Zn-SOD (SOD1) which also localizes in mitochondrial inner membrane space. It is thought to function in the detoxification of superoxide generated from respiration (Sturtz, Diekert et al. 2001) and a mitochondrial matrix manganese-containing SOD (MnSOD) (SOD2) which appear to play distinct roles during oxidative stress conditions (Yang, Cobine et al. 2006).

SOD1 deleted cells exhibit a variety of phenotypes including poor growth in respiratory conditions, hypersensitive against externally-added oxidants (such as paraquat or menadione), loss of viability in stationary phase and also display a number of oxidative stress-related phenotypes including vacuole damage and increased free iron concentrations (Schmidt, Kunst et al. 2000). Additionally, aerobic inactivation of [4Fe-4S] cluster enzymes in *SOD1* mutant results in auxotrophies for methionine and leucine (Slekar, Kosman et al. 1996);(Wallace, Liou et al. 2004).

SOD2 acts in the mitochondrial matrix and is particularly required during stationary phase growth which may be linked to superoxide generation from mitochondrial respiration (Longo, Gralla et al. 1996). Null *SOD2* mutant is hypersensitive to hyperoxia and is unable to grow under respiratory conditions. These mutants are less affected in growth and stress sensitivity compared with *SOD1* mutant but do show a reduced ability to grow under respiratory conditions (van Loon, Pesold-Hurt et al. 1986).

2.2.5. Glutathione transferases (Gsts)

Glutathione S-transferases (Gsts) are evolutionarily conserved enzymes, which are important in the detoxification of many xenobiotic compounds. Gsts play an important role in protecting cells from oxidative stress by detoxifying some of the secondary ROS produced when primary ROS react with cellular constituents.

In higher eukaryotes, Gsts are known to detoxify lipid hydroperoxides to their corresponding alcohols and water (Zhao, Singhal et al. 1999). Rat

hepatic Gst has been shown to catalyze the metabolism of ethanol to fatty acid ethyl esters (Bora, Spilburg et al. 1989).

Two genes *GTT1* and *GTT2* have been identified in *S. cerevisiae* (Choi, Lou et al. 1998). Strains deleted for *GTT1* and *GTT2* are viable and show no increased sensitivity to ROS, indicating that they are not required for protection against oxidative stress. These enzymes catalyze the conjugation of GSH to electrophilic substrates, producing compounds that are generally less reactive and more soluble. This facilitates GSH conjugates transportation to the vacuole by the YCF1 GS-X pump (Salinas and Wong 1999). The *GTT1 GTT2* mutant is sensitive to heat stress during stationary phase growth but, surprisingly, given the sensitivity of YCF1 mutants to the GST substrate, 1- chloro-24-dinitrobenzene (CDNB), is unaffected in resistance to CDNB (Li, Zhen et al. 1995). This may be explained by functional redundancy with GRX since yeast GRXs (GRX1 and GRX2) is active as GSTs with substrates such as CDNB (Collinson and Grant 2003). Mutant analysis have confirmed that GRX1 and GRX2 have an overlapping function with GTT1 and GTT2. It is due to simultaneous loss of all four genes substantially reduces the cellular GST activity and causes sensitivity to stress conditions, including exposure to xenobiotics, heat and oxidants (Collinson and Grant 2003; Collinson, Wheeler et al. 2002).

Three genes encoding Gsts in *S. pombe* are involved in oxidative stress response (Kim, Park et al. 2001; Cho, Park et al. 2002; Shin, Park et al. 2002). Gst1 and Gst2 encode proteins that are 79% identical, whereas the protein encoded by *gst3* gene is only 14% identical with GST1 and GST2. The identification of such a closely related gene pair suggests that the proteins have related functions. Gst1 and Gst2 are both distributed throughout the cell; however, Gst3 is excluded from the nucleus. The expression of the *S. pombe gst1 and gst2* genes is regulated by the Pap1-dependent signal pathway upon H_2O_2. However, in untreated conditions is regulated via mitogen activated protein kinase (MAPK) Sty1; because the basal levels of *gst1 and gst2* genes are up-regualted in cells lacking Sty1 (Veal, Toone et al. 2002). On the other hand, *gst3* is regulated in a Pap1-independent manner and Sty1-dependent manner (Veal, Toone et al. 2002).

The *gst1* mutant showed similar resistance to H_2O_2 than wild-type cells, consistent with it making a minor contribution to cellular GST activity. Although the *gst1 gst2* mutant had similar levels of Gst activity to the *gst2* single mutant, the *gst1 gst2* mutant was more sensitive to tBOOH and H_2O_2. This suggests that Gst1 may protect against oxidative stress by a mechanism that is independent of its GST activity (Hayes and McLellan 1999).

3. CYS HOMEOSTASIS

Cys lies at the interface between ROS targets of toxicity and ROS signaling, since the chemical properties that make specific SH redox-sensitive also predispose them to oxidative damage by reactive oxygen or nitrogen species during stress (Held and Gibson 2012).

Disulfide bonds formed between pairs of Cys are essential for the folding, activity and stability of many proteins exported from the cytoplasm. Based on *in vitro* studies of protein folding, in the 1960s, Anfinsen and his coworkers showed that the formation of disulfide bonds in proteins can occur spontaneously *in vitro*, using a model of reduced ribonuclease A and only in the presence of O_2 (Anfinsen, Haber et al. 1961). Firstly, it seemed that O_2 or other strong oxidant (e.g., GSSG) could be sufficient to promote disulfide bond formation in proteins *in vivo,* but this was not the case. It was not until thirty years later when genetic studies in bacteria and yeast revealed the importance of enzymatic systems in extra cytoplasmic compartments for the catalyzed formation of disulfide bonds in proteins (Kadokura 2003; Kadokura, Katzen et al. 2003).

Disulfide bonds are post-translational covalent modifications that occur between Cys residues (Sideris and Tokatlidis 2010),they enhance protein stability and regulate redox-dependent functions. In both prokaryotes and eukaryotes, proteins are compartmentalized thiol-disulfide oxidoreductases enzymes that use their redox active Cys to transfer electrons in oxidative and reductive pathways, and that can catalyze disulfide bond formation (Kadokura 2003).

3.1. Interplay between the thioredoxin and glutaredoxin systems

The major function of Trxs and Grxs systems are thought to be thecontrol of SH homeostasis by reduction of free SH_2 in target proteins. Although exceptions exist, both are reduced by different mechanisms. Grxs are reduced non enzymatically by GSH, whereas Trxs are reduced enzymatically by Trr (Holmgren 1985). The two enzymes are functionally interrelated and complement to each other, being involved in control over cell redox balance.

GSH is the major redox buffer in eukaryotic cells, and the redox potential of the GSSG/2GSH couple is considered as a measure of the redox cellular situation. GSH is obviously required as a substrate for Gpxs, Grxs and Gsts, but its levels are also influenced by the Trx system (Trotter and Grant 2003), evidencing the interplay between the Grx and Trx systems in yeast cells. In this emerging complex interplay between Trx and Grx glutathionylation can be included ; there are a lot of proteins identified as established or putative targets of Trx or Grx but also those undergoing glutathionylation. As an example, the transcription factors c-Jun and NF-κB are Trx targets and are glutathionylated (Michelet, Zaffagnini et al. 2006).

Also, in *E. coli*, Grx4 has been reported to act as a substrate for *E. coli* Trr after GSSG oxidation (Fernandes, Fladvad et al. 2005); in *S. cerevisiae,* GRX5 can be more efficiently reduced by Trx than by GSH (Tamarit, Belli et al. 2003).

The first indication of a functional link between the Trx and GSH/Grx systems, in yeast, came from the substitution of each other. Mutants lacking both Trx1 and Trx2 are unviable in the absence of Glr1, indicating that Trx mutants require the ability to reduce GSH for survival. A further overlap between the two systems is evident because the loss of both Trx1 and Trx2 results in elevated levels of GSSG, indicating a link between the Trx system and the redox status of GSH in the cell (Muller 1996).
It should be noted that Grx, like Trx plays an important role in development of drug resistance of tumor cells to pharmaceuticals, possessing pro-oxidant activity, by enhancement of the antioxidant defense system (Chen, Kamat et al. 2002; Kalinina, Chernov et al. 2007).

3.2. Cytosol: a reducing compartment

The reduced state inside the cell is due to the role of Trxs and Grxs. The major ubiquitous disulfide reductase is responsible for maintaining proteins in their reduced state, which is reduced by Trr using NADPH (Holmgren 1985).

It is very difficult to predict which Cys are oxidized, the type of modifications that are present, and the stability of the SH modification. Redox modifications are governed by complex kinetics and are dependent on a) which cellular compartment the protein is located in, b) which ROS are present, and c) the levels and localization of various antioxidant enzymes (Winterbourn 2008; Winterbourn and Hampton 2008). Moreover, antioxidant enzymes are known to act as oxidants under certain conditions (Delaunay, Pflieger et al. 2002; Vivancos, Castillo et al. 2005). Two chemical properties that primarily govern redox-activity are solvent accessibility and low SH pKa. The pKa of the Cys sulfidryl is heavily influenced by the local milieu and nearby amino acids. Therefore, the SH redox-activity is in part an intrinsic property of the protein structure. However, the environment also influences the reactivity via pH changes. Changing the thiol pKa affects the equilibrium constant between the SH and the redox-active deprotonated thiolate anion ($Cys\text{-}S^-$). In a cell, CyspKa can vary over 6 orders of magnitude. Typically, the pKa of a Cys sulfhydryl is ~8.6, yet it can be as low as 3.5 (Gan and Wells 1987; Grauschopf, Winther et al. 1995) or as high as 10 in some cases. The wide range of factors that contribute to a Cys´s reactivity and susceptibility to oxidation therefore are major determinants of the specificity of redox reactions in cellular systems.

3.3. Compartments oxidizing

The formation of disulfide bonds in specific compartments is catalyzed by proteins, which mediate disulfide exchange reactions. The electron transfer from a molecule with free sulfhydryl group goes to one, which is already disulfide bond. This process consists of four steps: 1) the $Cys\text{-}S^-$ of substrate attack on the disulfide bond of the oxidase, 2)both proteins

form an intermolecular mixed disulfide intermediate, 3) this intermediateis resolved by the attack of the second thiolate anion of the substrate, leading to 4) an oxidized substrate and the reduction of the oxidase (Sideris and Tokatlidis 2010).In prokaryotic cells, the formation of disulfide bonds occurs in the periplasm (see Fig. 12); while in eukaryotic cells SH oxidation pathways occurs in the lumen of the rough endoplasmic reticulum (ER) and in the inter-membrane mitochondrial space (IMS) of mitochondria. They share many similarities in the mechanism and the proteins employed (Sideris and Tokatlidis 2010). In contrast, the cytoplasm displays a network of enzymes and molecules dedicated to the reduction of disulfide bonds as detailed in section 3.2 (Aslund and Beckwith 1999).

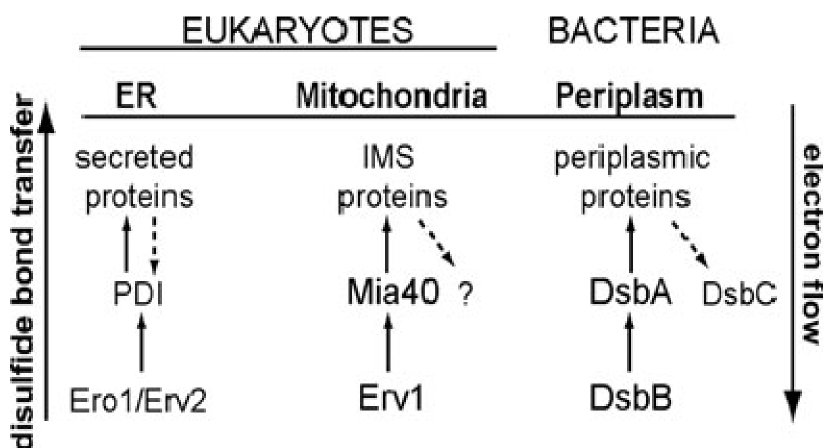


Figure 12. Oxidative pathways in the cell (Sideris and Tokatlidis 2010).

3.3.1. Bacterial periplasm

This is one of the best-characterized systems in the formation of disulfide bonds and is mediated by Dsb proteins (DsbA-DsbC system). Briefly, DsbA and DsbB introduce disulfide bonds in periplasmic proteins; while DsbC and DsbD isomerize some of these disulfide bonds to generate new others. This system consists of four proteins. The bacterial redox pathway is formed by the proteins DsbA and DsbB, which are involved in the

oxidation of periplasmic substrate proteins and the isomerization of these by the proteins DsbC and DsbD (Kadokura, Katzen et al. 2003).

DsbA is a primary oxidant of proteins exported to the cell envelope of *E. coli*. This is reflected by its redox potential (-120mV). It is a monomeric, 21-kDa periplasmic enzyme containing a redox active sequence, Cys30-Pro31-His32-Cys33, embedded in thioredoxin-like fold. DsbA is found exclusively in its oxidized form in cells and this is because of its re-oxidation by the cytoplasmic membrane protein DsbB (Martin, Bardwell et al. 1993)**.**

DsbB (20kDa) has two periplasmic domains, each one with two important Cys, Cys41 and 44 for the first one, and Cys104 and 130 for the second one. There is genetic and biochemical evidence that electron is transfered from the reduced DsbA to the oxidized quinone through the active site Cys of DsbB. There is a mixed disulfide bond between Cys30 of DsbA and Cys104 of DsbB. Both Cys pairs of DsbB are significantly less oxidized with redox potentials of -240mV and -267mV (Kadokura, Katzen et al. 2003).

Another two proteins belong to this system, DsbC and DsbD. DsbC is V-shaped homodimeric molecule (23.4-kDa for each monomer), which has a C-terminal thioredoxin fold with an active CxxC motif and is fundamental as a thiol disulfide isomerase and also has a chaperone activity. It is maintained in the reduced state and this state depends on the cytoplasmic membrane protein DsbD at the expense of Trx and NADPH oxidation in the cytoplasm (Rietsch, Belin et al. 1996). DsbA and DsbC have similar redox potential but with extreme different behaviour. The dimeric form of DsbC serves as a shield against DsbB-oxidation , leaving its active site reduced, which is a prerequisite for reduction by DsbD. DsbD is a 546 amino-acid protein with a three-domain structure: DsbDα (periplasmic domain with two Cys residues, Cys103 and Cys109, which interacts with DsbC), DsbDβ (8 transmembrane segments with Cys 163, required for interaction with Trx1 and Cys 285) and a carboxy-terminal Trx-like domain DsbDγ (it is also two Cys residues, Cys461 and Cys464 (Kadokura, Katzen et al. 2003). Its redox potential is -240mV, so the reduction of DsbC is thermodynamically favored (Collet, Riemer et al.

2002). It is important to mention that the redox environment plays an important role in determining the redox properties.

3.3.2. Eukaryotic cells

The endoplasmic reticulum (ER) is a cellular compartment in which proteins fold oxidatively, and intermembrane mitochondrial space (IMS) is thus the second eukaryotic cellular component found to promote disulfide bond formation in proteins (Gabriel, Milenkovic et al. 2007).

3.3.2.1. The endoplasmic reticulum (ER)

PDI and Ero1 are part of this group (Sevier, Cuozzo et al. 2001). Pdi1p contains four thioredoxin domains. It is used by yeast as a primary catalyst for the oxidation of substrate proteins. The redox state of PDI is determined by the essential protein Ero1. This is a flavin-bound membrane associated to protein Ero1p and it is an essential protein in yeast but not in higher eukaryotes (Pollard, Travers et al. 1998) although its absence does compromise disulfide formation. Ero1 recycles PDI for another round of substrate oxidation to FAD (Sevier, Cuozzo et al. 2001). In fungi, exist also the flavoenzyme Erv2. This protein can complement the lack of Ero1 and serve as a disulfide bond donor to PDI (Sevier and Kaiser 2006). At least four additional pathways have been described, such as Prx4, Gpx7 and Gpx8, quiescin sulfidryl oxidase (QSOXS) and vitamin Kepoxide reductase (VKOR) (Bulleid and Ellgaard 2011).

3.3.2.2. The intermembrane mitochondrial space (IMS)

The mitochondria are defined by four sub compartments: the outer membrane (OM), two IMS, the matrix and the inner membrane (IM) (Sideris and Tokatlidis 2010). This SH oxidizing system consists of two essential proteins, a disulfide carrier Tim40/Mia40 (40kDa) and a Flavin sulfhydryl oxidase Erv1 (Endo, Yamano et al. 2010). Mia40 has six conserved Cys that are essential for its function (Naoe, Ohwa et al. 2004). Moreover, Mia40 is a physiological substrate of the sulfhydryl oxidase Erv1. The precursor protein is imported in its reduced form into the IMS through TOM channel. This protein forms a disulfide bond with oxidized Mia40. Consequently, the precursor protein is oxidized and Mia40 is

reduced. Its reoxidation is achieved by Erv1. Mia40 depletion blocks the import of protein containing small Cys-rich domains. Cells without Erv1 have Mia40 reduced and they show a decrease of imported proteins in IMS (Mesecke, Terziyska et al. 2005).

4. H_2O_2 SENSING AND SIGNALING

4.1. Prokaryotic cells: *E. coli*

The adaptive response of bacterial antioxidant systems toward oxidative stress is coordinated mainly by two well-studied systems and grouped in two regulons: SoxR and OxyR. The first responds to the $O_2^{\cdot -}$, while the second responds to H_2O_2 (Aslund, Zheng et al. 1999).

4.1.1. OxyR

Christman et al. discovered the oxyR gene in a selection for *Salmonella* mutants that were very resistant to H_2O_2 (Christman, Morgan et al. 1985). OxyR belongs to LysR family of transcription factors and possesses a helix-turn-helix (HTH) DNA binding motif in its terminal domain (Zheng and Storz 2000). This polypeptide has a mass of 34 kD, and forms tetramers in its oxidized form (Choi, Kim et al. 2001).

In response to H_2O_2, the transcriptional activation of OxyR depends on two Cys residues (Cys199 and Cys208). The sulfur residue of Cys199 is oxidized to SOH followed by oxidation of Cys 208 with the consequent formation of intermolecular disulfide bond in OxyR (Zheng, Aslund et al. 1998). The S-S is for only a finite period of time with a half-time of 30s and responds to low doses, as submicromolar doses or nano molars of H_2O_2 (Gonzalez-Flecha and Demple 1997). OxyR is inactivated by GSH-glutaredoxin through reduction enzymatic of its S-S (see Fig. 13 a) (Aslund, Zheng et al. 1999).

The oxidized form of OxyR binds to near the −35 region of at least 20 regulon members (Hidalgo and Demple 1997) and it induces their transcription trough direct contact with RNA polymerase (Tao, Fujita et al. 1993). In this state, OxyR recognizes four ATAG elements with 10 bp spacing. The spacing of these elements allows to four subunits of OxyR to bind to four adjacent major grooves on one side of the DNA helix. On the

contrary, reduced OxyR, binds to just two major grooves (Toledano, Kullik et al. 1994). The oxyR regulon induces among others genes, *dps* (a DNA- and iron-binding protein), *gorA* (GSH reductase), *grxA* (glutaredoxin), *katG* (peroxidase), *ahpCF* (alkyl hydro peroxide-NADPH oxido-reductase), and *fur* (an iron-binding repressor of iron transport).

Many of these functions have clear roles in antioxidant defense of the cell, such as removal of H_2O_2 by catalase or the protection of DNA from oxidative attack by the Dps protein (Zheng, Aslund et al. 1998; Zheng, Doan et al. 1999). As well as, OxyR participates in protecting against different stresses, such as heat stress (Christman, Morgan et al. 1985), singlet oxygen (Kim, Kim et al. 2002) or lipid peroxidation-mediated cell damage (Yoon, Park et al. 2002) among others.

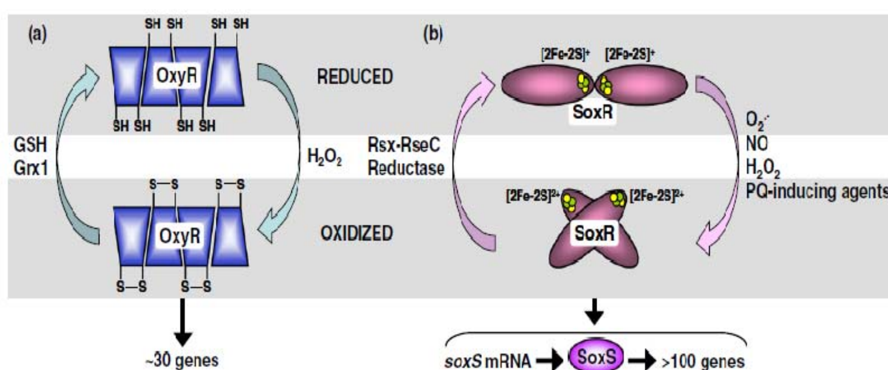


Figure 13. Oxidation/reduction schematic of the major oxidative stress regulators of *E. coli*, OxyR (a) and SoxR (b) (Chiang and Schellhorn 2012).

4.1.2. SoxR

In the mid-70's, Hassan and Fridovich discovered that the MnSOD is strongly induced when *E. coli* is exposed to redox-cycling antibiotics (Hassan and Fridovich 1977). Ten years later, Demple and Weiss labs independently determined that the transcriptional response to superoxide generators is governed by two proteins: SoxR, which is a sensor protein that detects redox stress, and SoxS, a transcriptional activator that positively regulates about two dozen genes around the chromosome.

SoxR is a 17KDa polypeptide with homology to the mercury-dependent MerR regulator of *E. coli* (see Fig. 13 b). It contains a HTH DNA binding motif in N-terminal domain. Moreover, SoxR forms a dimer in solution, each monomer containing a [2Fe–2S] cluster (Hidalgo, Bollinger et al. 1995; Wu, Dunham et al. 1995) and these [2Fe–2S] clusters are essential for the transcriptional activity of SoxR.

The ligands for metal binding in SoxR are only four Cys residues in the polypeptide located in a Cx2CxCx5C sequence near the carboxyl terminus (Bradley, Hidalgo et al. 1997). While both forms of SoxR, reduced and oxidized, binds to the *soxS* promoter, only univalent oxidation of the [2Fe–2S] clusters of SoxR activates *soxS* expression and SoxS dependent gene expression (Hidalgo and Demple 1994), such as *sodA* (SOD), *zwf* (glucose 6-fosfato dehydrogenase), *fpr* (NADPH: flavodoxine oxidorreductase), *fumC* (fumarase C), *acnA* (aconitase, *nfo* (endonuclease IV) and *micF* (a regulatory RNA) (Greenberg, Monach et al. 1990); (Tsaneva and Weiss 1990; Pomposiello and Demple 2001) among others.

Mainly, SoxR is a principal regulator of the superoxide radical (Greenberg, Monach et al. 1990).

4.2. Eukaryotic cells

4.2.1. *Saccharomyces cerevisiae*

4.2.1.1. YAP1

YAP1 is the primary determinant in the antioxidant response. It is a basic region-leucine zipper-containing positive transcriptional regulator. The bZIP: domain is a basic region that mediates sequence specific DNA binding properties and a leucine zipper that is required for the dimerization of two DNA binding regions. YAP1 belongs to AP-1 family (Moye-Rowley, Harshman et al. 1989).

This transcription factor has two Cys rich domains, N-terminal and C-terminal domain with three Cys residues each one. YAP1 subcellular localization is under redox control (Delaunay, Isnard et al. 2000) and the nuclear export receptor Crm1 (Xpo1) is involved in its nuclear

redistribution (Kuge, Jones et al. 1997; Kuge, Toda et al. 1998). YAP1 is in the cytoplasm before stress and goes to the nucleus upon H_2O_2, diamide, and diethylmaleate (DEM) by different mechanisms (Kuge and Jones 1994; Delaunay, Isnard et al. 2000). H_2O_2 stress leads to the formation of four sequential disulfide bonds between Cys within the C terminal Cys rich domain (c-CRD; Cys598, Cys620, Cys629) and the N terminal (n-CRD; Cys303, Cys310, Cys315) (Kuge and Jones 1994) causing conformational changes that prevent the interaction with CRM1 and result in nuclear accumulation of YAP1 (Kuge and Jones 1994; Yan, Lee et al. 1998). The thioredoxin system, TRX1 and TRR1 maintain YAP1 reduced in absence of stress (Carmel-Harel and Storz 2000). As a consequence, the formation of different disulfide bonds in YAP1 is correlated with four possible oxidized forms (Fig. 14) (Okazaki, Tachibana etal.2007)

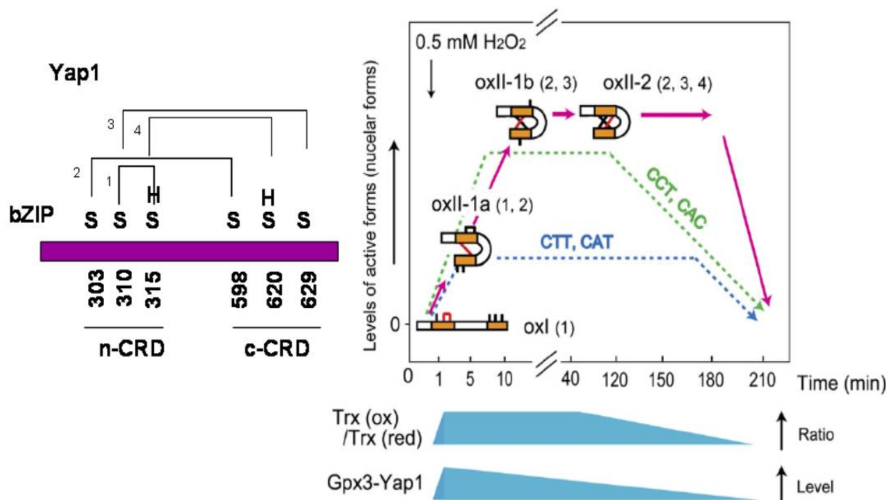


Figure 14: Scheme of different disulfides bonds in YAP1 and the relative levels of oxidized YAP1 and mutant forms of YAP1 that corresponded to each disulfide bond formed in YAP1 (Okazaki, Tachibana et al. 2007).

YAP1 oxidation by H_2O_2 requires the participation of another protein called GPX3, which acts as a sensor and can transfer the signal to YAP1 through a transient mixed disulfide via its Cys 36 and Cys 598 of YAP1 (Fig. 15) (Delaunay, Pflieger et al. 2002).

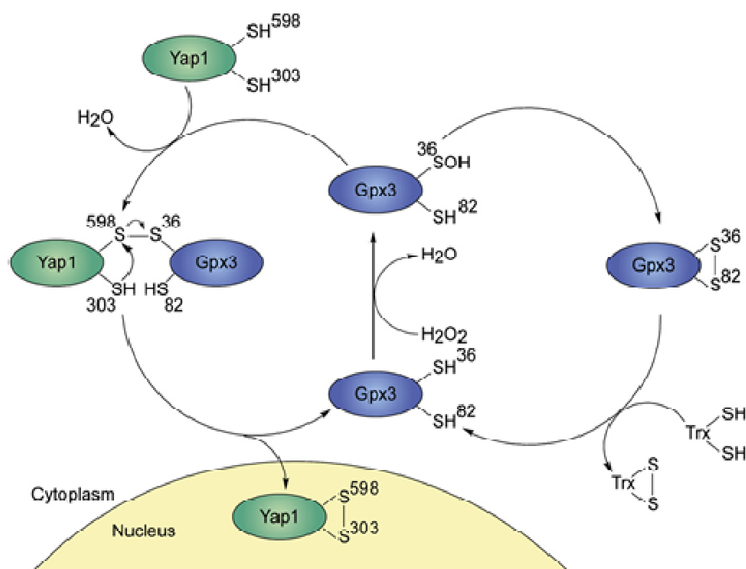


Figure 15: Proposed Molecular Mechanism for the YAP1-GPX3 Redox Relay (Paulsen and Carroll 2009).

It has also described that a protein designated YBP1 (YAP1-binding protein) is crucial for GPX3 - dependent disulfide bond formation in YAP1 in the presence of H_2O_2 (Veal, Ross et al. 2003; Gulshan, Rovinsky et al. 2004). Studies using a mutant allele of *YBP1* often found in laboratory yeast strains called *ybp1-1* (Okazaki, Naganuma et al. 2005); (Veal, Ross et al. 2003) demonstrated that peroxiredoxin TSA1 is capable of folding YAP1, albeit less effectively than the YBP1/GPX3 system (Tachibana, Okazaki et al. 2009). YBP1 represents a limiting component in H_2O_2 resistance, and its elevated doses improve the association of GPX3 and YAP1 in the cytoplasm cellular (Gulshan and Moye-Rowley 2011). In contrast to GPX3, where the C_R is not required, in TSA1 both Cys residues are necessary (Delaunay, Pflieger et al. 2002).

On the contrary, these disulfides bonds, C303 - C598, C310 – C315, C310 - C629 and C315 - C620 are not observed when cells are treated with diamide. The carboxy-terminus of YAP1 is enough for diamide-induced YAP1 nuclear localization.

Yap1 binds to the sequence T (T/G) ACTAA, termed the Yap1 response element (YRE) (Kuge and Jones 1994; Fernandes, Rodrigues-Pousada et al. 1997). Early work established that YAP1 was likely to be a positive regulator of gene expression involved in the response to oxidative stress, inducing transcription of genes such as those coding for *trx1*, *trr1* and *grx1* (Harshman and James 1998); (Drakulic, Temple et al. 2005) and was capable of conferring a multiple or pleiotropic drug resistance phenotype when it was overproduced (Hussain and Lenard 1991); (Leppert, McDevitt et al. 1990). Several groups found that YAP1 was critical for tolerance to oxidants such as H_2O_2 and diamide as well as heavy metals like cadmium leading to be an important determinant of oxidative stress tolerance (Schnell and Entian 1991; Kuge and Jones 1994); (Wemmie, Wu et al. 1994) (Wu and Moye-Rowley 1994).

4.2.1.2. SKN7

SKN7 was originally isolated as a high-copy-number suppressor of a strain defective in β-glycan synthesis (Brown, North et al. 1993). It was found in evolutionarily divergent fungi, whose DNA binding domain is highly similar to heat shock transcription factor Hsf1 (Morgan, Banks et al. 1997; Lee, Godon et al. 1999) which contains a receiver domain characteristic of response regulator of bacteria two component systems (Ohmiya, Kato et al. 1999). The receiver domain contains the aspartic acid residue (Asp 427) required for carrying out the Asp^- based phosphor acceptor function of SKN7p in SLN1-SKN7.

SKN7 can work with HSF1 leading to a possible means of modulating SKN7 transcriptional activity upon heat shock. SKN7 acted downstream of a histidine kinase involved in the osmotic stress response called SLN1 (Li, Ault et al. 1998). This stress response transcription factor is a constitutive nuclear protein and there is no evidence about any changes in the expression of this factor in the presence of oxidants The phosphorylation of the Asp residue is required for activation of genes induced in response to wall stress, then the mutation eliminates osmotic stress tolerance

(Brown, Bussey et al. 1994; Morgan, Bouquin et al. 1995); (Ketela, Brown et al. 1998).

YAP1 was also linked to oxidative stress tolerance by a genetic screen searching for mutations that cause sensitivity to peroxide (Krems, Charizanis et al. 1996). On the contrary, phosphorylation of Asp is not necessary upon oxidative stress (He, Mulford et al. 2009).

In addition, there is a convergence of YAP1 and SKN7 function; these two factors work together and induce the transcription of several antioxidant genes; such as *CTT1*, *SOD1*, *SOD2*, *TRR1*, *TRX2* o *TSA1* (see below) *(Lee, Godon et al. 1999).*

4.2.1.3. Association of SKN7 and YAP1

A likely explanation for the convergence of YAP1 and SKN7 function came from an analysis of transcriptional activation by these factors on promoters involved in thioredoxin homeostasis among others. (Gan 1991; (Muller, Wright et al. 1991); (Pedrajas, Kosmidou et al. 1999). For example, *TRX2* gene is target of both proteins. The loss of either YAP1 or SKN7 alone is sufficient to prevent H_2O_2 induction of TRX2 transcription. But these proteins bind to *TRX2* promoter at different sites (Morgan, Banks et al. 1997).

SKN7 and YAP1, have been shown to co-regulate several stress genes (Li, Zhen et al. 1995). The presence of binding sites for the YAP1 and SKN7 transcription factors in oxidative stress response promoters and also the sensitivity to oxidative stress of the deletion mutants leads to the possibility that these two factors work together (He and Fassler 2005; He, Mulford et al. 2009). They were both described to be important for wild-type tolerance toH_2O_2, but differentially required for cadmium resistance, and to cooperate in the induction of only a subset of H_2O_2-dependent proteins (Lee, Godon et al. 1999). Mutant strains lacking both YAP1 and SKN7 were no more sensitive to H_2O_2 than either single mutant(Krems, Charizanis et al. 1996);(Morgan, Banks et al. 1997) suggesting that these two transcriptional regulators act in the same genetic pathway. Several reports have been focused on the identification of SNK7 and YAP1 DNA binding sites (Fernandes, Rodrigues-Pousada et al. 1997; Toone and Jones 1999; He and Fassler 2005) or in the protein

domains involved in their putative hetero-dimerization. It has been published the importance of the receiver domain of SKN7 and the Cys-rich domains of YAP1 for their interaction. *In vivo* the SKN7-YAP1 complex forms only when conditions permit oxidized YAP1 to accumulate in the nucleus (Mulford and Fassler 2011).

5. OXIDATIVE STRESS RESPONSES IN S. *pombe*

5.1. *S. pombe* as a model organism in the study of stress responses

The yeast known as *Schizosaccharomyces pombe* is an eukaryotic unicellular organism. P. Linder first described it in 1893 after its isolation from an East Africa millet (see Fig. 16). The name has African descent, it derives from the Swahili word for beer, *pombe*. *S. pombe* divides by bipartition, forming a septum at a central position of the cell with a doubling time of 2-4 hours in simple culture conditions (Forsburg 2001), for this reason the organism is also known as fission yeast. The variety of cellular processes that are similar to those occurring in mammals, makes it ideal for the expression of proteins of higher eukaryotes (Holmgren 1989).

Furthermore, the fission yeast provides an ideal eukaryotic model for studying cellular processes because of the genetic and biochemical tractability of the organism and the availability of null mutants lacking non-essential genes. Moreover, the *S. pombe* genome has been fully sequenced. Its genetic material is organized in three chromosomes, containing 5036 genes scattered around 14 megabases (Mb) of total DNA making it the smallest free-living eukaryote (Wood, Poole et al. 2002).

A private Korean consortium generated a *S. pombe* deletion collection. A collection of *S. pombe* mutants deleted in each one of the non-essential genes. We screened a collection of around 2700 haploid mutant strains of this deletion collection for growth inhibition on H_2O_2, caffeine, menadione or respiratory-prone condition. Our aim was to identify new proteins necessary for the proper response of fission yeast to these four types of oxidative stress.

Firstly, using a double screen (for sensitivity both to H_2O_2 and to growth on respiratory–prone conditions), we isolated 51 deletion strains probably sensitive to the intrinsic oxidative stress produced by the high consumption of oxygen (or by the inability to detoxify ROS). 19 of these mutants where deleted in genes coding for mitochondrial components; in particular, 12 mutations affected the electron transport chain (see appendix paper 1 (Zuin, Gabrielli et al. 2008).

Secondly, in the genetic screening of the *S. pombe* deletion collection described before, we also isolated caffeine-sensitive mutants. Out of 98 isolated sensitive strains, we could demonstrate the importance of some oxidative stress pathway components on wild-type tolerance to this drug. In addition we also characterized some caffeine-resistant cells, and we saw that frequently the constitutive activation of oxidative stress pathways had been connected to caffeine tolerance (see below; (Calvo, Gabrielli et al. 2009).

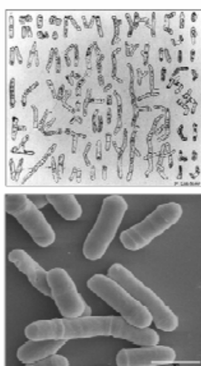


Figure 16. The fission yeast *S. pombe. Upper* panel, Lidner's draw of the isolated strain. Lower panel, scanning electron microscopy image of some *S. pombe* cells.

5.2. General response against stress: map kinase sty1 pathway

The MAPK Sty1 (also known as Phh1/Spc1) gives name to main general pathway against stress in *S. pombe*. Its homologue in mammalian cells is p38 and HOG1 in *C. albicans* and *S. cerevisiae.* Like its homologue p38, Sty1 responds to different stresses, such as high osmolarity, heavy metals, heat shock, cold shock and nutrient limitation, as well as to a variety of agents inducing oxidative stresses, such as H_2O_2, paraquat,

arsenite, alkylating agents and UV radiation (Millar, Buck et al. 1995);(Shiozaki and Russell 1995); (Shiozaki and Russell 1996). Mutants defective in *sty1*, or in other components of the pathway, are hypersensitive to ROS and the other stress agents listed above. This mutant is almost sterile due to a markedly reduced induction of *ste11* (the master regulator of sexual differentiation in fission yeast (Shiozaki and Russell 1996). In addition to this, Δ*sty1* cells have also problems in the G2-M transition, displaying a long cell size phenotype (Shiozaki and Russell 1995). Although Sty1 is not essential for the growth of *S. pombe*, the activation of this pathway is crucial for cell survival after stress.

The activity of the Sty1 MAPK cascade has to be precisely regulated. *S. pombe* achieves this by three different means: the kinases that transmit the environmental signals, some changes in subcellular localization and phosphatases to turn off the pathway.

The MAPK module is composed of the MAPK Sty1, which is activated by the MAPK Kinase Wis1 phosphorylating Sty1 at threonine 171 (Thr-171) and tyrosine 173 (Tyr-173) residues, and two MAPK Kinase Kinase Wak1 (also known as Wis4) and Win1, which are functionally redundant. Their only reported substrate is Wis1, which becomes phosphorylated in serine 469 (Ser-469) and Thr-437 (Shiozaki and Russell 1996);(Wilkinson, Samuels et al. 1996);(Samejima, Mackie et al. 1997); Sty1 is kept dephosphorylated under basal conditions by phosphatase protein Pyp1 and after stress by Pyp2; Tyr-173 is dephosphorylated by these two tyrosine-specific phosphatases (Millar, Buck et al. 1995); (Shiozaki and Russell 1995); (Samejima, Mackie et al. 1997; Shieh, Wilkinson et al. 1997); (Shiozaki and Russell 1997); (Shiozaki 1998) andThr-171 dephosphorylation is achieved by two specific Ser/Thr phosphatases of the 2C type class (PP2C), Ptc1 and Ptc3 (Nguyen and Shiozaki 1999).A His-to-Asp phosphorelay system acts upstream of the Sty1 pathway. This relay is composed of three histidine kinases (Mak1/Phk3, Mak2/Phk1 and Mak3/Phk2) acting as sensors of the different types of insults; one phosphor relay protein (Spy1/Mpr1) (Aoyama, Mitsubayashi et al. 2000) (Aoyama, Aiba et al. 2001) and two response regulators (Mcs4 and Prr1) (Aoyama *et al*., 2001). It is still unclear how the sensor histidine kinases recognize and respond to different types of stresses. However, several lines of evidence suggest that the phosphorelay protein Spy1 is activated

by phosphorylation and negatively regulates the response regulator Mcs4 by binding and transferring its phosphate group. Finally, the anchor of the phosphorelay protein, Mcs4, binds to the MAPK Kinase Kinase and initiates the activation of the Sty1 cascade (Fig. 17).

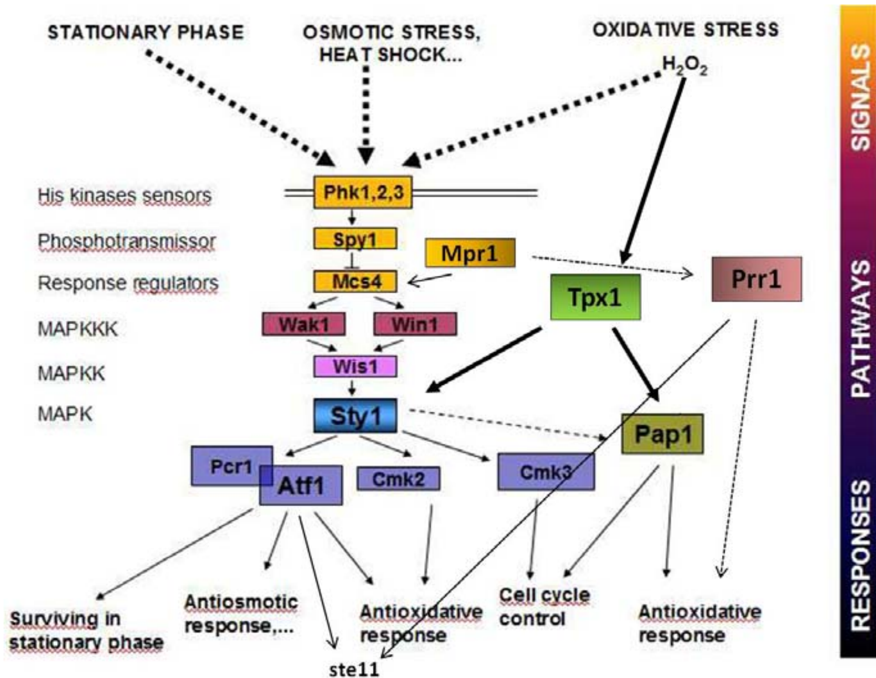


Figure 17. *S. pombe* stress response pathway.

Once Sty1 is activated by phosphorylation, the kinase accumulates in the nucleus, where most of its stress roles are defined. The nuclear localization of Sty1 is not enough to induce transcription, it must be phosphorylated. The mechanism by which Sty1 migrates into the nucleus is still unclear, since Sty1 lacks a typical nuclear localization signal.

The main target of Sty1 pathway is the transcription factor Atf1; it has a conserved sequence at the C-terminus, containing the bZIP region (Takeda, Toda et al. 1995). *In vitro* studies (Wahls and Smith 1994) and coimmunoprecipitation (Co-IP) experiments (Kanoh, Watanabe et al.

1996)suggest that Atf1 heterodimerizes with Pcr1, another bZIP transcription factor.

Furthermore, other substrates of phosphorylated Sty1 are the RNA-binding protein Csx1 (Rodriguez-Gabriel, Burns et al. 2003) and the kinases Cmk2 and Srk1 (Sanchez-Piris, Posas et al. 2002); (Lopez-Aviles, Grande et al. 2005).

The nuclear localization and phosphorylation of Sty1 leads to the activation of Atf1; and subsequent activation of battery genes implicated in the defense of stress. Genome-wide transcription studies revealed that there is a large number of genes (~700 out of 4970) whose expression levels changed two fold or greater after one stressor out of five that had been analyzed, (H_2O_2, sorbitol, cadmium, heat shock and MMS; (Chen, Toone et al. 2003). The authors of this study found that in four out of five of those stresses 140 genes were commonly induced and 100 repressed. This shared outcome was called the Common Environmental Stress Response (CESR). Most of them are Sty1-dependent (see section 5.2). Each stress activates transcription of certain genes, which are the specific response to a particular type of stress, which is defined as SESR (Specific Environmental Stress Response) (Chen, Toone et al. 2003; Chen, Wilkinson et al. 2008).

5.3. The response regulator Prr1

The *prr1* gene encodes for the homologue of *S. cerevisiae* SKN7. It is part of a two-component response system, that in *S. pombe* consists on three histidine kinases, a unique phosphotransmitter protein Mpr1 and two response regulators, Mcs4 and Prr1 see above). It was found in evolutionarily divergent fungi, and contains a receiver domain characteristic of response regulator proteins (see Fig. 18). The nuclear response regulator Prr1 most likely acts as a direct transcriptional regulator of some oxidative stress response genes, independently or not of the Sty1 and Pap1 pathways (Ohmiya, Kato et al. 1999); (Ohmiya, Yamada et al. 2000) (Buck, Quinn et al. 2001).

Figure 18. The response regulator Prr1.

The two-component regulatory systems, common in bacteria, have been identified in fungi (Santos and Shiozaki 2001) and plants (Urao, Yamaguchi-Shinozaki et al. 2001), but not in animals. Prr1 contains a typical receiver domain found in response regulators, including the predicted conserved phosphorylatable Asp 418residue (Ohmiya, Kato et al. 1999). It has recently been reported that the upstream two-component regulation of Prr1 and its Asp 418 in the receiver domain plays a role in the response to high levels of peroxide stress, which is mediated by the Sty1-Atf1 pathway at least for the induction of some genes (Quinn, Malakasi et al. 2011). The expression of non-phosphorylatable (D418N) mutant version of Prr1 complements the sensitivity of *Δprr1* cells to low levels of t-BOOH, suggesting that regulation of Prr1 is independent of two-component phosphorylation (Ohmiya, Yamada et al. 2000). Prr1 is also required for tolerance to cadmium; mutating D418 had no detectable effect on the sensitivity of cells to this heavy metal.

Recent transcript-profiling studies have revealed H_2O_2concentration dependent differences in the requirement for Prr1 (Chen, Wilkinson et al. 2008). In particular, is essential for activation of the core oxidative stress response genes (Quinn, Malakasi et al. 2011), also cells without *prr1* gene show sensitivity to low levels of t-BOOH. Prr1 regulates the expression of antioxidant genes, such as *ctt1* or *trr1,* which are also Pap1 dependent (Ohmiya, Kato et al. 1999). In addition, it regulates the expression of *ste11,* which is necessary for the transactivation of genes implicated in sexual development leading to sterile strain in cells without *prr1* gene (Ohmiya, Yamada et al. 2000).

5.4. Fission yeast specific sensors:

5.4.1. Pap1 and Tpx1

S. pombe is an ideal model to study stress responses due to the diversity of mechanisms it uses to respond to environmental stresses. In addition to

a specific oxidative stress response (as found in bacteria), relying on the Pap1 transcription factor and the Tpx1 sensor, the fission yeast holds specific factors involved in the response to different kind of stresses, like heat shock, heavy metals, etc.

Pap1 and its *S. cerevisiae* homologue YAP1 belong to the AP-1 family of transcriptional regulators, showing homology in their bZIP DNA binding domain to the mammalian c-Jun protein and it is essential for normal tolerance to peroxides (Moye-Rowley, Harshman et al. 1989; Toda, Shimanuki et al. 1991).

Pap1 was identified in the Sipiczki laboratory in a screen were caffeine-resistant mutants were identified (Benko, Sipiczki et al. 1998). These mutants displayed pleiotropic, albeit slightly different phenotypes: caffeine resistance, increased sensitivity to UV-irradiation, a reduction in fertility, lengthening of the cell cycle and some morphological aberrations. Most caffeine-resistant mutants isolated in Sipiczki´s laboratory do trigger accumulation or constitutive nuclear localization of Pap1 (Benko, Sipiczki et al. 1998; Benko, Fenyvesvolgyi et al. 2004). Thus, *caf2–3* carries a loss-of-function mutation at *crm1*, which is the nuclear exporter of Pap1 (Kumada, Yanagida et al. 1996; Benko, Sipiczki et al. 1998).

Pap1 activity is modulated at the level of cellular localization; Pap1 has a hypothetical double nuclear import signal (NLS) and a nuclear export signal (NES), which are recognized by the importin-α Imp1 (Umeda, Izaddoost et al. 2005) and the exportin Crm1 (Kudo *et al.,* 1999) respectively. The Crm1 - dependent export of Pap1 prevails over the import, and therefore the transcription factor displays cytosolic localization prior to stress imposition. Pe-existing Pap1 accumulates in the nucleus upon oxidative treatments, as a consequence of inhibition of Crm1 dependent Pap1 nuclear export (Toone, Kuge et al. 1998) Kudo *et al.,* 1999). Mutation at *hba1*, coding a protein with Ran-binding domain and cofactor of the Crm1, also triggers constitutive nuclear localization of Pap1 and resistant to caffeine (Castillo, Vivancos et al. 2003).

Pap1 contain two clusters of three and four Cys residues each one. One located at the centre of the protein (n-CRD) with four Cys residues and the second at the carboxy-terminal region (c-CRD) with three Cys and where the NES is also located (Fig. 19) (Wemmie, Wu et al. 1994; Toone, Kuge et al. 1998).

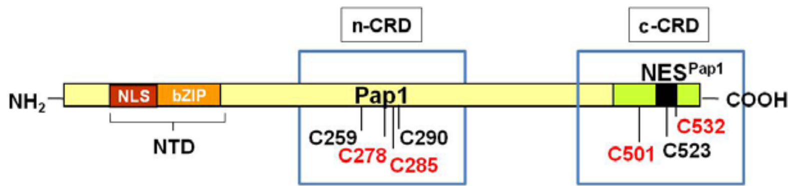


Figure 19. Scheme of the transcription factor Pap1 in *S. pombe.*

Conditions suspected to alter the intracellular redox state of Pap1 Cys residues, such as H_2O_2, diamide, diethylmaleate (DEM), heavy metals and a variety of toxic drugs, induce the expression of Pap1-dependent genes (Toone, Kuge et al. 1998). DEM irreversibly modifies Cys of the c-CRD by direct covalent modification of their SH groups impeding access of Crm1 to the NES. This modification is an irreversible post-translational modification of Pap1 (Fig. 20).

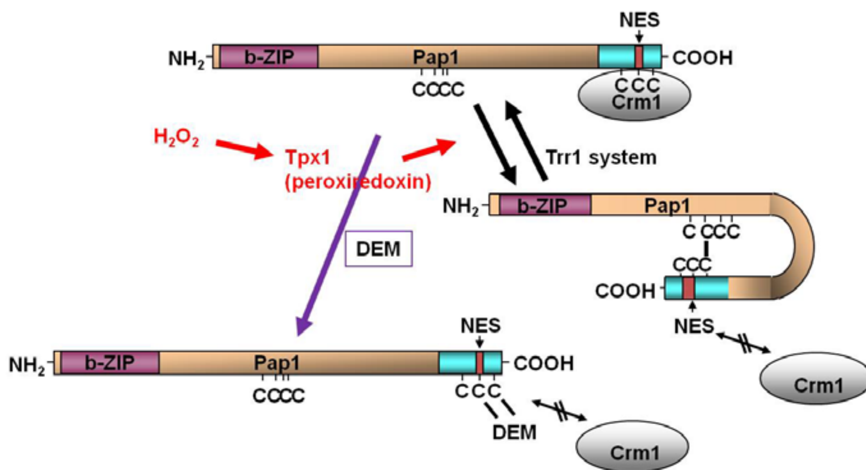


Figure 20. A model for Pap1 nuclear accumulation upon H_2O_2 vs. DEM stress (Castillo *et al.,* 2002).

In response to low doses of H_2O_2, at least one intramolecular disulfide bond in is formed Pap1between two distant Cys residues, one from the n-CRD (Cys278) and the other one from the c-CRD (Cys 501 or Cys 532).

This disulfide bond causes a conformational change, which impairs the access of Crm1, leading to its dissociation from the Crm1–Hba1 nuclear export machinery and its nuclear accumulation (Fig. 21) **(**Kudo *et al.,* 1999**;** (Castillo, Ayte et al. 2002; Vivancos, Castillo et al. 2004).

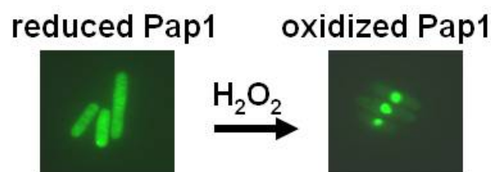


Figure 21. The cellular distribution of GFP-Pap1 before and after stress.

Oxidized Pap1 can be reduced by Trx system; Trx1 contributes to maintain the reduce state of Pap1 before and after stress which is in turn up-regulated at the transcriptional level by activated Pap1 as a negative feed-back mechanism (Song and Roe 2008). The deletion of the *trr1* gene, necessary for Pap1´s reduction, results in the constitutive oxidation and therefore nuclear localization of Pap1(Vivancos, Castillo et al. 2004). Once Pap1 is stress-activated and accumulates in the nucleus, it induces the expression of the following oxidative stress genes among others: *ctt1* (Nakagawa, Yamada et al. 2000), *trx2* (encoding mitochondrial Trx; (Kuge, Jones et al. 1997), *trr1* (Casso and Beach 1996), *pgr1* (encoding reductase (Lee, Dawes et al. 1997), *tpx1* and efflux pumps coding genes *hba2/bfr1, caf5* and *pmd1* (Kuge, Jones et al. 1997). All these genes are part of the cellular adaptive response to oxidants. Then, the ability of Pap1 to be activated by low levels of oxidants prepares the cell to survive to higher degrees of this stress (Quinn, Findlay et al. 2002).

In *S. pombe*, the Pap1 and Sty1 pathways constitute the key protective responses to oxidative stress. Pap1 transcription factor is more sensitive to H_2O_2 than the MAP kinase Sty1 pathway, and thus Pap1 is known to induce adaptation, whereas Sty1 promotes survival responses. The role of Sty1 in Pap1 activation has been unclear in recent years, because it was thought that this MAPK was required for Pap1 nuclear accumulation; and the strain deleted in the MAPK Sty1 is not able to activate Pap1 after H_2O_2 (Toone, Kuge et al. 1998). However, Sty1 seems

only to be required for Pap1 activation at high doses of the oxidant. This can be explained by the role of peroxiredoxin Tpx1 in the H_2O_2-dependent activation of Pap1 (Vivancos, Castillo et al. 2004) (see below).

Tpx1 is a 2-Cys Prx in fission yeast. Tpx1 is an essential H_2O_2 scavenger in *S*. *pombe* with an extremely high affinity for its substrate and this peroxidase activity is required during aerobic metabolism (Jara, Vivancos et al. 2007). It has been previously reported to participate in signal transduction in response to mild extracellular oxidative stress and in aerobic growth, because Δ*tpx1* cells shifted from anaerobic to aerobic conditions suffered growth arrest (Jara, Vivancos et al. 2007). In contrast, the deletion of the glutathione peroxidase-like enzyme *ORP1/GPX3* in *S. cerevisiae* shows no growth defects (Toledano, Delaunay et al. 2004; Vivancos, Castillo et al. 2005).

Tpx1 is the real H_2O_2 sensor and acts as the upstream activator of the Pap1 pathway; it transfers the redox signal to Pap1 under mild H_2O_2 stress. Thus, Pap1 can only become oxidized to a disulfide bond in cells expressing Tpx1, which is in the cytoplasm and seems to be the only component of the pathway able to interact directly with the H_2O_2 signal. Both Cys residues of Tpx1 are required for Pap1 activation in response to low H_2O_2 stress (Bozonet, Findlay et al. 2005; Vivancos, Castillo et al. 2005). This means that Tpx1 provides the molecular basis for the temporary shutdown of Pap1 pathway in response to elevated levels of H_2O_2 (see Fig. 22). The formation of a SO_2Hin Tpx1, as a consequence of high doses of H_2O_2 that reversibly inactivates Tpx1 and, therefore, postpones Pap1 activation until the Sty1-dependent transcriptional response is activated. Tpx1 is required for activation of Sty1 through Tpx1-Sty1 disulfide formation only in aH_2O_2-dependent manner (Veal, Findlay et al. 2004). The oxidation of Cys 48 to SO_2H occurs when Tpx1 exists as a covalent dimer, before the accumulation of the inactive Tpx1 monomer (Jara, Vivancos et al. 2007). This hyper oxidation is resistant to reduction by Trx (Wood, Poole et al. 2003); (Yang, Kang et al. 2002); however can be reversed to a SH group by ATP-dependent sulfiredoxin Srx1. The Sty1 response includes the *srx1* gene; Srx1 restores the Tpx1-Pap1 redox relay, and Pap1 activation by recycling oxidized Tpx1 (Fig. 22). The Sty1-dependent H_2O_2 scavenging activities Gpx1 and catalase (Quinn, Findlay et al. 2002) might also contribute to this regulation by decomposing peroxide excess, thus preventing reinactivation of Tpx1.

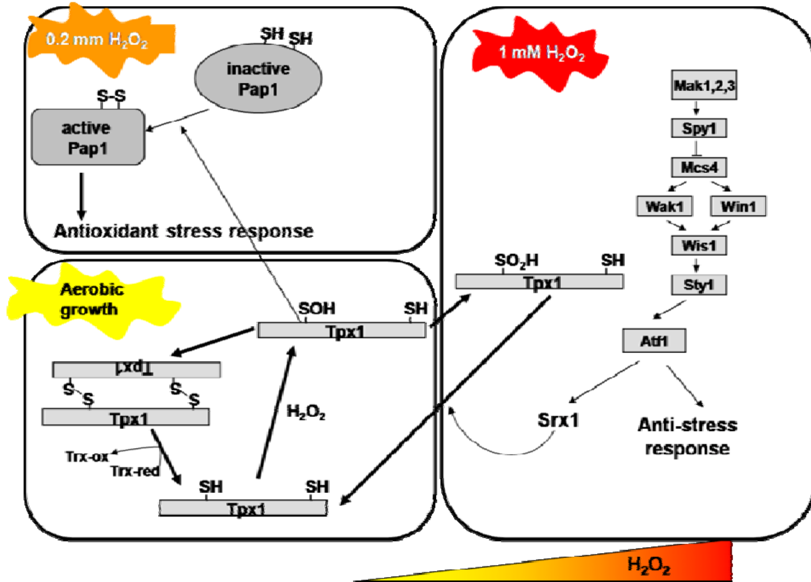


Figure 22: Levels of oxidative stress in *S. pombe (Giorgio, Trinei et al. 2007).*

Tpx1 is also a main substrate for Trx in *S. pombe*. Trx1 serves as the electron donor for the catalytic breakdown of H_2O_2 by Tpx1 (Fig. 23) (Jara, Vivancos et al. 2007).

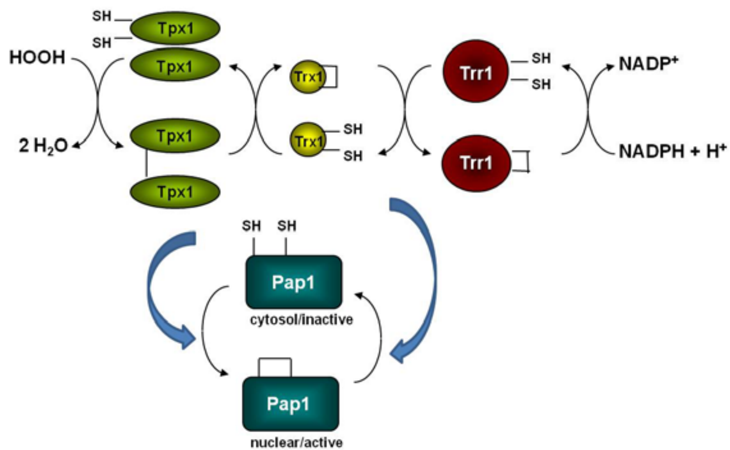


Figure 23. Schematic representation of the thioredoxin system.

OBJECTIVES

"Los objetivos son sueños con fechas límites"

Diana Scharf Hunt

Our first goal was to decipher the mechanism of activation and inactivation of Pap1 after low H_2O_2 concentrations. Our second goal was to study the role of Pap1 as a transcription factor and the Pap1 - dependent gene response upon oxidative stress in the fission yeast *S. pombe.*

The specific objectives of this PhD project were:

1. Involvement of the Pap1 pathway in the response to H_2O_2 and cellular tolerance to caffeine.

2. Characterization of Pap1 as a sensor of H_2O_2.

3. Role of different components of the thioredoxin system in activation and inactivation of Pap1.

4. Elucidate how Pap1 is activating the transcriptional response inside the nucleus, and role of the transcription factor Prr1 in the regulation of pap1-dependent genes. In addition to understand the needing of two different transcription factors, Pap1 and Prr1, in the antioxidant transcription regulation.

CHAPTER 2: RESULTS

"Imagination is the highest form of research"

Albert Einstein

The results will be divided in four sections.

The first one describes a genetic screening of a mutant library, which contains two research papers included in appendices (Appendix 1 and Appendix 2).

In the second and third section we describe the characterization of Pap1 as a sensor of H_2O_2 and the different molecular events leading to its activation. In particular, the role of the thioredoxin system in the activation and inactivation of Pap1 is the focus of section. Both parts are presented as manuscripts in preparation.

Lastly, the fourth section explains the characterization of Pap1 as a transcription factor and the study of Pap1-dependent gene expression program. It is presented as a published manuscript.

1. GENOME WIDE SCREEN OF *S. pombe* GENES ESSENTIAL FOR ROS HOMEOSTASIS AND FOR WILD-TYPE TOLERANCE TO CAFFEINE

In 2008, our laboratory received an *S. pombe* collection of viable open reading frame deletion mutants. We spread the collection and designed different screenings to isolate genes required for survival upon both exogenous and endogenous oxidative stress, and to elucidate the importance of some oxidative stress pathways on wild-type tolerance to caffeine. The *pap1* gene was initially isolated in a screen to caffeine (see below).

In our first screening, we identified new deletion strains very important for H_2O_2 tolerance (see section 1.2). When we combined the list of deletion mutants sensitive to extracellular H_2O_2 with that of mutants with defective growth on respiratory growth conditions, we obtained a number of genes encoding mitochondrial proteins (see section 1.3). Thirdly, we searched for *S. pombe* mutants with inhibited growth on caffeine containing plates (see section 1.4).

Some of the results of these screenings have been published in two manuscripts: (Calvo, Gabrielli et al. 2009) where I participated as a first co-author.

1.1. Description of the screening performed with the deletion collection

ROS homeostasis plays an important role in chronological aging processes and some associated diseases (Johnson, Sinclair et al. 1999; Finkel and Holbrook 2000; Dawson and Dawson 2003; Lin and Beal 2006; Giorgio, Trinei et al. 2007). ROS originate mainly from the mitochondria due to incomplete reduction of oxygen at several sites along the electron transfer chain (Fridovich 2004). The sensitivity to caffeine was also used to study the relationship with oxidative stress pathways.

We decided to isolate *S. pombe* genes required for wild-type tolerance to oxidative stress, applied either extracellular (exogenous

oxidative stress) or by promoting growth under respiratory-prone conditions (endogenous oxidative stress). To do this, the 2,700 haploid yeast deletion mutants were grown in liquid complex media containing a selective antibiotic for 2 days. Cultures were replicated on 4 types of solid plates for the first screen: complex media with or without H_2O_2 (5 mM), defined media, or glycerol-containing media (defined media with 1% glycerol, 0.1% glucose). To study the sensitivity to caffeine, we added 10 mM caffeine to YE complex media. The plates were incubated at 30ºC for 3–4 days.

1.2. Identification of the *S. pombe* genes required for wild-type tolerance to extracellular peroxides

In eukaryotic cells, ROS sensors and other pathway components have been identified and characterized by treatment of cultures with extracellular peroxides, which rapidly increase the intracellular steady-state levels of H_2O_2. In *S. pombe*, there are different signaling pathways depending of the severity of the oxidative stress applied, the main ones being the Pap1 and Sty1 pathways (Vivancos, Castillo et al. 2004; Chen, Wilkinson et al. 2008).

From the *S. pombe* collection, we performed twice screenings on H_2O_2-containing plates, and isolated 18 genes, which did not grow at all in H_2O_2-containing fermentable media plates (see Table I). We also isolated a huge number of deletion strains (approximately 215 genes), which can grow partially in H_2O_2 plates. We classified the genes mutated in these sensitive clones in several families depending on the cellular role in which they are implicated. We confirmed that cells lacking Pap1 and Sty1 are very sensitive to H_2O_2 and we used them as internal controls in further screenings. We could identify other strains lacking genes which are essential for H_2O_2 tolerance. Studies of some of these strains are on their way by other members of the laboratory.

NAME	FUNCTION
Chain Electron Transport	
coq5	C-methytransferase. ubiquinone biosynthetic process and aerobic respiration
Ribosomal	
rpl4301	60S ribosomal protein L37a
DNA repair	
pnk1	DNA kinase/phosphatase, DNA 3'-phosphatase, DNA 5' kinase, involved in DNA repair, involved in DNA damage response (by gamma radiation or camptothecin) (required), localization nucleus (GFP)
Meiosis	
mfm2	M-factor precursor, pheromone, no apparent orthologs
zfs1	zinc finger protein, zf-CCCH type, mating pheromone recognition pathway, non-essential, required to prevent septation if mitotic progression is inhibited
Vesicle-mediated transport	
gpi17	GPI-anchor transamidase complex (predicted)
vps17	sorting nexin, PX domain protein, localization Pik3p dependent
Oxidoreductase	
hmt2	sulfide-quinone oxidoreductase, involved in sulfide metabolism, involved in heavy metal resistance, localization mitochondrion
Oxidative stress	
sty1	serine/threonine protein kinase, Sty1 SAPK cascade, interacts physically with SPBC9B6.10, MAP kinase (MAPK)
pap1	transcription factor, ap-1-like, bZIP, leucine zipper, involved in response to oxidative stress (required)
mcs4	mitotic catastrophe suppressor, response regulator receiver domain, involved in response to stress, involved in cell cycle regulation, Sty1 SAPK cascade
wis1	serine/threonine protein kinase, MAP kinase kinase (MAPKK), Sty1 SAPK cascade, involved in cell cycle regulation, involved in regulation of mitotic cell cycle
atf1	transcription factor, DNA binding (recombination hotspot), bZIP (basic leucine zipper) transcription factor family
Signal Transduction	
cgs2	3',5'-cyclic-nucleotide phosphodiesterase, cAMP phosphodiesterase class II. nucleoside 3',5'-cyclic phosphate + H2O = nucleoside 5'-phosphate. cAMP catabolic process.
calcineurin (b subunit)	(regulatory subunit) (predicted), protein phosphatase regulatory subunit, EF hand, calcium binding protein
Lipids Metabolism	
choline kinase	(predicted), involved in phosphatidylcholine biosynthesis (predicted)
Urea cycle and metabolism of aminoa groups; aa metabolism	
met6	homoserine O-acetyltransferase
cys11	cysteine synthase, involved in cysteine metabolism
Sulfur metabolism	
sir1	sulfite reductase NADPH flavoprotein subunit (predicted), catalyzes the 6-electron reduction of sulfite to sulfide. This is one of several activities required for the biosynthesis of L-cysteine from sulfate (By similarity).
Mitochondrial protein synthesis	
mitochondrial translation regulator	(predicted), PPR domains, predicted N-terminal signal sequence

Table I. Identification of 18 genes required for survival extracellular oxidative stress.

1.3. Identification of genes required for growth on both H_2O_2-containing plates and on respiratory-prone conditions

With this second screening we isolated several genes, which regulate endogenous oxidative stress (see Appendix 1). As a consequence of the high respiratory rates in defined, low glucose media, cells are more sensitive to extracellular peroxides when grown in defined medium. As we expected, an example is the activation of the transcription factor Pap1 (pombe AP-1-like). Pap1 is oxidized (activated) faster in lower concentrations of H_2O_2 defined than in complex media, once it is activated, it goes to the nucleus and induces the antioxidant response.

1.3.1. Mitochondrial Dysfunction Increases Oxidative Stress and Decreases Chronological Life Span in fission yeast

From the *S. pombe* deletion collection, we isolated 51 strains which are sensitive to growth under respiratory-prone conditions (defined, low glucose medium) and to H_2O_2 on complex medium (high glucose). Out of the 51 strains, 19 of these were related to mitochondrial function, and 12 coded directly for components of the electron transfer chains. The mitochondrial mutants displayed reduced oxygen consumption and increased intracellular ROS levels due to enhanced electron leakage from their defective transport chain. This alteration of the electron transfer chain causes a reduction of the life span. Lastly, the growth defects of these mutants can be improved with the help of antioxidants or if they grow under anaerobic conditions which points to intrinsic oxidative stress as the origin of the phenotypes observed. We confirmed that none of the 12 mutants that coded directly for components of the electron transfer chain grow on glycerol-containing defined medium (non-fermentable as a carbon source) because respiration is the only energy source in these plates.

The results of this work have been published in the manuscript entitled: Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast. PLoS One. 2008 Jul 30;3 (7):e2842. Zuin A, Gabrielli N, Calvo IA, García-Santamarina S, Hoe KL, Kim DU, Park HO, Hayles J, Ayté J, Hidalgo E. (Appendix 1).

1.4. Genome-wide screen of genes required for caffeine tolerance in fission yeast

In *S. pombe*, caffeine has been demonstrated to participate in a diverse number of processes (Butcher and Sutherland 1962; Fabre 1972; Loprieno, Barale et al. 1974; Gentner and Werner 1975; Osman and McCready 1998). Different concentrations of caffeine can inhibit the growth of *S. pombe*, in a similar way as H_2O_2 does (Benko AND SIPICZKI 1993). We aim to study how cells become tolerant to a toxic dose of this drug and the relationship between caffeine and oxidative stress pathways. It is worth to point out that the *pap1* gene was initially isolated in a screen for resistance to caffeine (Toda, Shimanuki et al. 1991). Thus, oxidative stress pathways and normal tolerance to caffeine are mutually connected.

1.4.1. Genome-wide screen of caffeine-sensitive mutants

In order to paint a global picture of the cellular mechanisms used by *S. pombe* to cope with toxic doses of caffeine, we carried out a genome-wide isolation of mutants strains displaying growth defects on YE plates containing 10 mM of caffeine. We obtained 98 putative isolates with impaired growth. Among these 98 deletion strains sensitive to caffeine, 59 were confirmed by sequential spotting. The genes mutated in these sensitive clones were involved in several cellular processes including the H_2O_2-induced Pap1 and Sty1 stress signaling pathways, the integrity and calcineurin pathways, cell morphology and chromatin remodeling. We also explored additional cellular pathways involved in caffeine resistance (see Fig. 24). We could investigate the role of oxidative stress pathways in sensing and promoting survival to caffeine.

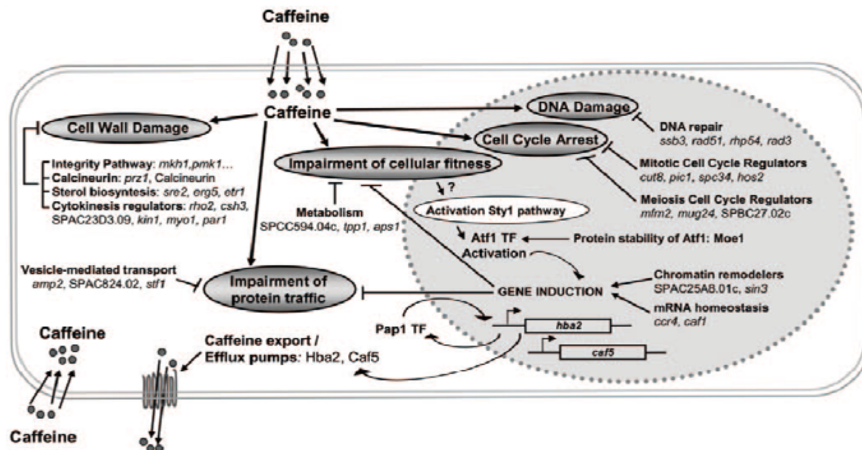


Figure 24. Proposed model for the cellular targets and defense response mechanisms to caffeine in fission yeast.

The results of this work have been published in the manuscript entitled: Genome-wide screen of genes required for caffeine tolerance in fission yeast. PLoS One. 2009 Aug 12;4(8):e6619. Calvo IA, Gabrielli N, Iglesias-Baena I, GarcÍa-Santamarina S, Hoe KL, Kim. Sansó M, Zuin A, Pérez P, Ayté J, Hidalgo E (Appendix 2).

1.4.2. Involvement of the Pap1 pathway in cellular tolerance to caffeine

In 1998, the Sipiczki laboratory isolated a number of caffeine-resistant mutants, which defined single loci. These mutants displayed pleiotropic, albeit slightly different phenotypes with regard to multidrug resistance. Indeed, all five mutations have finally been reported to be connected to the Pap1 pathway (Benko, Sipiczki et al. 1998). They could conclude that all the *caf* genes had related functions and define a single caffeine-responsive ‘‘tolerance’’ pathway.

Overexpression or constitutive activation of Pap1 was identified as enhancer of resistance to this drug. Active Pap1 up-regulates *hba2* and other genes coding for efflux pumps and that confers resistance to caffeine. We determined that cells lacking Pap1 are sensitive to 10 mM caffeine because *hba2* coding for the major caffeine exporter in *S. pombe*,

its basal transcription is 3-fold lower in Δ*pap1* than in wild-type cells, which explains the sensitivity to caffeine of cells lacking Pap1. As a consequence, Δ*hba2* cells are also sensitive to caffeine.

We also determined that the Pap1 pathway is not activated at any concentration of the drug. On the contrary, the MAPK Sty1 stress pathway is activated by 10 mM caffeine. Then, we cannot conclude that any of the toxic effects of caffeine is mediated through direct generation of reactive oxygen species (see Appendix 2).

As a consequence of these results, we use routinely the resistance to caffeine as a reporter of constitutive activation of the Pap1 pathway. Thus, genetic interventions known to modulate Pap1 activity will be also characterized by their ability to modulate the tolerance to caffeine (see section 3 of results).

2. GENETIC AND PROTEOMIC CHARACTERIZATION OF ACTIVE Pap1

Cys residues are target of H_2O_2-dependent oxidation, and such post-translational modification is both a mark of oxidative damage and a mechanism of activation of signaling cascades. We wanted to demonstrate here the role of all seven Cys residues of Pap1 in the response to H_2O_2 by genetic, biochemical and proteomic analysis. Before the work that I present here in, was initiated with genetic data indicated that 2 Cys residues in Pap1 were essentials for its nuclear accumulation upon stress, and for the formation of a faster migrating band under non-reducing electrophoresis, suggesting of the formation of a disulfide bond in Pap1. This disulfide bond in Pap1 is in a Tpx1-dependent manner. Then, the peroxirredoxin Tpx1 acts as a sensor and upstream activator of the Pap1 pathway (Vivancos, Castillo et al. 2004; Chen, Wilkinson et al. 2008). The mutated *GFP*-tagged genes were integrated downstream of an inducible promoter to determine the subcellular localization of the mutants proteins by fluorescence microscopy.

2.1. Genetic analysis and identification of new cys residues essential for Pap1 activation

We performed a detailed mutagenesis analysis of all cysteine residues in Pap1, and substituted the endogenous *pap1* locus with the mutant open reading frames to yield wild-type levels of the transcription factor. As a consequence, the otherwise different protein levels could not interfere in the phenotype instead of the previous mutants where Pap1 was over-expressed from a regulated heterologous promoter and its effect on peroxide sensitivity and the induction of antioxidant response could not be determined.

Pap1 contains seven Cys residues clustered in two Cys-rich domains: on the center of the protein called amino terminal (nCRD) with four Cys and the second, at the carboxy terminal part (cCRD) with three Cys. We could identify new residues essential for Pap1 activation. Four Cys

substitutions (Cys278 to alanine, Cys285 to serine, Cys501 to alanine and Cys532 to threonine) abolished the nuclear localization, oxidation and activation of Pap1 after stress. In conclusion, our results indicate that Cys278, Cys285 and Cys532 are essential to trigger the conformational change observed with non-reducing electrophoresis, for nuclear accumulation and for wild-type resistance to peroxides. Cys501 is also important, but this mutant shows partial phenotype (see below).

Furthermore, some mutants (C523D, C523A C532T among others) with alteration of the NES located at the carboxy terminal part of Pap1 protein show constitutively nuclear accumulation due to the alteration of its export, and as a result, they are resistant to caffeine in an Hba2-dependent manner by up-regulating efflux pumps (see section 1.4.2 of results).

2.2. Proteomic characterization of active and inactive Pap1

Moreover, we did the proteomic characterization of Pap1 based on the detection of disulfide bonds in protein extracts and differential alkylation of free thiols *versus* oxidized thiols to verify our genetic results. The reduced cysteines are blocked with N-ethilmaleimide and oxidized cysteines are blocked with iodoacetamide. We confirmed the reversible oxidation of these four Cys residues.

The genetic, biochemical and proteomic analysis indicates that the active form of Pap1 contains an intramolecular disulfide bond between Cys285 and Cys532. Cys278 and Cys501 are also oxidized in the active form, but they have not identified by proteomic analysis with a covalently linked peptide.

The detailed description of this section 2 is provided in the format of a manuscript submitted for publication (see below).

Role of thiol oxidation in the H_2O_2-dependent activation of the fission yeast transcription factor Pap1

Isabel A. Calvo, José Ayté and Elena Hidalgo*

Oxidative Stress and Cell Cycle Group, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, C/ Dr. Aiguader 88, E-08003 Barcelona, Spain;

*Corresponding author: Elena Hidalgo, Universitat Pompeu Fabra, C/ Dr. Aiguader 88, E-08003 Barcelona, Spain (elena.hidalgo@upf.edu; Tel: 34-93-316-0848; Fax: 34-93-316-0901)

Keywords: H_2O_2 sensor, Pap1, disulfide bond, redox cascade, fission yeast

ABSTRACT

Thiol oxidation is both a mark of H_2O_2 toxicity and an initiator of signalling events. The Pap1 transcription factor senses H_2O_2 to induce an antioxidant response. We have dissected the role of all seven cysteine residues in Pap1 using genetic and proteomic approaches. We show that four of them are required for Pap1 activation by H_2O_2. The mutant proteins do not become oxidized by H_2O_2 and cannot bind to promoters or trigger the Pap1-dependent gene expression program. Our study suggests that two disulfide bonds are required to promote the important conformational changes that promote Pap1 activation and nuclear accumulation.

INTRODUCTION

Reactive oxygen species can damage all biomolecules. In particular, hydrogen peroxide (H_2O_2) has reactivity towards many amino acids in proteins, and cysteine (Cys) residues are a classical target or peroxide reactivity. Thus, the thiol group is susceptible to oxidation towards sulfenic acid (SOH), which is then prone to react with a nearby thiol group forming a disulfide bond, or to over-oxidation to either sulfinic (SO_2H) and sulfonic acid (SO_3H) forms, both of which are considered in general irreversible modifications.

H_2O_2 sensors can also use thiol oxidation as a gain-of-function modification leading to the activation of a signalling pathway. That was initially proposed for the bacterial sensor OxyR (Zheng, Aslund et al. 1998). OxyR, the *Escherichia coli* sensor of H_2O_2, suffers thiol oxidation as a result of a mild H_2O_2 stress, which transforms it into a potent transcriptional activator of an antioxidant response. The modification proposed by Storz and colleagues was an intramolecular disulfide bond, even though it has also been postulated that oxidation of the thiol to SOH or to S-nitrosothiol (SNO) (when treated with nitric oxid) also activates OxyR as a transcription factor (Kim, Merchant et al. 2002).

YAP1 is the more extensively studied H_2O_2-responding transcription factor in eukaryotes. It contains two clusters of Cys residues in the center and the carboxi-terminal region of the polypeptide, with three Cys present in each domain. YAP1 becomes oxidized and activated by H_2O_2 through the formation of an internal disulfide bond , which can be detected *in vitro* by a change in electrophoretic mobility in non-reducing gels (Delaunay, Isnard et al. 2000). Genetic analysis demonstrated that two Cys residues are essential for the H_2O_2-dependent oxidation and activation of Yap1. Later work also showed that several disulfides do occur in Yap1 upon H_2O_2 exposure, some preceding the others, and leading to different activation kinetics (both regarding levels of activity and duration of the signal) of the transcription factor (Okazaki, Tachibana et al. 2007).

The *Schizosaccharomyces pombe* Pap1 transcription factor is the homolog of budding yeast YAP1 (Toda, Shimanuki et al. 1991; Toone, Kuge et al. 1998). It also responds to moderate levels of H_2O_2 by rapidly

adopting a conformation that blocks its nuclear export and allows its transient accumulation at the nucleus (Vivancos, Castillo et al. 2004). Pap1 contains seven Cys residues clustered in two domains, one at the center of the polypeptide called amino terminal Cys rich domain (nCRD) and the second at the carboxy terminal domain (cCRD). Upon H_2O_2 stress, the transcription factor accumulates at the nucleus, and again a disulfide bond has been hypothesized to contribute to the dissociation between Pap1 and the Hba1-Crm1 nuclear export machinery (Castillo, Ayte et al. 2002; Castillo, Vivancos et al. 2003; Vivancos, Castillo et al. 2004; Bozonet, Findlay et al. 2005; Vivancos, Castillo et al. 2005). Two Cys substitutions, Cys-278-Ala and Cys-501-Ala, fully avoid the H_2O_2-dependent nuclear accumulation of GFP-fused Pap1 (Castillo, Ayte et al. 2002). However, the exact role of the other five Cys residues is unknown. Furthermore, in previous studies the mutant proteins were over-expressed from a regulated heterologous promoter, and their effect on peroxide sensitivity could not be determined since high levels of Pap1 can induce the antioxidant response in a H_2O_2-independent manner.

In an attempt to decipher the exact mechanism of activation by H_2O_2 of a eukaryotic transcription factor, we have here mutated all seven Cys residues of Pap1 and substituted the endogenous *pap1* locus with the mutant open reading frames. Our results indicate that Cys278, Cys285 and Cys532 are essential to trigger Pap1 activation. Cys501 is also required for Pap1 oxidation and for nuclear accumulation, although some residual transcriptional activity is still present in this mutant. We have confirmed by proteomic analysis the reversible oxidation of these four Cys residues upon H_2O_2 stress. The identification of all Cys residues involved in the response to peroxides opens new perspectives regarding the dissection of the molecular events that define a redox cascade.

RESULTS AND DISCUSSION

Cys278 and Cys285 of the nCRD of Pap1 are essential for the activation of Pap1

We decided to mutate each Cys residues of Pap1 to test their role in the H_2O_2-responding redox cascade. We generated two sets of mutant strains. On one hand, the mutated *GFP*-tagged genes were integrated downstream of an inducible promoter to determine the subcellular localization of the mutant proteins by fluorescent microscopy. On another hand, the untagged mutagenized genes were integrated at the chromosomal *pap1* locus to yield wild-type levels of the transcription factor, so that the otherwise different protein levels could not interfere with the phenotypic characterization of strains harbouring the mutations. The nCRD of Pap1 contains four Cys residues (Fig. 1A), and we performed several individual substitutions to serine or alanine, which are summarized in Fig. 1B. Fluorescent microscopy analysis of the GPF-tagged mutant proteins clearly demonstrated that not only Cys278 but also Cys285 are fully required for the H_2O_2-dependent nuclear accumulation of Pap1, whereas Cys259 and Cys290 are fully dispensable (Fig. 1B). We confirmed this last observation by generating a double Cys259Ser Cys290Ser mutant, which also showed to be wild-type regarding subcellular localization (Fig. 1B). Importantly enough, cells expressing Pap1.C259, 290S display wild-type tolerance to peroxides, while mutant cells expressing Pap1.C278A or Pap1.C285S are as sensitive to peroxides as *Δpap1* cells (Fig. 1C).

We obtained extracts from cells expressing each one of the mutant forms of Pap1, before and after stress imposition. As shown in Fig. 1D, wild-type Pap1 became oxidized upon H_2O_2 stress, as reflected by its faster mobility in non-reducing electrophoresis. Pap1.C259, 290S behaved as wild-type Pap1 regarding this conformational change, whereas Pap1.C278A or Pap1.C285S did not show any change in electrophoretic mobility upon stress. Consistent with the above characterization, while wild-type Pap1 was recruited to stress promoters in a H_2O_2-dependent manner, mutant Pap1.C278A or Pap1.C285S proteins were not detected at these chromosomal loci, as determined by chromatin immuno-precipitation (Fig. 1E). Furthermore, cells expressing the mutant

Pap1.C278A or Pap1.C285S were unable to engage the Pap1-dependent antioxidant response, as shown by Northern blot analysis (Fig. 1F). The conclusion from these experiments is that two Cys residues at the nCRD, Cys278 and Cys285, are absolutely required to sense and transduce the H_2O_2 signal.

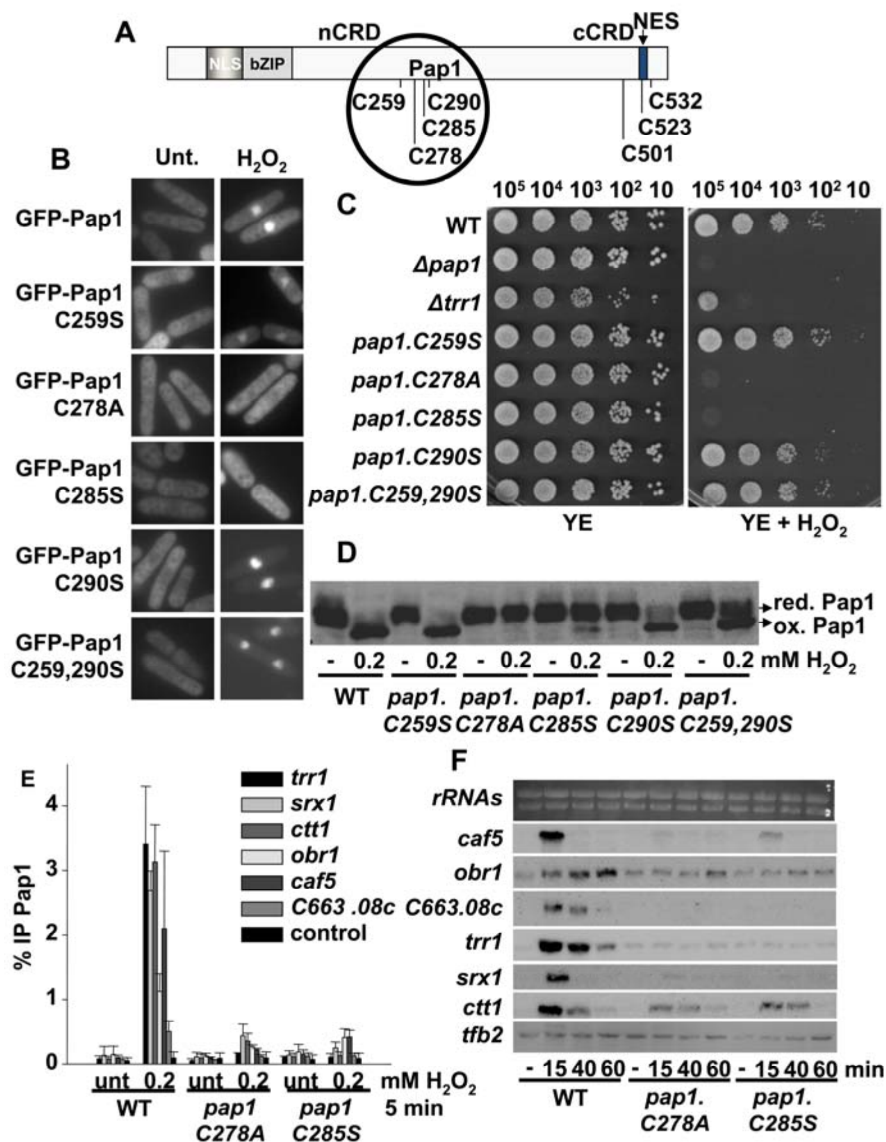


Figure 1. Characterization of the role of the three Cys residues located at the nCRD of Pap1. Cys278 and Cys285 are required for H_2O_2 sensing. **A.** Schematic representation of Pap1. **B.** The cellular distribution of GFP-Pap1 in wild-type (EHH14) and mutant strains treated or not with 0.2 mM H_2O_2 for 5 min. **C.** Sensitivity to H_2O_2 exposure. **D.** In vivo oxidation of Pap1. Reduced/inactive (red.) and oxidized/active (ox.) Pap1 forms are indicated with arrows. **E and F.** Reduced and cytoplasmic Pap1 mutants are not recruited to promoters and do not induce the transcription after stress.

While both Cys532 and Cys501 of the cCRD are essential for Pap1 nuclear accumulation, Cys501 is only partially required for the activation of the antioxidant response

We performed the same analysis for the Cys residues of the cCRD of Pap1. This domain surrounds the nuclear export signal (NES) of Pap1, and some specific substitutions alter the interaction with the export machinery even under untreated conditions (Castillo, Ayte et al. 2002). This is the case of Pap1.C523D, which displays constitutive nuclear localization (Fig. 2B) (Castillo, Ayte et al. 2002), high sensitivity to peroxides (Fig. 2C) and constitutively reduced conformation (Fig. 2D); this may be due to the exclusion of the upstream sensor Tpx1 from the nucleus (Calvo, Garcia et al. 2012). When mutated to alanine, Cys523 does not seem to have any role on the H_2O_2-dependent redox rely: GFP-Pap1.C523A accumulates at the nucleus upon stress (Fig. 2B), and cells expressing Pap1.C523A display wild-type sensitivity to peroxides (Fig. 2C), and the mutant protein displays a shift in mobility under non-reducing electrophoresis, as wild-type Pap1 does (Fig. 2D).

Pap1 carrying a Cys-532-Thr mutation behaves very similar to the above characterized mutants Cys278A and Cys285S: it does not respond to peroxides (Fig. 2). Similarly, a Cys-501-Ala mutation does not allow the nuclear accumulation of Pap1 at the nucleus upon stress (Fig. 2B), and Pap1.C501A does not display a mobility shift upon exposure to H_2O_2 (Fig. 2D). However, while the sensitivity to peroxides of cells expressing Pap1.C532T is similar to that of *Δpap1* cells, *pap1.C501A* cells only display moderate sensitivity to peroxides (Fig. 2C). Concomitantly, Pap1.C532T is not recruited to stress promoters upon H_2O_2 nor triggers an antioxidant transcriptional response (Fig. 2EF), whereas cells expressing Pap1.C501A are partially able to accumulate mutant Pap1 at promoters and to induce gene activation. We conclude that while Cys532 is also indispensable for the H_2O_2-dependent signal transduction, Cys501 has a secondary role which may be redundant to the other Cys residue left at the cCRD, Cys523. We can only hypothesize that in this particular mutant the other two Cys at the cCRD (Cys523 and Cys532) can substitute the role of Cys501. Unfortunately, accumulation of several mutations at the cCRD alters the structure of the NES, disrupts the interaction of the transcription factor with the export machinery and leads to constitutively nuclear Pap1 mutants (Castillo, Ayte et al. 2002).

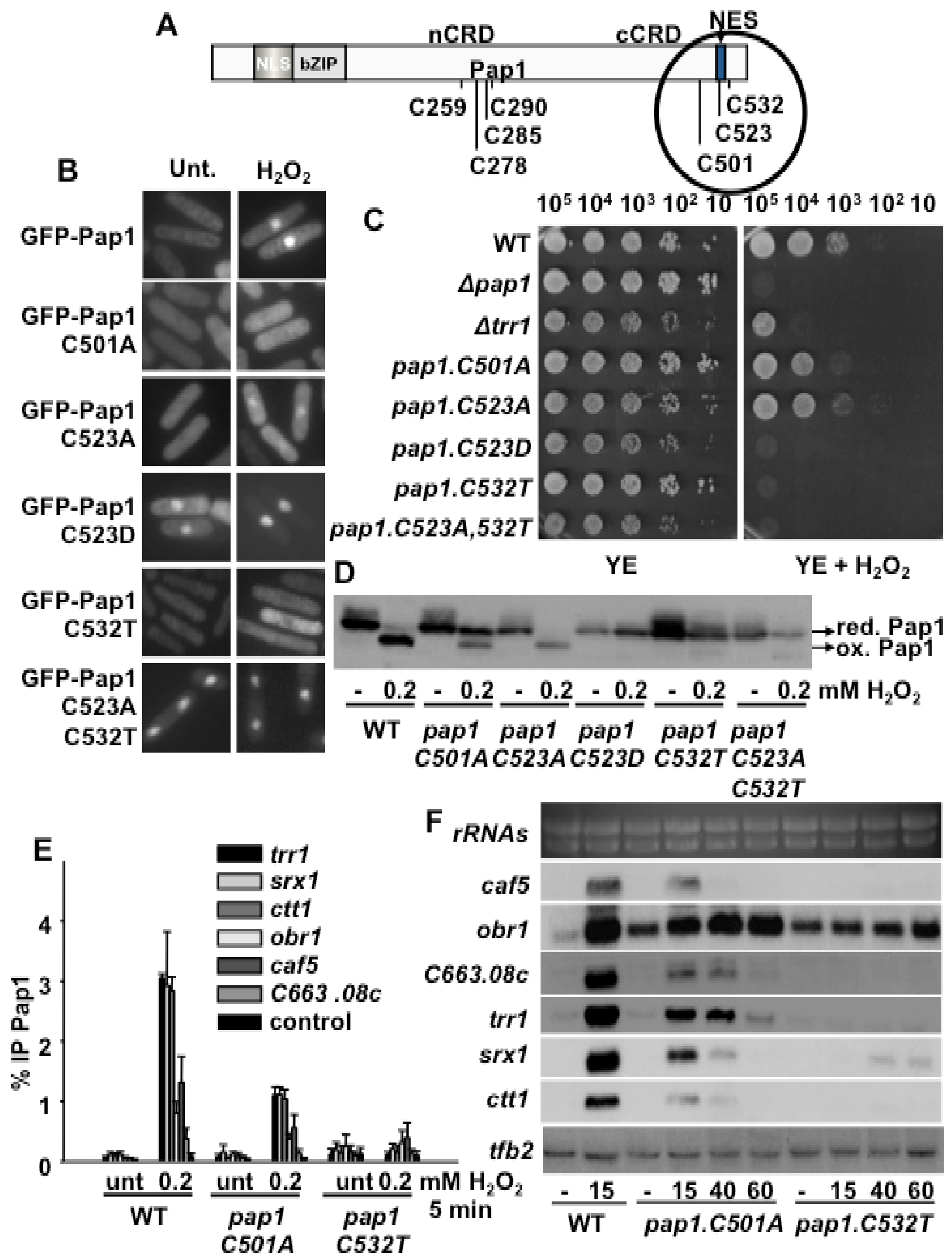


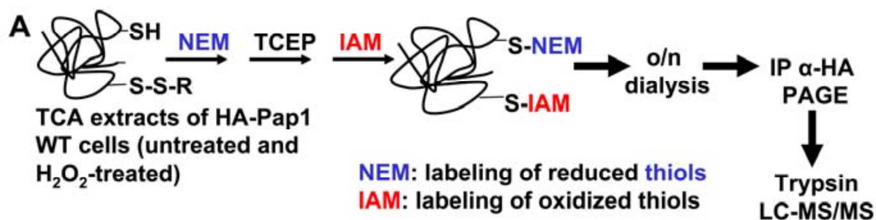
Figure 2. Characterization of the role of the three Cys residues located at the cCRD of Pap1: Cys501 and Cys532 are required for H_2O_2 sensing. A. Schematic representation of Pap1**. B** The cellular distribution of GFP-Pap1. **C.** Sensitivity to H_2O_2 exposure. **D.** In vivo oxidation of Pap1. **E and F.** Pap1.C501A mutant is sometimes recruited to all Pap1-dependent promoters and partially induces gene activation upon stress.

Proteomic identification of reduced and reversibly oxidized thiols in Pap1 before and after stress

To confirm the relevant role of Cys residues 278, 285, 501 and 532 of Pap1 in redox transduction, we purified wild-type GFP-HA-Pap1 from fission yeast cells exposed or not to H_2O_2, and analysed the redox state of all seven Cys residues. To do so, we obtained acidic cell extracts, to freeze the redox state of Cys residues, and blocked all free thiols with N-ethylmaleimide. Then, after sequential reduction of reversibly oxidized thiols and further alkylation with iodoacetamide of the newly exposed groups, we dialyzed the extracts to solubilize denatured proteins, and then immuno-precipitated GPF-HA-Pap1 with anti-HA anitbodies. We loaded the immuno-precipitates in SDS-PAGE gels, and gel slices corresponding in molecular weight to GFP-HA-Pap1 were trypsinized and peptides were analysed by LC/MS-MS (Fig. 3A). The results of this analysis is depicted in Fig. 3B and Fig. 3C. While all seven Cys residues in Pap1 appear as reduced under untreated conditions, four of them were alkylated with iodoacetamide (and therefore were reversibly oxidized) after exposure to H_2O_2: Cys278, 285, 501 and 532. These experiments confirm the essential role of four Cys residues in Pap1 activation.

The study we have performed here is essential for understanding the molecular events that rule a redox signalling cascade. It has been proposed that only Cys residues from glutathione peroxidases and peroxiredoxins have the capacity to initiate these processes in response to H_2O_2 (Winterbourn 2008; Winterbourn and Hampton 2008). In the case of Pap1, a transcription factor that indirectly responds to H_2O_2, the peroxiredoxin Tpx1 is the upstream sensor of H_2O_2 (Bozonet, Findlay et al. 2005; Vivancos, Castillo et al. 2005). While it is likely that only a peroxiredoxin such as Tpx1 can sense fluctuations in H_2O_2 and initiate this signalling process, it remains unclear how the transduction of the signal towards Pap1 takes place. We suspect that an internal disulfide in Tpx1 could oxidize Pap1 by thiol-to-disulfide exchange, since both Cys residues in the peroxiredoxin are required for Pap1 activation (Bozonet, Findlay et al. 2005; Vivancos, Castillo et al. 2005). Thiol disulfide exchange takes place by the transient formation of a mixed disulfide between the oxidative and reducing substrates, which could be trapped with a particular mutation in the resolving Cys of the electron donor of this

redox couple exchange. Therefore, we expect that in extracts from cells expressing one of the four Cys mutants (Pap1.C278A, Pap1.C285S, Pap1.C501A or Pap1.C532T) the mixed disulfide with Tpx1 could be exacerbated. The corresponding experiments are on their way.



B

SH-NEM S-S-IA	untreated	treated
K.SITHLHHDC259(NEM)SALSNGENGEDVADGK.Q	2.0 m	
K.SITHLHHDC259(NEMmetox)SALSNGENGEDVADGK.Q		17.5 m
K.QFC278(IA)QK.L		20 m
SITHLHHDC259(NEM)SALSNGENGEDVADGKQFC278(IA)QK		20 m
K.LSTAC285(IA)GSIAC290(NEMmetox)SMLTK.T	5.0 m	38.2 m
K.LSTAC285(NEM)GSIAC290(NEMmetox)SMLTK.T	142.5 m	17.5 m
RAYLSC501(NEM)PKV	665 m	171 m
RAYLSC501(IA)PKV	23 m	129 m
R.FESFDID DLC523(NEM)SK.L	86.8 m	3.3 m
R.FESFDID DLC523(IA)SK.L	3.6 m	1.3 m
K.AKC532(NEM)SSSGVLLD ER.D	246 m	20 m
K.AKC532(IA)SSSGVLLD ER.D	1.6 m	10.3 m

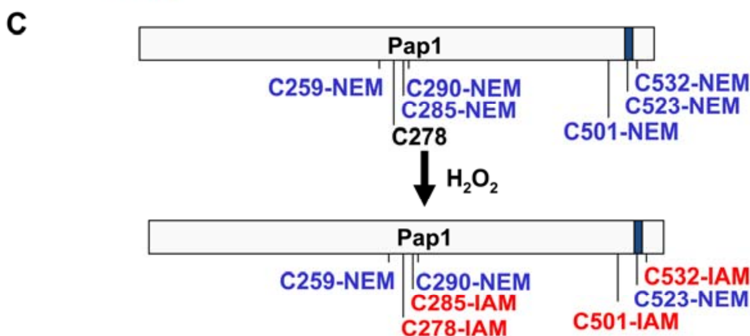


Figure. 3. Proteomic analysis of H_2O_2-dependent reversible thiol oxidation in Pap1. A. TCA protein extracts were obtained and thiols (SH) were initially alkylated with NEM. Upon reduction of oxidized thiols (SSR) with TCEP, resulting thiols (SH) were biotin-labeled with IAM. After renaturing the protein extracts by dialysis, GFP-HA-Pap1 was immuno-precipitated with anti-HA antibody (crosslinked with beads previously) and further enriched by non-reducing PAGE and analysed by LC-MS/MS. **B.** Identification of four cysteine residues essentials for activity of Pap1. Table with the areas of peptides alkylated with NEM (SH peptides) or IAM (SS peptides) in two samples (treated or not with 0.2 mM H_2O_2 for 5min). **C** Scheme depicting the final outcomes of the analysis of the redox-state of Pap1 Cys residues by LC-MS/MS.

METHODS

Yeast strains and growth conditions

The origins and genotypes of strains used in this study are outlined in Supplementary Table S1. Cells were grown in rich medium (YE) or in synthetic minimal medium as described previously (Alfa, Fantes et al. 1993).

Plasmids

The plasmid p85.41x' and its mutant derivatives (Castillo, Ayte et al. 2002), containing the *GFP-pap1* coding region at the *leu1* locus were used. In order to construct new *pap1* mutant alleles, p85.41x' was used as a template for PCR reactions using pairs of mutagenic and complementary primers containing the codon change of interest (Castillo, Ayte et al. 2002), yielding plasmids p85.41x'.C259S, p85.41x'.C285S and p85.41x'.C290S. Double and triple Cys substitutions were generated using the single-codon mutants as templates of second or third mutagenesis reactions. All the mutations were confirmed by sequencing. The p85.41x' plasmid derivatives were used to generate chromosomal insertions of *GFP*-tagged *pap1* genes into the *leu1* locus of strain EHH108, as described before (Castillo, Ayte et al. 2002).

Fluorescence microscopy

Fluorescence microscopy and image capture was performed as described before (Vivancos, Castillo et al. 2004).

H_2O_2 sensitivity assay

For survival on solid plates, *S. pombe* strains were grown, diluted and spotted in YE5S agar plates, containing 2 mM H_2O_2 or 15 mM caffeine, as described previously (Calvo, Gabrielli et al. 2009).

Preparation of *S. pombe* TCA extracts and immunoblot analysis.

To analyze the *in vivo* redox state of Pap1, trichloroacetic acid (TCA) extracts were prepared as described elsewhere (Vivancos, Castillo et al. 2005). Pap1 was immuno-detected using polyclonal anti-Pap1 antibodies (Vivancos, Castillo et al. 2004).

RNA analysis.

Total RNA from *S. pombe* minimal media cultures was obtained, processed and transferred to membranes as described previously (Castillo, Ayte et al. 2002). Membranes were hybridized with [α-32P] dCTP-labelled *caf5*, *obr1*, SPCC663.08c, *trr1*, *srx1* or *ctt1* probes, containing the complete open reading frames. We used ribosomal RNA or *tfb2* as loading controls.

Chromatin immuno-precipitation.

The *in vivo* binding of Pap1 to stress promoters was analysed as described previously (Calvo, Garcia et al. 2012), using polyclonal antibodies againt Pap1 (Vivancos, Castillo et al. 2004).

TCA extracts to detect Cys modifications in Pap1 by proteomics analysis

300-ml cultures of strain EHH14, expressing GFP-HA-Pap1, at on OD_{600} of 0.5, were treated or not with 0.2 mM H_2O_2 for 5 min. The redox state of free thiols was frozen by addition of 100% TCA to a final concentration of 10% to the cell cultures. Each 300 ml-culture was distributed in six aliquots and each of these aliquots was pelleted, washed with 20% TCA and lysed with glass beads in a BioSpec Minibeadbeater with 750 μl of 12.5% TCA. Cell lysates were then pelleted, washed twice in cold acetone and dried. Free thiols were blocked by resuspending each pellet in 750 μl of alkylating buffer (100 mM Tris–HCl pH 8, 1 mM EDTA and 1% SDS) containing 100 mM N-ethylmaleimide (NEM, Sigma-Aldrich, St. Louis, MO, USA), and incubated at 30°C for 60 min in the dark. Aggregates were spun down by centrifugation and proteins were precipitated by addition of 1 vol of 20% TCA and incubated at −20°C for 10 min. A TCA protein pellet was obtained, washed twice in cold acetone and dried. The resulting pellet was dissolved in alkylating buffer. At this point, the six aliquots from a single treatment were pooled and protein concentration was determined by Bradford protein assay (BioRad, Hercules, CA, USA). The dialysis (for renaturing our protein extracts) was performed using Slide-A-Lyzer G2 dialysis cassettes of 3 ml-capacity (Thermo Scientific). We used two cassettes for each point, using 300 times the volume of the sample of dialysis buffer (NET, 20 mM Tris-HCl

pH 8, 100 mM NaCl and 1 mM EDTA). Briefly, we dialyzed for 1 h at 4ºC, changed the buffer, dialyzed for another hour, and change the buffer and dialyzed overnight. 3-mg of renatured protein extracts were immune-precipitated with monoclonal anti-HA antiserum (12CA5); the antibodies were previously cross-linked to protein G sepharose. After 2 h of incubation, immuno-precipitates were washed three times with the same buffer and eluted from beads with PAGE sample buffer without DTT and electrophoretically separated by non-reducing SDS/PAGE. After Coomassie colloidal staining, the gel slice corresponding to Pap1 was send to proteomics to detection of disulfide bonds. To verify to Pap1 migration on gels, 10 µg of total extracts before and after dialysis and 100 ug of the immunoprecipitates were also resolved on SDS/PAGE, but transferred to nitrocellulose membranes and Pap1 was immune-detected using polyclonal anti-Pap1 antibody (Vivancos, Castillo et al. 2004). Perform the reduction and the second alkylation of the sample only if needed to differential alkylation of free thiols *versus* oxidized thiols. Reversibly oxidized Cys were then reduced by resuspending each pellet in 500 µl of a solution containing 10 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Sigma-Aldrich, St. Louis, MO, USA) in alkylating buffer instead only alkylating buffer followed by incubation at 30°C for 30 min. TCA protein precipitation and acetone washing removed excess of TCEP. To label the newly reduced thiols, the resulting pellet was resuspended in 500 µl of alkylating buffer containing 100 mM iodoacetamide (IAM, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 25°C in the dark for 1 h. TCA protein precipitation and acetone washing removed unbound IAM.

Mass Spectrometry Analysis

Gel slices of GFP-HA-Pap1 (see above) were in-gel digested with trypsin. After digestion, samples were analysed by LC-MS/MS on an LTQ-Orbitrap Velos fitted with a nanospray source previous nanoLC separation in an EasyLC system (Proxeon). Peptides were separated in a reverse phase column, 100 µm x 150 mm (Nikkyo Technos Co.,L) with a gradient of 12 to 36% ACN with 0.1% FA** in 58 min at a flow of 0.5µl/min. The Orbitrap Velos was operated in positive ion mode with nanospray voltage set at 2.1 kV and source temperature at 250°C. The instrument was externally calibrated using Ultramark 1621 for the FT mass analyzer. An internal

calibration was performed using the background polysiloxane ion signal at m/z 445.120025 as the calibrant. The instrument was operated in DDA mode. In all experiments, full MS scans were acquired over a mass range of m/z 350-2000 with detection in the Orbitrap mass analyzer at a resolution setting of 30,000. For each MS scan, the twenty most intense ions with multiple charged ions above a threshold ion count of 5,000 were selected for fragmentation at normalized collision energy of 35% in the LTQ linear ion trap. All data were acquired with Xcalibur 2.1 software. Data was searched against Gene_DB_SPombe database using an internal version of the search algorithm Mascot v.2.2 (http://www.matrixscience.com/). Peptides were filtered using a Mascot Ion Score of 20.

ACKNOWLEDGEMENTS

We thank Mercè Carmona for technical assistance. We are grateful to the joint CRG-UPF Proteomic Facility (Barcelona, Spain), where the mass spectrometry experiments were performed, and especially thank Cristina Chiva and Guadalupe Espadas for their in-deep participation in the project. This work was supported by the Spanish Ministry of Science and Innovation (BFU2009-06933, BFU2012-32045), PLAN E and FEDER, by the Spanish program Consolider-Ingenio 2010 Grant CSD 2007-0020, and by SGR2009-196 from Generalitat de Catalunya (Spain) to E.H. and J.A. are recipients of ICREA Academia Awards (Generalitat de Catalunya).

AUTHOR CONTRIBUTIONS

I.A.C. performed all the experiments. E.H. and J.A. guided the research and E.H. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

REFERENCES

1. Zheng M, Aslund F, Storz G (1998) Activation of the OxyR transcription factor by reversible disulfide bond formation [see comments]. *Science* **279:** 1718-1721

2. Kim SO, Merchant K, Nudelman R, Beyer WF, Jr., Keng T, DeAngelo J, Hausladen A, Stamler JS (2002) OxyR: a molecular code for redox-related signaling. *Cell* **109:** 383-396

3. Delaunay A, Isnard AD, Toledano MB (2000) H(2)O(2) sensing through oxidation of the Yap1 transcription factor. *EMBO J* **19:** 5157-5166

4. Okazaki S, Tachibana T, Naganuma A, Mano N, Kuge S (2007) Multistep disulfide bond formation in Yap1 is required for sensing and transduction of H2O2 stress signal. *Mol Cell* **27:** 675-688

5. Toda T, Shimanuki M, Yanagida M (1991) Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. *Genes Dev* **5:** 60-73

6. Toone WM, Kuge S, Samuels M, Morgan BA, Toda T, Jones N (1998) Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1 [published erratum appears in Genes Dev 1998 Aug 15;12(16):2650] [see comments]. *Genes Dev* **12:** 1453-1463

7. Vivancos AP, Castillo EA, Jones N, Ayte J, Hidalgo E (2004) Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration. *Mol Microbiol* **52:** 1427-1435

8. Castillo EA, Ayte J, Chiva C, Moldon A, Carrascal M, Abian J, Jones N, Hidalgo E (2002) Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. *MolMicrobiol* **45:** 243-254

9. Castillo EA, Vivancos AP, Jones N, Ayte J, Hidalgo E (2003) Schizosaccharomyces pombe cells lacking the Ran-binding protein Hba1 show a multidrug resistance phenotype due to constitutive nuclear accumulation of Pap1. *J Biol Chem* **278:** 40565-40572

10. Vivancos AP, Castillo EA, Biteau B, Nicot C, Ayte J, Toledano MB, Hidalgo E (2005) A cysteine-sulfinic acid in peroxiredoxin regulates H2O2-sensing by the antioxidant Pap1 pathway. *Proc Natl Acad Sci U S A* **102:** 8875-8880

11. Bozonet SM, Findlay VJ, Day AM, Cameron J, Veal EA, Morgan BA (2005) Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide. *J Biol Chem* **280:** 23319-23327

12. Calvo IA, Garcia P, Ayte J, Hidalgo E (2012) The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H2O2. *Nucleic Acids Res* **40:** 4816-4824

13. Winterbourn CC (2008) Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol* **4:** 278-286

14. Winterbourn CC, Hampton MB (2008) Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med* **45:** 549-561

15. Alfa C, Fantes P, Hyams J, McLeod M, Warbrick E (1993) *Experiments with Fission Yeast: A Laboratory Course Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

16. Calvo IA *et al* (2009) Genome-wide screen of genes required for caffeine tolerance in fission yeast. *PLoS One* **4:** e6619.

Calvo et al, Supplementary information includes: Supp. Table S1 and references there-in.

Supplementary Table S1. Strains used in this study

Strain	Genotype	Origin
NG25	h^+ *caf4/trr1::ura4*$^+$ *ura4-D18*	[1]
EHH14	h^- *his2 ura4 pap1::ura4*$^+$ *leu1–32 nmt::GFP-HÁ-pap1::leu1*$^+$	[2]
EHH14.C523D	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C523D::leu1*$^+$	[2]
EHH14.C278A	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C278::leu1*$^+$	[2]
EHH14.C501A	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C501A::leu1*$^+$	[2]
EHH14.C523A	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C523A::leu1*$^+$	[2]
EHH14.C532T	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C532T::leu1*$^+$	[3]
EHH14.C523A,C532T	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C523AC532T::leu1*$^+$	This work
EHH14.C259S	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C259S::leu1*$^+$	This work
EHH14.C285S	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C285S::leu1*$^+$	This work
EHH14.C290S	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C290S::leu1*$^+$	This work
EHH14.C259S,C290S	h^- *his2 ura4 pap1::ura4-D18 leu1–32 nmt::GFP-HÁ-pap1C259SC290S::leu1*$^+$	This work
IC2	h^- *pap1 ura4-D18 leu1-32*	[4]
IC1	h^- *pap1::ura4*$^+$ *ura4-D18 leu1-32*	[4]
IC2.C523D	h^- *pap1.C523D ura4-D18 leu1-32*	[4]
IC2.C259S	h^- *pap1.C259S ura4-D18 leu1-32*	This work
IC2.C278A	h^- *pap1.C278A ura4-D18 leu1-32*	This work
IC2.C285S	h^- *pap1.C285S ura4-D18 leu1-32*	This work
IC2.C290S	h^- *pap1.C290S ura4-D18 leu1-32*	This work
IC2.C259S,C290S	h^- *pap1.C259SC290S ura4-D18 leu1-32*	This work
IC2.C501A	h^- *pap1.C501A ura4-D18 leu1-32*	This work
IC2.C523A	h^- *pap1.C523A ura4-D18 leu1-32*	This work
IC2.C532T	h^- *pap1.C532T ura4-D18 leu1-32*	This work
IC2.C523A,C532T	h^- *pap1.C523AC532T ura4-D18 leu1-32*	This work

REFERENCES

1. Calvo IA *et al* (2009) Genome-wide screen of genes required for caffeine tolerance in fission yeast. *PLoS One* **4:** e6619

2. Castillo EA, Ayte J, Chiva C, Moldon A, Carrascal M, Abian J, Jones N, Hidalgo E (2002) Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. *MolMicrobiol* **45:** 243-254

3. Vivancos AP, Castillo EA, Biteau B, Nicot C, Ayte J, Toledano MB, Hidalgo E (2005) A cysteine-sulfinic acid in peroxiredoxin regulates H2O2-sensing by the antioxidant Pap1 pathway. *Proc Natl Acad Sci U S A* **102:** 8875-8880

4. Calvo IA, Garcia P, Ayte J, Hidalgo E (2012) The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H2O2. *Nucleic Acids Res* **40:** 4816-4824.

3. ROLE OF Tpx1 AND THE THIOREDOXIN SYSTEM IN Pap1 ACTIVATION

In fission yeast, Pap1 suffers H_2O_2-dependent oxidation that promotes its nuclear accumulation and the activation of an antioxidant gene program. Tpx1 is essential to transduce the H_2O_2 signal to Pap1, since in cells lacking the peroxiredoxin, Pap1 oxidation does not occur and its gene activation is inhibited (Bozonet, Findlay et al. 2005; Vivancos, Castillo et al. 2005). Thioredoxin reductase also seems to be required for the inactivation of Pap1, since cells lacking the *trr1* gene constitutively accumulate nuclear Pap1 (Vivancos, Castillo et al. 2004). The molecular bases of these activation/inactivation processes have been the focus of study of my PhD project.

3.1. Tpx1 IS THE MAIN ACTIVATOR OF Pap1

During the course of our project, we have generated a large number of strains lacking one or several components of the Tpx1 and thioredoxin system to understand better the mechanism of activation of Pap1. As will be shown below, our experiments clearly indicate that the peroxiredoxin Tpx1 is essential for Pap1 activation: Pap1 oxidation, Pap1 recruitment to stress promoters and Pap1-dependent gene activation after H_2O_2 stress are completely abolished in Δ*tpx1* cells. This requirement applies to all the different strain backgrounds that we have generated during the last years. However, only two strain backgrounds are able to bypass this strict requirement.

On the one hand, cells lacking *trr1* gene is the first exception to this rule: cells lacking both Trr1 and Tpx1 display constitutive activation of Pap1 and consequently constitutive induction of Pap1-dependent genes. Furthermore, we believe that cells lacking Trr1 suffer from disulfide stress, and that eliminates the need for proper H_2O_2-dependent signalling cascades.

On another hand, the lack of Tpx1 can be overcome through the over-expression of another peroxiredoxin, Gpx1. The lack of specific interactions between Pap1 and Gpx1 is bypassed by the massive over-expression of Gpx1. Furthermore, since this peroxiredoxin has a lower Km

for H_2O_2 than Tpx1, the activation of Pap1 only occurs upon high concentrations of peroxides.

3.2. Trx1 CAN BE REGULATING THE H_2O_2-INDUCED ACTIVATION AND INACTIVATION OF Pap1

The thioredoxin system was proposed to mediate the maintenance of Pap1 in the reduced protein conformation and to inactivate the cascade once the response has been triggered Vivancos, Castillo et al. 2004)We first decided to determine which one of the three thioredoxins presents in *S. pombe* was the actual electron donor in this inactivation system. We only saw some differences with respect to wild-type strain in cells lacking Trx1. Our surprise was that the depletion of Trx1 did not trigger a constitutive oxidation of Pap1 according to non-reducing electrophoresis. In fact, oxidation of Pap1 upon H_2O_2 in Δ*trx1* strain was clearly impaired. However, even though the gene expression program profile was changed in cells lacking Trx1 (a partial activation under untreated conditions and a prolonged activation after stress imposition), the response was there, as well as the binding of Pap1 to promoters. The same phenotype was observed in the double mutant Δ*trx1* Δ*trx3*. To determine whether the basal and H_2O_2-triggered activation of the Pap1 gene response in cells lacking Trx1 was dependent on Tpx1, we analyzed the activation of Pap1 by TCA protein extracts, ChIP and Northern blot in Δ*trx1* Δ*tpx1* strain, and everything was completely eliminated in this strain background.

To explain the important, but not indispensable, role of Trx1 on Pap1 activation, we have carefully analyzed which thioredoxin performs the recycling of Tpx1 *in vivo*. We have fully dissected the Tpx1 cycle and the primary role of Trx1 in its reduction, as well as the secondary role of other Tpx1 electron donors, such as Trx3 and the same Pap1.

We conclude that Trx1 modulates, but it is not essential, for Pap1 activation. Its role in the Tpx1 cycle may explain the effects on the activity of the trasncription factor.

The detailed description of this section 3 is provided in the format of a manuscript submitted for publication (see below).

Dissection of a redox relay: the peroxiredoxin Tpx1 and the transcription factor Pap1 are oxidized by H_2O_2 and reduced by thioredoxin

Isabel A. Calvo, Susanna Boronat, Sarela García-Santamarina, Alba Domenech, José Ayté and Elena Hidalgo[1,*]

[1]Oxidative Stress and Cell Cycle Group, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, C/ Dr. Aiguader 88, E-08003 Barcelona, Spain;

*Corresponding author: Elena Hidalgo, Universitat Pompeu Fabra, C/ Dr. Aiguader 88, E-08003 Barcelona, Spain (elena.hidalgo@upf.edu; Tel: 34-93-316-0848; Fax: 34-93-316-0901)

Running title:

Keywords: h2o2 sensor, peroxiredoxin, Pap1, thioredoxin, fission yeast

ABSTRACT

H_2O_2 can act as a toxic molecule that inactivates proteins, but is also a potent activator of redox signalling cascades by oxidizing specific cysteine residues in target proteins. In fission yeast, the transcription factor Pap1 suffers H_2O_2-dependent oxidation that promotes its nuclear accumulation and the activation of an antioxidant gene program. However, the mechanisms that regulate the sensitivity and selectivity of Pap1 activation by peroxides are not fully understood. Here, we demonstrate that the peroxiredoxin Tpx1, the real sensor of this signalling cascade, activates the otherwise unresponsive Pap1 protein once the main cytosolic reduced thioredoxin, Trx1, becomes transiently depleted. In other words, Pap1 is an alternative electron donor for oxidized Tpx1. Recycling of Tpx1 by Trx1 is required for the efficient signalling towards the transcription factor, what suggests that the complete cycle of H_2O_2-scavenging by Tpx1 and further recycling of oxidized Tpx1 by Trx1 is required for full downstream activation of the redox cascade.

INTRODUCTION

Cysteine (Cys) residues in proteins are essential mediators of catalytic reactions. Thus, many enzymes experience the reversible oxidation of catalytic Cys, often to form intra or intermolecular disulfides, as part of their enzymatic activities. That is the case for enzymes such as ribonucleotide reductase, methionine sulfoxide reductases, PAPS reductases and glutathione or thioredoxin peroxidases. Their catalytic cycles are completed through disulfide reduction by electron donors of the thioredoxin and glutaredoxin families, which recycle the enzymes back to their reduced thiol-stages (for recent reviews on thioredoxins and glutaredoxins, see (Meyer, Buchanan et al. 2009; Collet and Messens 2010; Stroher and Millar 2012).

Thiol groups in Cys residues had also been proposed to become accidentally oxidized by hydrogen peroxide (H_2O_2) and other oxidants towards higher oxidation states, such as sulfenic acid (SOH), disulfides, sulfinic and sulfonic acids, leading to the inactivation of the oxidized Cys-containing proteins. However, recent biochemical observations indicate that Cys in general are not very reactive towards peroxides, and that only highly reactive thiols in proteins may respond to physiological fluctuations of H_2O_2. Therefore, oxidation of these highly reactive Cys could constitute a signalling event that allows activation of antioxidant cellular responses to peroxide stress. These so-called H_2O_2 sensors should then count on an exposed and reactive Cys, with its signal-mediated oxidation triggering a downstream redox cascade.

In particular, glutathione peroxidases and thioredoxin peroxidases (peroxiredoxins) seem to have ideal Cys residues to respond to H_2O_2. Kinetic parameters of reactivity towards oxidants suggest that these enzymes, which are peroxide scavengers, are likely to be the only direct targets for moderate concentrations of H_2O_2. That allows their action, detoxification of peroxides, but also proposes the basis of oxidative stress-dependent signalling. Thus, glutathione peroxidases and peroxiredoxins have been proposed to mediate the oxidation/activation of downstream components of antioxidant cascades, and provide the molecular bases of sensitivity and specificity in H_2O_2 signalling.

The thioredoxin peroxidase Gpx3/Orp1 of *Saccharomyces cerevisiae* was described by Toledano and colleagues to initiate a signalling cascade in response to H_2O_2 (Delaunay, Pflieger et al. 2002). The authors proposed that upon peroxide treatment a reactive thiol group in Gpx3 oxidizes to SOH, which would then react with a Cys residue in the transcription factor Yap1. The intermolecular disulfide is then resolved by another Cys residue in Yap1, yielding an intramolecular disulfide affecting the protein structure and inducing its nuclear accumulation (Delaunay, Isnard et al. 2000; Delaunay, Pflieger et al. 2002). This transcription factor triggers a gene expression program mediating the cellular defence to oxidative stress.

Similarly, *Schizosaccharomyces pombe* responds to moderate doses of H_2O_2 by activating the transcription factor Pap1 (*pombe* AP-1), which accumulates at the nucleus upon stress. Genetic and biochemical evidences suggest than Pap1 is cytosolic prior to stress imposition, and that at least one disulfide bond is formed upon peroxide addition, which triggers a conformational change impairing Crm1/exportin-mediated nuclear export (Kudo, Taoka et al. 1999; Castillo, Ayte et al. 2002; Vivancos, Castillo et al. 2004). The transcription factor is then able to transiently bind and activate 40-80 genes, whose encoded proteins are meant to counteract the stress imposed and induce and adaptation response (Chen, Toone et al. 2003; Chen, Wilkinson et al. 2008; Calvo, Garcia et al. 2012). Importantly enough, the peroxiredoxin Tpx1 is essential to transduce the H_2O_2 signal to Pap1, since in cells lacking the peroxiredoxin Pap1 oxidation does not occur and its gene activation is inhibited (Bozonet, Findlay et al. 2005; Vivancos, Castillo et al. 2005).

A remarkable difference between the budding and the fission yeast signal transduction systems relies on the mechanisms of peroxidase-mediated oxidation of the transcription factor. The system proposed for the Gpx3/Yap1 relay is based on the formation of one SOH in Gpx3 which would then react with a Cys residue in Yap1, forming a mixed disulfide intermediate. This transient intermolecular disulfide is then rearranged into an intramolecular one in Yap1, which is the active transcription factor, and the concomitant release of reduced Gpx3 (Delaunay, Pflieger et al. 2002). Alternatively to this SOH-to-disulfide relay, the fission yeast system could be using a thiol-disulfide exchange reaction, so that an

internal disulfide in Tpx1, of course preceded by oxidation of its peroxidatic Cys to an SOH, can be transferred to Pap1 as part of a redox cascade; the main evidence suggesting the need of an intramolecular disulfide, and not just an SOH in Tpx1 prior to activation of Pap1 is the fact than a mutant lacking the resolving Cys in the peroxiredoxin is not able to transduce the redox signal to Pap1 (Bozonet, Findlay et al. 2005; Vivancos, Castillo et al. 2005).

As any other signalling cascade, proper activation of the pathway also relies on negative feedback loops, both to maintain the initiators/sensors in the reduced conformation and to inactivate the cascade once the response has been triggered. In these particular Cys-mediated events, the thioredoxin system has been proposed to mediate the maintenance of the reduced protein conformations. Thus, in the budding yeast system cells devoid of the thioredoxin system, but not the glutaredoxin system, do display partial basal activation of Yap1, and upon peroxide treatment the activity of the pathway is more sustained than in a wild-type background (Delaunay, Isnard et al. 2000). In the case of fission yeast, deletion of the thioredoxin reductase gene leads to a complete activation of Pap1, as determined by the isolation of a faster migrating band under non-reducing electrophoresis corresponding to oxidized Pap1, and by the analysis of its transcriptome (Benko, Sipiczki et al. 1998; Vivancos, Castillo et al. 2004; Calvo, Garcia et al. 2012). It is worth to point out that a main substrate of reduced thioredoxins in *S. pombe* is the peroxiredoxin Tpx1, which requires this electron donor to be recycled after H_2O_2 scavenging.

In an attempt to dissect the redox relay ruling the antioxidant response to H_2O_2 in an eukaryotic organism, we have carefully analyzed the effects of mutations on the Tpx1 and thioredoxin pathways with regard to Pap1 activation. Our experiments suggest that Tpx1 is normally scavenging peroxides and uses thioredoxins to start the cycle again, and only when reduced thioredoxin becomes limiting Pap1 become an alternative electron donor for oxidized Tpx1.

RESULTS

The absence of Tpx1, thioredoxin reductase or the cytoplasmic thioredoxin Trx1 has different effects on Pap1 activation

As explained in the Introduction, both Tpx1 and the only *S. pombe* thioredoxin reductase Trr1 had been shown mainly by genetic evidences to participate in the H_2O_2-dependent activation and basal inactivation, respectively, of Pap1 (Fig. 1A). Thus, the conformational change observed in wild-type Pap1 upon mild H_2O_2 stress using non-reducing electrophoresis of TCA protein extracts and Western blot analysis is not observed at all in cells lacking Tpx1, whereas cells lacking Trr1 display constitutive activation/oxidation of Pap1 according to these SDS-PAGE gels (Fig. 1B) (Vivancos et al., 2004). Consistently, activation of the H_2O_2- and Pap1-dependent gene expression program is completely abolished in strain Δ*tpx1*, and is constitutively engaged in cells lacking Trr1 (Fig. 1C).

We decided to determine which thioredoxin mediates Pap1 reduction under basal conditions. The *S. pombe* genome contains three thioredoxin-coding genes (Wood et al., 2002). Trx1 is the main cytoplasmic thioredoxin (Song and Roe, 2008); Trx3/Txl1 has been described to be associated to ribosomes, but is also important in the antioxidant defense (Andersen et al., 2011; Jimenez et al., 2007; Kim et al., 2007); Trx2 has mitocondrial localization and function (Song et al., 2008). We confirmed the cytoplasmic localization of Trx1 and Trx3 by fluorescent microscopy of GFP-tagged proteins (data not shown) and by immuno-fluorescence of HA-tagged proteins using monoclonal antibodies against HA (Fig. 1D). Deletion of the Trx3 or Trx2-coding genes did not affect the oxidation of Pap1 (Fig. 1E). Depletion of Trx1 did not trigger a constitutive oxidation of Pap1 according to non-reducing electrophoresis. In fact, in Δ*trx1* cell extracts the H_2O_2-dependent shift in electrophoretic mobility of Pap1 was clearly impaired (Fig. 1E). To dismiss that the other cytosolic thioredoxin, Trx3, contributed to reduce Pap1 at the basal level we analyzed a Δ*trx1* Δ*trx3* strain. As shown in Fig. 1F, the pattern of Pap1 oxidation in extracts of this strain did not differ from that of Δ*trx1* extracts.

These biochemical data seemed to indicate that not only Tpx1, but also Trx1, are required for Pap1 oxidation. We then deleted *tpx1*, or *trx1*, or both in the Δ*trr1* background. In all cases Pap1 was fully oxidized and its gene expression program engaged (Fig. 2). As we have recently shown, deletion of the *trr1* gene induces general disulfide formation in approximately 50% of the Cys-containing peptides of the *S. pombe* proteome; this massive thiol oxidation is probably dependent on the generation of oxidizing redox couples (García-Santamarina *et al.,* submitted). Thus, depletion of Trr1 leads to constitutive activation of Pap1 irrespective of the presence of Tpx1 or Trx1, probably due to the generation of H_2O_2-independent disulfide stress in this strain background.

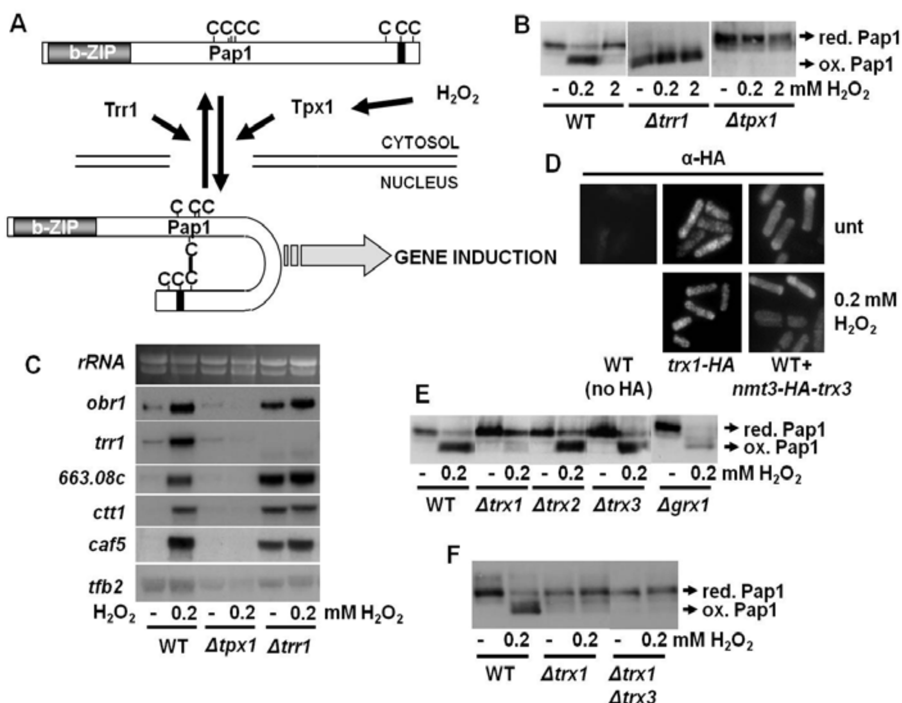


Figure 1. Pap1 oxidation state: an equilibrium between the thioredoxin system and Tpx1-H_2O_2 (A) Schematic representation of Pap1. **(B)** *In vivo* oxidation of Pap1 in wild-type and mutant strains. (**C)** Cells lacking Tpx1 abolish Pap1-dependent gene expression program **(D)** Cytosolic Localization of Trx1 and Trx3 by immuno-fluorescence. **(E and F)** Trx1 is the only thioredoxin implicated in the activation of Pap1.

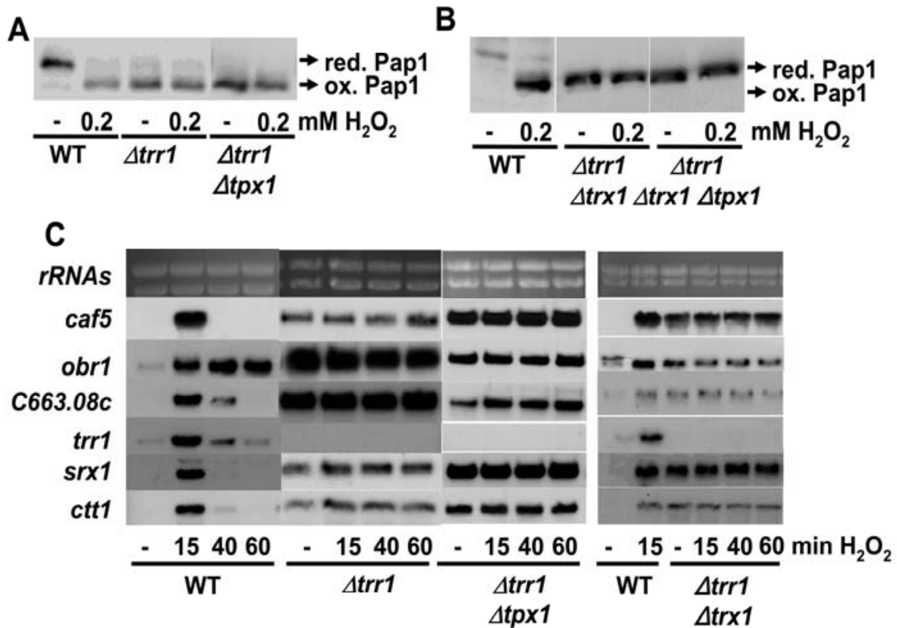


Figure 2. Pap1 is active in *Δtrr1* cells–in a Tpx1-and Trx1-independent manner. (A and B) *In vivo* oxidation of Pap1 in wild-type and mutant strains. **(C)** Constitutive activation of Pap1 dependent genes in Δ*trr1* background. Northern blot analysis of several Pap1-dependent genes.

Trx1 is the main electron donor of Tpx1; trapping of a mixed disulfide between Trx1 and Tpx1

The peroxiredoxin Tpx1 is not only a H_2O_2 sensor in the Pap1 pathway, but it is also essential to scavenge peroxides during aerobic metabolism (Jara et al., 2007). In order to dissect the Tpx1-Pap1 signalling cascade, we first decided to analyze the Tpx1 cycle. Peroxiredoxins such as Tpx1 are dimers arranged head-to-tail, which upon reaction with H_2O_2 suffer oxidation of the peroxidatic Cys48 to SOH, disulfide formation with the resolving Cys169 of the anti-parallel monomer, and release of a molecule of H_2O. Reduced thioredoxins are then acting as electron donors to recycle the covalently linked peroxiredoxin dimers to the thiol form, through the transient formation of a mixed disulfide.

To determine which thioredoxin performs the recycling of Tpx1 *in vivo*, we analyzed the basal levels of covalently linked Tpx1 dimers by non-reducing electrophoresis and Western blot analysis of TCA extracts. In wild-type cells, most Tpx1 is reduced upon basal conditions, even though is detoxifying peroxides. In extracts from 0.2 mM H_2O_2-treated wild-type cultures, two slowly migrating bands are observed, which correspond to the covalently linked dimers with one (upper) or two (lower band) disulfides (Fig. 3A). Cells lacking Trx2, Trx3 or Grx1 did show a pattern of Tpx1 oxidation identical to that of a wild-type strain. However, in Δ*trx1* cells most of Tpx1 has one intra-dimer disulfide bridge under basal conditions, and H_2O_2 stress allows the accumulation of the two-disulfide-containing dimers (Fig. 3A). Since Tpx1 is not fully oxidized in this strain background under basal conditions, we tested whether the other cytosolic thioredoxin, Trx3, could be an alternative electron donor to Tpx1 in Δ*trx1* cells. Indeed, cells lacking both Trx1 and Trx3 display full oxidation (two-disulfide bonds per Tpx1 dimer) under basal conditions (Fig. 3B). We conclude that Trx1 is the main electron donor of Tpx1, and that Trx3 can also partially reduce Tpx1 disulfides in the absence of Trx1. Confirming the important role of Trx1 in Tpx1 recycling, strain Δ*trx1* is the only deletion strain with a severe growth defect under H_2O_2 stress (Supplementary Fig. 1A).

We then decided to trap the Trx1-Tpx1 intermediates by eliminating the resolving Cys residue in Trx1. Thioredoxins interact with their substrates through the N-terminal Cys of the thioredoxin fold, which includes the sequence Cys-X-X-Cys, and the transient mixed disulfides are then resolved by nucleofilic attack with the thiol group of the C-terminal Cys of this sequence. We substituted the chromosomal *trx1* locus with mutant alleles (*trx1.C30S* or *trx1.C33S*), and demonstrated that they were both as sensitive to H_2O_2 as cells lacking Trx1 (Supplementary Fig. 1B). As shown in Fig 3C, the pattern of Tpx1 oxidation was identical in Δ*trx1* and *trx1.C30S* strains. However, two DTT-sensitive bands corresponding to Trx1-Tpx1 mixed disulfides, with or without an additional disulfide inside the Tpx1 dimer, could be detected in extracts from *trx1.C33S* cells, either using antibodies against Trx1 or Tpx1 (Fig. 3C). Interestingly, extracts from cells lacking thioredoxin reductase Trr1 also accumulated, although to a lesser extend, these mixed disulfides between Tpx1 and Trx1 (Fig. 3C). We postulated that in this Δ*trr1* background,

where Trx1 is fully oxidized, we could force the formation of this intermediate in the backwards direction by expressing a form of Tpx1 lacking the Cys not involved in the formation of the bridge. Indeed, in extracts from Δ*trr1* cells expressing Tpx1.C48S, lacking the peroxidatic Cys in Tpx1, we could clearly detect the Trx1-Tpx1 intermediate, whereas this mixed disulfide was not formed in Δ*trr1* cells expressing Tpx1.C169S (Fig. 3D). We conclude that in the normal Tpx1 cycle, the thioredoxin Trx1 breaks the disulfide between Cys48 and Cys169 of Tpx1 by disulfide exchange, and a Trx1-Cys30-Cys169-Tpx1 is transiently generated to finally release oxidized Trx1 and reduced Tpx1 (Fig. 3E).

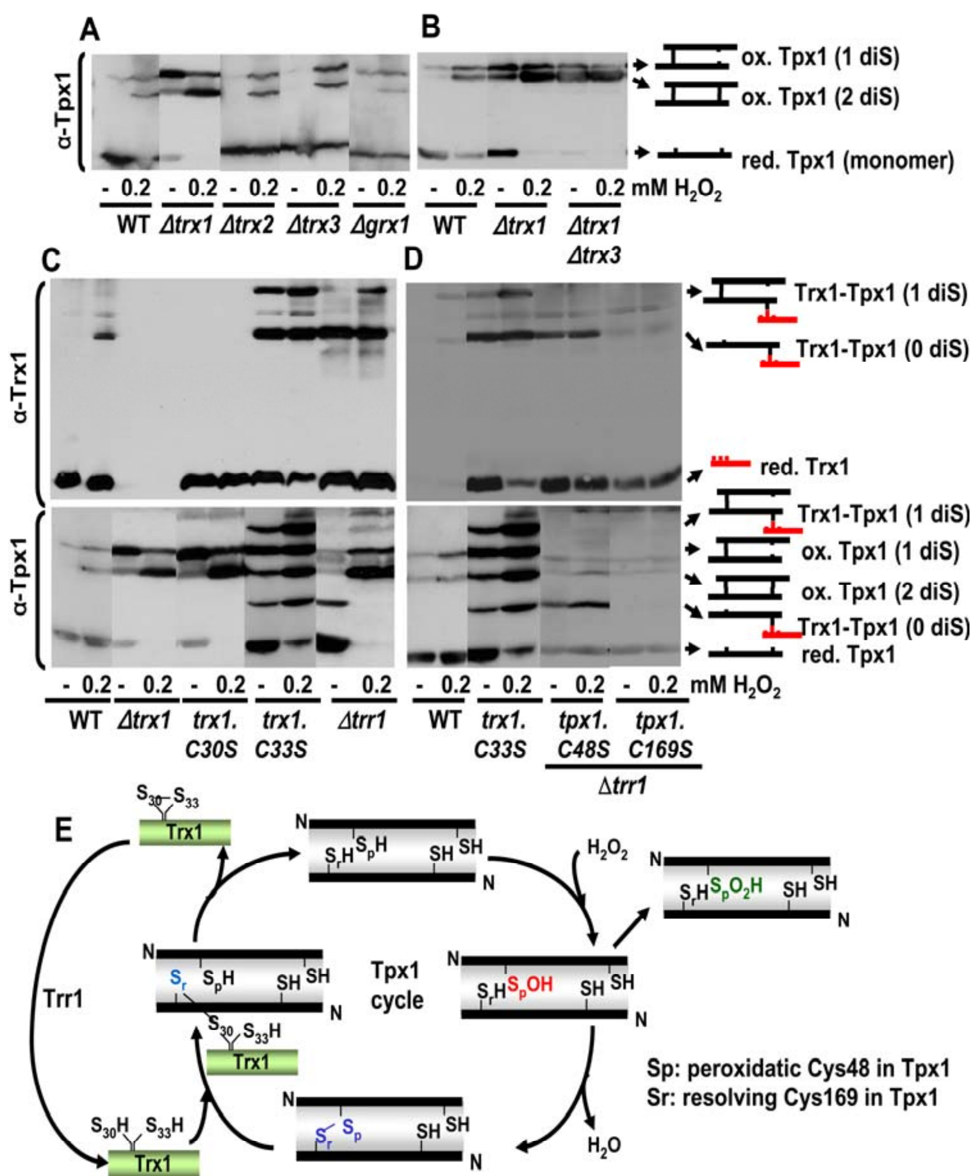


Figure 3. Trx1 is the main electron donor of Tpx1. (A and B) *In vivo* oxidation of Tpx1 in wild-type and mutant strains. Reduced and oxidized Tpx1 with one or two disulfide bonds are indicated by different schemes. **(B)** Trx3 can reduce Tpx1 in absence of Trx1. **(C and D)** Trapping of a mixed disulfide between Tpx1 and Trx1. **(C)** Cysteine 30 of Trx1 is interacting with Tpx1**. (D)** Resolving cysteine of Tpx1 is necessary for interaction with Trx1. **(E)** Trx1 breaks the intramolecular disulfide of Tpx1 by disulfide exchange leading to a transiently disulfide between Trx1-Cys30-Cys169-Tpx1 to finally release oxidized Trx1 and reduced Tpx1.

Deletion of the thioredoxin gene changes the dynamics of Pap1 oxidation by H_2O_2

Once characterized at the molecular level the Tpx1 cycle, we went back to analyze the role of Trx1 in Pap1 activation. Mild oxidative stress induced a transient oxidation of Pap1 as determined by non-reducing electrophoresis; the faster migrating band could be detected in extracts from cells lacking Trx1 even prior to stress, and H_2O_2 treatment slightly enhanced its formation (Fig. 4A). Immuno-fluorescence of untagged Pap1 demonstrated that Pap1 accumulates at the nucleus in a wild-type background upon H_2O_2 stress, and constitutive nuclear localization is observed in Δ*trr1* cells (Fig. 4B). An intermediate pattern is observed in cells lacking Trx1: a fraction of Pap1 can already be visualized at the nucleus prior to stress (Fig. 4B). Further deletion of *trx3* in the Δ*trx1* background did not exacerbate basal Pap1 nuclear localization (Fig. 4B, Δ*trx1* Δ*trx3*). Similar results were obtained with a GFP-Pap1 fusion protein using fluorescence microscopy, although Pap1 accumulation in Δ*trx1* cells was less pronounced (Supplementary Fig. 2A). The Pap1-dependent gene expression program was not eliminated in Δ*trx1* cells; basal expression of most genes was slightly up-regulated, and their mRNA levels were delayed and sustained; a similar transcriptome profile was observed for strain Δ*trx1* Δ*trx3* (Fig. 4C). In fact, Pap1 protein was detected at the Pap1-dependent promoters prior to stress imposition in a Δ*trx1* background as determined by chromatin immuno-precipitation (ChIP), and never reached the same levels as in wild-type cells upon H_2O_2 stress (Fig. 4D). The activation of the Pap1-dependent gene expression program in Δ*trx1* cells was due to Pap1 oxidation, since it was completely eliminated in Δ*trx1* cells expressing the non-oxidizable Pap1.C278A or Pap1.C285S proteins (Supplementary Fig. 3A-C). This result dismisses a putative role of Trx1 on nuclear trafficking and not directly on Pap1 oxidation levels. We conclude that Trx1 modulates, but is not essential for, the H_2O_2–dependent activation of Pap1.

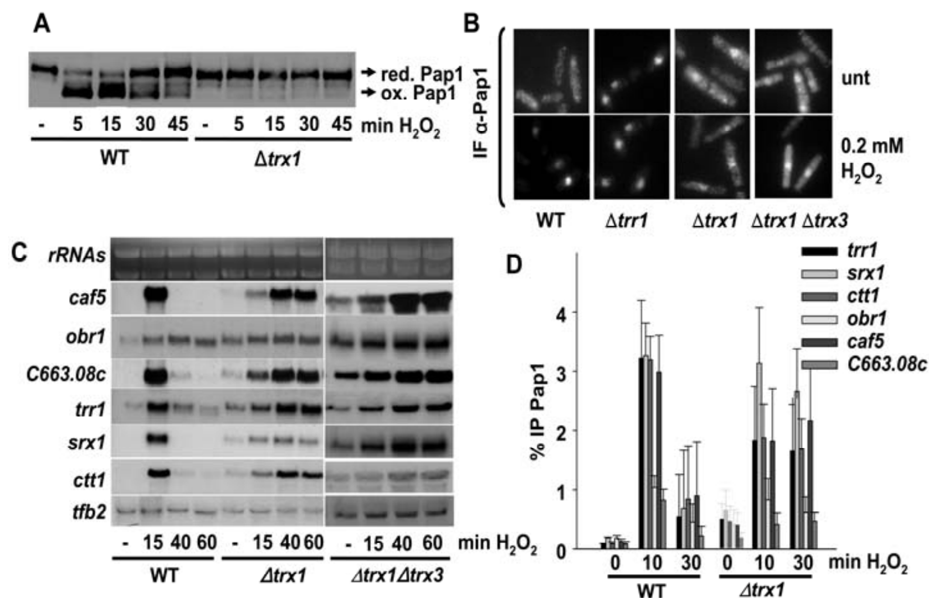


Figure 4. Loss of Trx1 prolongs H_2O_2-induced Pap1-dependent gene expression. (A) Deletion of Trx1 changes the dynamics of Pap1 oxidation by H_2O_2.. **(B)** Localization of Pap1 in wild-type and Δ*trx1* strain. The cellular distribution of GFP-Pap1 was determined by fluorescence microscopy . **(C)** Northern blot analysis of Pap1-dependent genes. **(D)** Pap1 is recruited to all Pap1-dependent promoters in Δ*trx1* background prior to stress. ChIP experiments using anti-Pap1 antibody, were performed using primers covering only promoter SA1 is capa*ble of folding YAP1, albeit* less *effectively* than the **The slow and**

The slow and sustained activation of Pap1 in cells lacking Trx1 is fully dependent on Tpx1

To determine whether the basal and H_2O_2-triggered activation of the Pap1 gene response in cells lacking Trx1 was still dependent on Tpx1, we analyzed by ChIP and Northern blot a Δ*trx1* Δ*tpx1* strain. The recruitment of Pap1 to promoters (Fig. 5B) and the activation of the gene expression program (Fig. 5A) were completely eliminated in this strain background.

We speculated that an electron donor of the thioredoxin system, either Trx1 or Trx3, could be maintaining Pap1 in the reduced conformation and that the enhanced levels of the Pap1-dependent gene expression program could be just a consequence of the depletion of this reducing power. In other words, Trx1 and Trx3 could be required to keep

Pap1 thiols reduced under aerobic conditions, and H_2O_2 oxidation of Tpx1 could be only required to overcome this reducing power. This has already been proposed for the budding yeast Gpx3-Yap1 redox relay (Okazaki et al., 2007). However, our experiments clearly show that Pap1 activation in a strain depleted of cytosolic thioredoxins requires Tpx1 both for the basal levels and for the H_2O_2-dependent activation of the Pap1 pathway (Fig. 5C-E). Therefore, oxidation of Pap1 is dependent on Tpx1, even in the absence of thioredoxins.

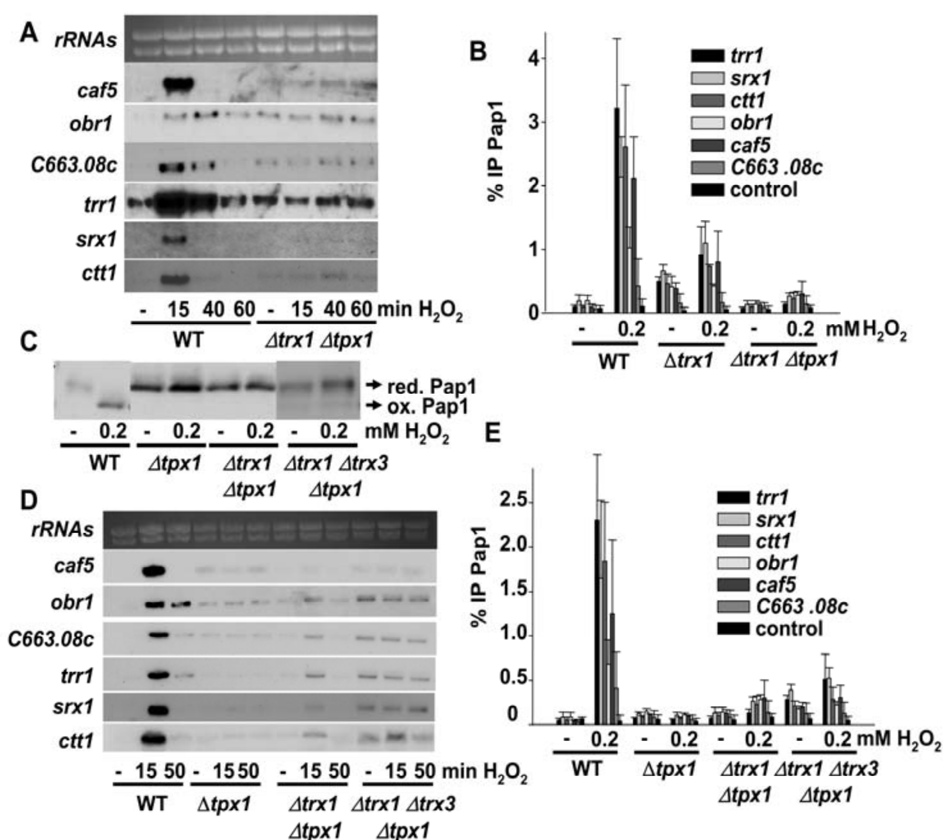


Figure 5. The late activation of Pap1 *in* Δ*trx1 strain* is Tpx1-dependent. (A) Northern blot analysis of Pap1-dependent genes.. (**B**) The recruitment of Pap1 to promoters is Tpx1-dependent. **(C, D and E)** The late activation of Pap1 in Δ*trx1* Δ*trx3* strain is abolished in Δ*tpx1* background. (C) *In vivo* oxidation of Pap1. (D) Northern blot analysis of Pap1-dependent genes. (E) ChIP analysis of recruitment of Pap1.

Pap1 as an alternative electron donor for Tpx1 upon transient oxidation of Trx1

As stated in the Introduction, previous experiments suggest that Tpx1 transfers the redox signal to Pap1 by thiol-disulfide exchange. However, Tpx1 is cycling during normal aerobic metabolism, since cells devoid of the peroxiredoxin are unable to grow on aerobic plates (Jara et al., 2007). We hypothesize that Pap1 could be a secondary electron donor for disulfide-bridged oxidized Tpx1, but only when its natural electron donor, reduced Trx1, becomes transiently limiting. If that would be the case, Tpx1 basal oxidation could be exacerbated in a strain lacking both Trx1 and Pap1, similarly to what we observed in Δ*trx1* Δ*trx3* cells (Fig. 3B). Indeed, we detected maximum oxidation of Tpx1 (two-disulfide bonds per Tpx1 dimer) under basal conditions in extracts from strain Δ*trx1* Δ*pap1* (Fig. 6A). Similarly, Δ*trx1* strains expressing non-oxidizable mutant Pap1 proteins (Pap1.C278A and Pap1.C285S) displayed the same pattern of Tpx1 oxidation (Fig. 6B). Therefore, Pap1 can act as an electron donor to Tpx1 only when reduced Trx1 becomes limiting.

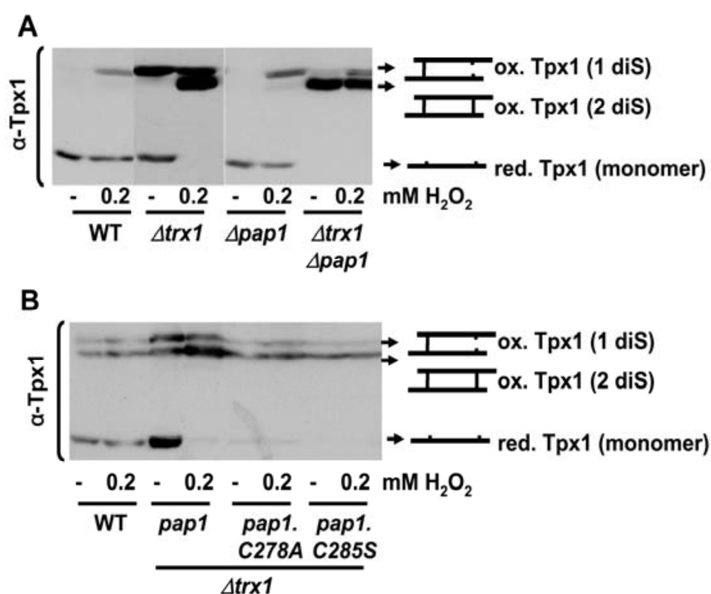


Fig. 6. Pap1 is an alternative electron donor for Tpx1 in Δ*trx1* cells. (A and B) The redox state of Tpx1 in IC2 (WT), MJ15 (Δ*trx1*), IC1 (Δ*pap1*), SG69 (Δ*trx1* Δ*pap1*), IC75 (Δ*trx1 pap1.C278A*) and IC82 (Δ*trx1 pap1.C285S*) was analyzed as described in Figure 3A.

The role of Tpx1 in Pap1 activation can be bypassed by over-expression of another thioredoxin peroxidase, Gpx1

Our experiments indicate that the peroxiredoxin Tpx1 is sensing and scavenging peroxides with the participation of Trx1 as the electron donor in the Tpx1 cycle (Fig. 3E). Only when reduced Trx1 becomes limiting, the disulfide of Tpx1 is transferred to an alternative electron donor, Pap1. We then tested whether the role of the peroxiredoxin Tpx1 could be exerted when another peroxiredoxin, able to sense and reduce H_2O_2, accumulates in the oxidized form upon peroxide addition. The glutathione peroxidase Gpx1 of fission yeast has been described to possess thioredoxin-dependent peroxidase activity (Kim et al., 2010). Obviously, either the endogenous Gpx1 levels or its low peroxidatic activity are not sufficient to scavenge H_2O_2 produced during aerobic metabolism, since cells devoid of Tpx1 cannot grow on aerobic plates (Jara et al., 2007) and are not able to modify the levels of oxidized Trx1 as shown by non-reducing electrophoresis of TCA extracts (Fig. 7A) and modifying Trx1 in TCA extracts with AMS; this Cys alkylating agent only interacts with reduced Cys residues, and each AMS moiety enhances the size of the alkylated protein by 0.5 kDa, what can be detected in reducing electrophoresis (Fig. 7B). Over-expression of HA-tagged Gpx1 overcome these limitations, since Δ*tpx1* cells transformed with an episomal plasmid carrying the *gpx1* gene are able to grow on aerobic plates (data not shown) and induce Trx1 oxidation (Fig. 7C, upper panel). In fact, HA-Gpx1 oxidation could be detected by non-reducing electrophoresis (Fig. 7C, middle panel). It is worth to mention that Gpx1 can only trigger significant Trx1 oxidation when H_2O_2 is added at high concentrations to the media (2 mM; Fig. 7C), probably due to a higher K_m for H_2O_2 than Tpx1; at this concentration, Tpx1 cannot scavenge peroxides due to over-oxidation of its peroxidatic Cys to sulfinic acid (Bozonet et al., 2005; Vivancos et al., 2005) (Fig. 3E). Importantly enough, in Δ*tpx1* cells over-expressing HA-Gpx1, Pap1 is oxidized at the concentration of H_2O_2 that is sensed by Gpx1, as shown by non-reducing electrophoresis (Fig. 7C, lower panel) and by analysis of the Pap1-dependent gene expression program (Fig. 7D). Thus, we conclude that accumulation of the oxidized form of a thioredoxin peroxidase, either Tpx1 or Gpx1, can transduce the redox signal towards Pap1. Similarly, over expression of another thioredoxin substrate in Δ*tpx1* cells such as PAPS reductase to the same

levels as HA-Gpx1 was able to deplete the pool of reduced Trx1, but did not mimic the effect of Gpx1 regarding Pap1 activation and could not trigger Pap1 oxidation (Supplementary Fig. 4).

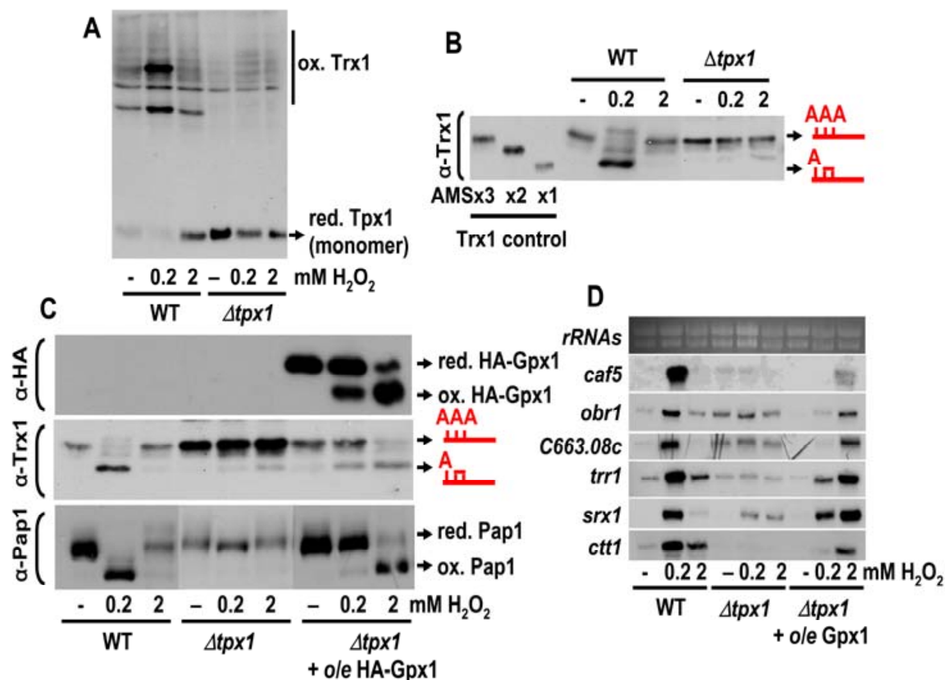


Figure. 7. Oxidation of the peroxiredoxin, Gpx1, induces Pap1 oxidation in cells lacking Tpx1. (A and B) Absence of oxidation of Trx1 in cells lacking Tpx1. **(A)** *In vivo* oxidation of Trx1. **(B)** Western blot analysis of Trx1 oxidation. TCA extracts were labeled with AMS, and separated using reducing electrophoresis. The labelling of Trx1 with three, two or one AMS molecules are indicated in the figure in control extracts. In WT untreated, 2 mM H_2O_2 and in Δt*px1* strain, three cysteines can be labeled with AMS (scheme with AAA). On the contrary, in WT strain after 0.2 mM H_2O_2 Trx1 is oxidized and then two cysteines are only labeled with AMS (scheme with A). **(C)** Over-expression of HA-tagged Gpx1 induces Trx1 and Pap1 oxidation at high concentrations of H_2O_2.. **(D)** Over-expression of Gpx1 induces Pap1-dependent gene expression program. Northern blot analysis of Pap1-dependent genes.

DISCUSSION

The presence of several redox couples in antioxidant redox relays, with inter-dependent cycles of oxidation-reduction, constitute the bases of redox signalling cascades. We have here investigated the role of the main components of a simple antioxidant pathway: the peroxiredoxin Tpx1 as a H_2O_2 sensor, Trx1 as the principal electron donor for the recycling of oxidized Tpx1, and Pap1 as the transcription factor which acts as an alternative electron donor to Tpx1 which becomes activated by a thiol-disulfide exchange mechanism.

The unambiguous outcome of all the experiments shown here is that Tpx1 is absolutely essential for Pap1 activation. Tpx1 is precisely suited to sense and scavenge low concentrations of peroxides, and is the main activity detoxifying H_2O_2 during aerobic growth on solid plates (Jara et al., 2007). Other peroxidases in *S. pombe*, such as Gpx1, BCP or Pmp20, may contain Cys also able to react with H_2O_2 with high sensitivity and transfer the redox signal to other Cys in proteins, since cells lacking Tpx1 still display accumulation of general disulfides after H_2O_2 treatment (G.-S, S.B. and E.H., data not shown). However, the disulfides in Pap1 cannot be formed unless Tpx1 is present, what suggest that Tpx1-Pap1 protein interactions may provide the specificity to this redox cascade. Only massive over-expression of another peroxiredoxin, Gpx1, can overcome the absence of specific interactions between this protein and Pap1.

Three putative models could explain the role of Tpx1 and Trx1 in Pap1 activation: (i) H_2O_2 oxidizes Tpx1, which then oxidizes the pool of Trx1; oxidized Trx1 would be the direct activator of Pap1; (ii) oxidized Tpx1 directly activates Pap1, although to a lower pace than it oxidizes Trx1; (iii) a mixed Trx1-Tpx1 intermediate in the Tpx1 cycle (Fig. 3E) would be the inducer of Pap1 oxidation. We have dismissed the last hypothesis because cells expressing Trx1.C33S accumulate the Trx1-Tpx1 intermediate (Fig. 3C), and Pap1 follows the same activation kinetics as in cells lacking Trx1 (data not shown). We have also discarded the first option (oxidized Trx1 being the inducer of Pap1), because cells lacking Trx1 are still able to activate Pap1, as demonstrated by Northern blot and ChIP analysis (Fig. 4CD), whereas further deletion of Tpx1 on this background fully eliminates the Pap1 gene expression program (Fig. 5).

Therefore, we are left with the hypothesis than oxidized Tpx1 is the direct inducer of Pap1.

We also believe that Tpx1 activates Pap1 from the double disulfide form, when reduced Trx1 becomes limiting (Fig. 3E), and by thiol-disulfide exchange. Another alternative, the activation of Pap1 from the Tpx1-S_{48}OH form, is unlikely, since cells expressing Tpx1 with a mutation in the resolving Cys, Tpx1.C169S, which should allow the accumulation of Tpx1-S_{48}OH, are not able to activate Pap1 in response to peroxides (Vivancos et al., 2005).

It is still intriguing the fact that in cells lacking Trx1 the oxidation of Pap1 by non-reducing electrophoresis is not easily detectable (Fig. 4A). We are aware that at least two disulfides in Pap1 are required for the activation of Pap1 as a transcription factor (I.A.C. and E.H, manuscript in preparation). It may well be that Trx1 participates in the isomerization of these disulfides. A second alternative is that Tpx1 has to be engaged in the catalytic cycle (and Trx1 participates in the reduction of oxidized Tpx1) for Pap1 to be activated, as it has previously been described for over-oxidation of peroxiredoxins to the sulfinic form (Yang et al., 2002). Lastly, a third alternative is that Trx1, which reduces Tpx1 and may reduce Pap1, also works as a chaperone that holds both proteins together. So far, we have been able to co-immuno-precipitate Pap1 and Tpx1 with Trx1 in a complex, but the same two proteins seem to remain together in cells lacking Trx1 (Patricia García, I.A.C. and E.H., data not shown).

It is important to point out that cells lacking thioredoxin reductase seem to generate an intracellular environment which fully disassembles the wild-type thiol homeostasis. In fact, a proteome-wide approach indicates than almost 50% of all Cys-containing *S. pombe* peptides are over-oxidized more than 2-fold in this strain background. We believe that several non-physiological oxidized species accumulate in Δ*trr1* cells, such as oxidized Trx1, Trx3 and Tpx1, and induce this massive, H_2O_2-independent thiol oxidation. In this genetic background, H_2O_2-signalling is fully disturbed.

MATERIALS AND METHODS

Yeast strains and growth conditions

The origins and genotypes of strains used in this study are outlined in Supplementary Table S1. Cells were grown in rich medium (YE) or in synthetic minimal medium as described previously (Alfa, Fantes et al. 1993).

Plasmids

The plasmid p85.41x' and its mutant derivatives (Castillo, Ayte et al. 2002), containing the *GFP*-*pap1* coding region at the *leu1* locus were used. In order to construct new *pap1* mutant alleles, p85.41x' was used as a template for PCR reactions using pairs of mutagenic and complementary primers containing the codon change of interest (Castillo, Ayte et al. 2002), yielding plasmids p85.41x'.C278A and p85.41x'.C285S. All the mutations were confirmed by sequencing. The p85.41x' plasmid derivatives were used to generate chromosomal insertions of *GFP*-tagged *pap1* genes into the *leu1* locus of strain EHH108, as described before (Castillo, Ayte et al. 2002). We used plasmids p145.C48S (p*tpx1´*::*tpx1.C48S*) and p145.C169S (p*tpx1´*::*tpx1.C169S*) (Vivancos, Castillo et al. 2005). We used some plamids for overexpression of different peroxiredoxins. The *gpx1* coding sequence was obtained by digestion from p101.42x (Vivancos, Castillo et al. 2004) and cloned into the *nmt* (*n*o *m*essage in *t*hiamine)-driven expression vector pRep.3x (Maundrell 1993) to yield plasmid p432.3x (p*gpx1.3x*) or into pRepHA.3x (Maundrell 1993) to yield plasmid p440.3x (pHA*gpx1.3x*). The *met16* coding sequence was PCR-amplified from S. pombe cDNA using specific primers and cloned into the *nmt* (no message in thiamine)-driven expression vector pRepHA.3x (Maundrell 1993) to yield plasmid p441.3x (pHA*met16.3x*).

Fluorescence microscopy

Fluorescence microscopy and image capture was performed as described before (Vivancos, Castillo et al. 2004).

H_2O_2 sensitivity assay

For survival on solid plates, *S. pombe* strains were grown, diluted and spotted in YE5S agar plates, containing 2 mM H_2O_2 as described previously (Calvo, Gabrielli et al. 2009).

Preparation of *S. pombe* TCA extracts and immunoblot analysis

To analyze the *in vivo* redox state of Pap1, Tpx1 and Trx1, *S. pombe* cultures were obtained and total TCA extracts were prepared as previously described (Vivancos, Castillo et al. 2005). We used already described specific polyclonal antibodies for Pap1 (Vivancos, Castillo et al. 2004), for Tpx1 (Jara, Vivancos et al. 2007) and for Trx1 (Garcia-Santamarina, Boronat et al. 2011).

TCA protein extracts labeled with AMS

To determine the number of oxidized Cysteines in Trx1, TCA extracts were prepared as described (Vivancos, Castillo et al. 2005) with the following modifications. Acetone washed TCA pellets corresponding to 1.25 ml of cultures were resuspended in 25 μl 20 mM AMS (4-acetamido-4'-maleimidylstilbene-2, 2'-disulfonic acid) freshly prepared in 200 mM Tris-HCl, pH 7.5, 1mM EDTA, 1% SDS. Derivatization reactions were then allowed to proceed for 2 hours at 37°C. The exact number of cysteines oxidized in Trx1 was determined by using TCA extracts of different Trx1 mutants. Briefly, TCA extracts were obtained from 10 ml cultures of wild-type strains, Trx1.C33S and Trx1.C16SC33S mutants and resuspended in 200 μl 20 mM TCEP (Tris (2-carboxyethyl) phosphine hydrochloride) freshly prepared in 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS. After incubation at 30°C during 30 minutes, extracts were TCA precipitated and washed twice with acetone. Extracts were then resuspended in 50 μl with 20 mM AMS and labeled as described above. AMS labeled protein extracts were subjected to electrophoresis under reducing conditions using 15% poly-acrylamide gels (50:1), and detected by Western blotting using a previously described Trx1 antibody (Garcia-Santamarina, Boronat et al. 2011).

RNA analysis

Total RNA from *S. pombe* minimal media cultures was obtained, processed and transferred to membranes as described previously (Castillo, Ayte et al. 2002). Membranes were hybridized with [α-32P] dCTP-labelled *caf5*, *obr1*, SPCC663.08c, *trr1*, *srx1* or *ctt1* probes, containing the complete open reading frames. We used ribosomal RNA, *tfb2* or act1 as loading controls.

Chromatin immuno-precipitation

The *in vivo* binding of Pap1 to stress promoters was analyzed as described previously (Calvo, Garcia et al. 2012) using polyclonal antibodies against Pap1 (Vivancos, Castillo et al. 2004).

Immuno-fluorescence assay

Ten ml of cells grown in minimal media were grown till an OD_{600} of 0.3 and harvested. Cells were fixed with 1-2% of formaldehyde during 20 min at 25ºC in a water bath. Pelleted cells were washed twice in cold PBS 1%. Cells were resuspended in 0.4 ml of preincubation buffer (20 mM citric acid, 20 mM Na_2HPO_4, 40 mM EDTA pH 8.0) with 30 mM of β-mercaptoethanol and were incubated at 30ºC for 10 min in a water bath. Cells were collected by centrifugation for 1 min at 8,000 rpm, and pellets were resuspended in 0.2 ml sorbitol-Tris buffer (1 M sorbitol, 50 mM Tris-HCl pH 7.4) with 10 mM β-mercaptoethanol and 2.25 mg of zymolyase 20T (ICN biochemical) at 30ºC for 40 min in a water bath. Cell wall digestion was confirmed by adding 1% SDS to 2 µl of cells and observing lysis at the microscope. Cells were pelleted at 6,000 rpm for 1 min, and pellets were washed three times with 1 ml of cold PEMS (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 1.2 sorbitol, pH 6.5-6.9). Then, cells were incubated in 1 ml of cold PEMS with 1% Triton X-100 during 1 min at room temperature to permeabilize nuclear membranes. Cells were pelleted at 6,000 rpm for 1 min, and washed three times with 1 ml of PEM (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$). Then, cells were resuspended in 1 ml of PEMBAL (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 3% BSA, 0.1% NaN3, 100 mM lysine hydrochloride) and incubated rocking during 20 min at room temperature. Cells were pelleted again and resuspended in 100 µl of PEMBAL plus 1/200 dilution of polyclonal anti-Pap1 or monoclonal anti-HA (12CA5) antibodies. Incubation proceeded rocking overnight at room temperature. Cells were

pelleted, washed three times in PEMBAL, rocking during 10 min at room temperature each time. Pelleted cells were then resuspended in 100 µl of PEMBAL plus 1/500 dilution of secondary antibody [Cy2 AffiniPure Donkey anti-rabbit IgG (H+L) (ref. 711-225-152, Jackson) or Alexa Fluor 555 goat anti-mouse IgG (ref. A21424, Invitrogen), and incubated by rocking 5-7 h at room temperature in the darkness. Cells were pelleted and washed three times in PEMBAL, rocking them during 10 min at room temperature each time. Cells were resuspended in 100 µl of PEMBAL, and analyzed directly by fluorescence microscopy.

ACKNOWLEDGEMENTS

We thank Alberto Jiménez and Jose Luis Revuelta for kindly providing strain SP2 (Δ*trx3*). We thank Michel Toledano and Elizabeth Veal for helpful discussions. The authors thank Mercè Carmona for technical assistance. This work was supported by the Spanish Ministry of Science and Innovation (BFU2009-06933, BFU2012-32045), PLAN E and FEDER, by the Spanish program Consolider-Ingenio 2010 Grant CSD 2007-0020, and by SGR2009-196 from Generalitat de Catalunya (Spain) to E.H. E. H. and J.A. are recipients of ICREA Academia Awards (Generalitat de Catalunya).

REFERENCES

Alfa, C., Fantes, P., Hyams, J., McLeod, M. and Warbrick, E. (1993) *Experiments with Fission Yeast: A Laboratory Course Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Andersen, K.M., Jensen, C., Kriegenburg, F., Lauridsen, A.M., Gordon, C. and Hartmann-Petersen, R. (2011) Txl1 and Txc1 are co-factors of the 26S proteasome in fission yeast. *Antioxid Redox Signal*, **14**, 1601-1608.

Benko, Z., Sipiczki, M. and Carr, A.M. (1998) Cloning of caf1+, caf2+ and caf4+ from Schizosaccharomyces pombe: their involvement in multidrug resistance, UV and pH sensitivity. *Mol.Gen.Genet.*, **260**, 434-443.

Bozonet, S.M., Findlay, V.J., Day, A.M., Cameron, J., Veal, E.A. and Morgan, B.A. (2005) Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide. *J Biol Chem*, **280**, 23319-23327.

Calvo, I.A., Gabrielli, N., Iglesias-Baena, I., Garcia-Santamarina, S., Hoe, K.L., Kim, D.U., Sanso, M., Zuin, A., Perez, P., Ayte, J. and Hidalgo, E. (2009) Genome-wide screen of genes required for caffeine tolerance in fission yeast. *PLoS One*, **4**, e6619.

Calvo, I.A., Garcia, P., Ayte, J. and Hidalgo, E. (2012) The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H2O2. *Nucleic Acids Res*, **40**, 4816-4824.

Castillo, E.A., Ayte, J., Chiva, C., Moldon, A., Carrascal, M., Abian, J., Jones, N. and Hidalgo, E. (2002) Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. *Mol.Microbiol.*, **45**, 243-254.

Collet, J.F. and Messens, J. (2010) Structure, function, and mechanism of thioredoxin proteins. *Antioxid Redox Signal*, **13**, 1205-1216.

Chen, D., Toone, W.M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N. and Bahler, J. (2003) Global transcriptional responses of fission yeast to environmental stress. *Mol.Biol.Cell*, **14**, 214-229.

Chen, D., Wilkinson, C.R., Watt, S., Penkett, C.J., Toone, W.M., Jones, N. and Bahler, J. (2008) Multiple pathways differentially regulate global oxidative stress responses in fission yeast. *Mol Biol Cell*, **19**, 308-317.

Delaunay, A., Isnard, A.D. and Toledano, M.B. (2000) H(2)O(2) sensing through oxidation of the Yap1 transcription factor. *EMBO J.*, **19**, 5157-5166.

Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J. and Toledano, M.B. (2002) A thiol peroxidase is an h(2)o(2) receptor and redox-transducer in gene activation. *Cell*, **111**, 471-481.

Garcia-Santamarina, S., Boronat, S., Espadas, G., Ayte, J., Molina, H. and Hidalgo, E. (2011) The oxidized thiol proteome in fission yeast--optimization of an ICAT-based method to identify H2O2-oxidized proteins. *J Proteomics*, **74**, 2476-2486.

Jara, M., Vivancos, A.P., Calvo, I.A., Moldon, A., Sanso, M. and Hidalgo, E. (2007) The peroxiredoxin Tpx1 is essential as a H2O2 scavenger during aerobic growth in fission yeast. *Mol Biol Cell*, **18**, 2288-2295.

Jimenez, A., Mateos, L., Pedrajas, J.R., Miranda-Vizuete, A. and Revuelta, J.L. (2007) The txl1+ gene from Schizosaccharomyces pombe encodes a new thioredoxin-like 1 protein that participates in the antioxidant defence against tert-butyl hydroperoxide. *Yeast*, **24**, 481-490.

Kim, J.S., Bang, M.A., Lee, S., Chae, H.Z. and Kim, K. (2010) Distinct functional roles of peroxiredoxin isozymes and glutathione peroxidase from fission yeast, Schizosaccharomyces pombe. *BMB Rep*, **43**, 170-175.

Kim, S.J., Jung, E.M., Jung, H.J., Song, Y.S., Park, E.H. and Lim, C.J. (2007) Cellular functions and transcriptional regulation of a third thioredoxin from Schizosaccharomyces pombe. *Can J Microbiol*, **53**, 775-783.

Kudo, N., Taoka, H., Toda, T., Yoshida, M. and Horinouchi, S. (1999) A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor Pap1. *J.Biol.Chem.*, **274**, 15151-15158.

Maundrell, K. (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene*, **123**, 127-130.

Meyer, Y., Buchanan, B.B., Vignols, F. and Reichheld, J.P. (2009) Thioredoxins and glutaredoxins: unifying elements in redox biology. *Annu Rev Genet*, **43**, 335-367.

Okazaki, S., Tachibana, T., Naganuma, A., Mano, N. and Kuge, S. (2007) Multistep disulfide bond formation in Yap1 is required for sensing and transduction of H2O2 stress signal. *Mol Cell*, **27**, 675-688.

Song, J.Y., Kim, K.D. and Roe, J.H. (2008) Thiol-independent action of mitochondrial thioredoxin to support the urea cycle of arginine biosynthesis in Schizosaccharomyces pombe. *Eukaryot Cell*, **7**, 2160-2167.

Song, J.Y. and Roe, J.H. (2008) The role and regulation of Trxl, a cytosolic thioredoxin in Schizosaccharomyces pombe. *J Microbiol*, **46**, 408-414.

Stroher, E. and Millar, A.H. (2012) The biological roles of glutaredoxins. *Biochem J*, **446**, 333-348.

Vivancos, A.P., Castillo, E.A., Biteau, B., Nicot, C., Ayte, J., Toledano, M.B. and Hidalgo, E. (2005) A cysteine-sulfinic acid in peroxiredoxin regulates H2O2-sensing by the antioxidant Pap1 pathway. *Proc Natl Acad Sci U S A*, **102**, 8875-8880.

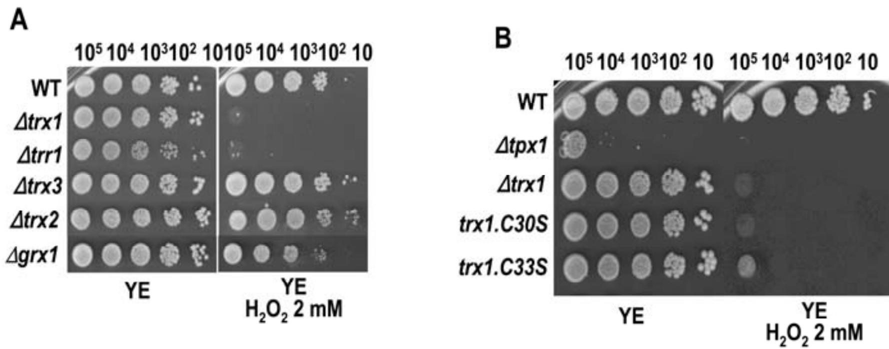
Vivancos, A.P., Castillo, E.A., Jones, N., Ayte, J. and Hidalgo, E. (2004) Activation of the redox sensor Pap1 by hydrogen peroxide

requires modulation of the intracellular oxidant concentration. *Mol Microbiol*, **52**, 1427-1435.

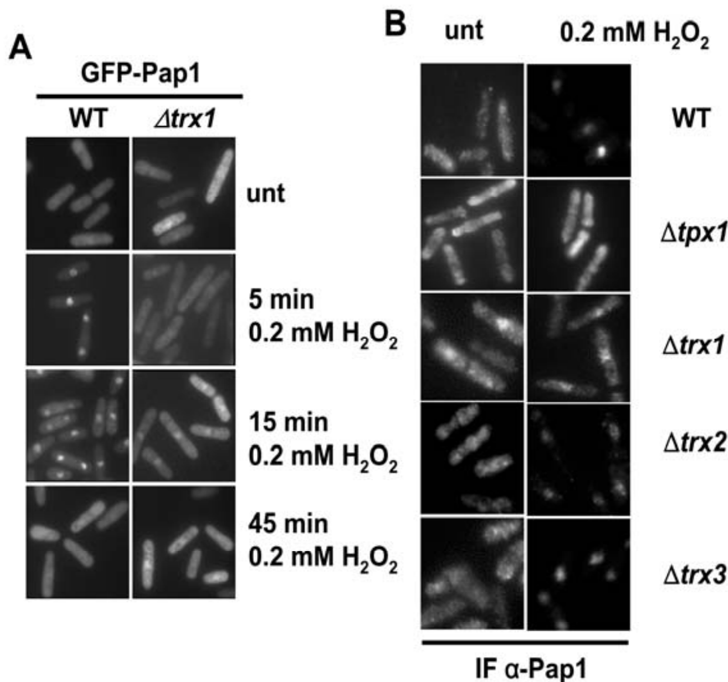
Wood, V., Gwilliam, et al; (2002) The genome sequence of Schizosaccharomyces pombe. *Nature*, **415**, 871-880.

Yang, K.S., Kang, S.W., Woo, H.A., Hwang, S.C., Chae, H.Z., Kim, K. and Rhee, S.G. (2002) Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. *J Biol Chem*, **277**, 38029-38036.

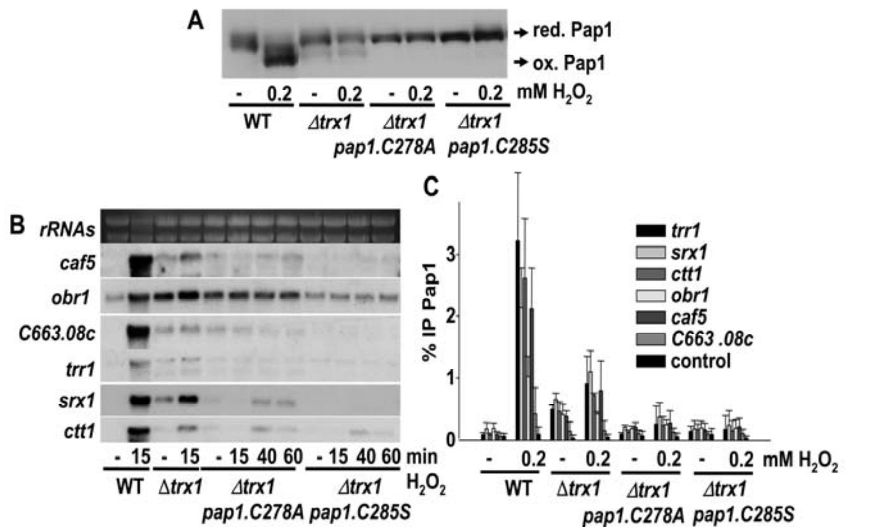
Calvo et al, Supplementary information includes: Supp. Figs. 1-4 and Supp. Table S1 and references there-in.



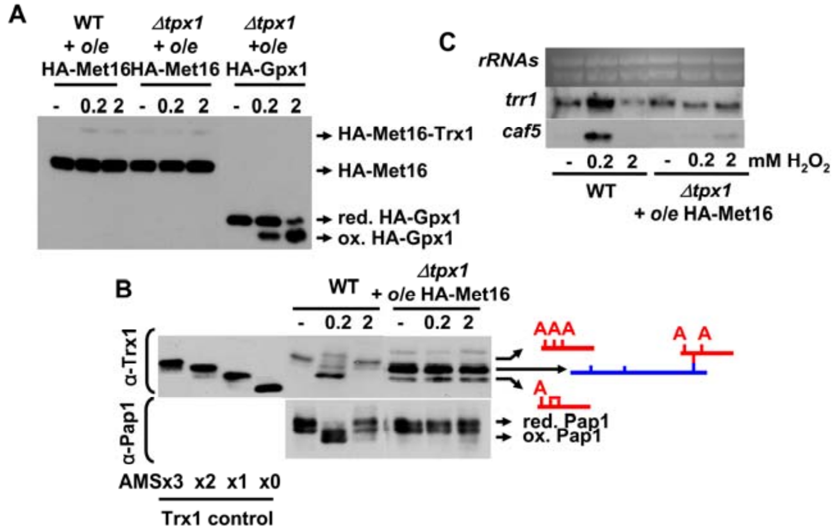
Supplementary Figure S1. Δ*trx1* and Trx1 mutant strains are sensitive to H_2O_2 on solid plates. (A and B) Strains were grown in liquid YE media, and the indicated number of cells were spotted onto plates with or without 2 mM H_2O_2.



Supplementary Fig. S2. Localization of Pap1 in different strains backgrounds. (A) GFP-Pap1 accumulation in Δ*trx1* cells was less pronounced than untagged Pap1. **(B)** Localization of untagged wild-type Pap1 by immunofluoresecence in some strains, before and after 0.2 mM H_2O_2 for 5 minutes.



Supplementary Figure S3. The oxidation of Pap1 is eliminated in Δ*trx1* cells expressing the non-oxidizable Pap1 mutants. (A) *In vivo* oxidation of Pap1. TCA extracts were treated or not with 0.2 mM H_2O_2 for 5 minutes. **(B)** Northern blot analysis of Pap1-dependent genes.. **(C)** ChIP analysis of recruitment of Pap1. Cultures were treated with 0.2 mM H_2O_2 for 5 minutes.



Supplementary Fig. S4. Overexpression of PAPS reductase was able to deplete the pool of reduced Trx1 and cannot activate Pap1 after 2 mM H_2O_2. **(A)** Overexpression of the same levels of PAPS reductase (Met16) to Gpx1. **(B)** Over-expression of HA-Met16 does not induce Trx1 and Pap1 oxidation at high concentrations of H_2O_2. In Δ*tpx1* overexpressing HA-Met16 strain two AMS (AA) of Trx1 are labeled with AMS because Trx1 is trapped with HA-Met16. (C) Over-expression of HA-Met16 does not induce the Pap1-dependent transcription.

Supplementary Table S1. Strains used in this study

Strain	Genotype	Origin
IC2	*h^- pap1 ura4-D18 leu1-32*	(1)
IC71	*h^- trr1::NatMX6 ura4-D18 leu1-32*	This work
MJ11	*h^- tpx1::kanMX6*	This work
MJ15	*h^+ trx1::kanMX6*	This work
MJ16	*h^- trx2::kanMX6*	This work
SP2	*h^+ txl1(trx3)::kanMX6 leu1-32*	(2)
IC38	*h^- grx1::kanMX6*	This work
MJ3	*h^- trx1::HA:: kanMX6 ura4-D18 leu1-32*	This work
IC76	*h^+ trx1::NatMX6 txl1::kanMX6 ura4-D18 leu1-32*	This work
SG164	*h^- trr1::kanMX6 tpx1::NatMX6*	This work
SG170	*h^- trr1::kanMX6 tpx1::NatMX6 trx1::ura4-D18 ade6-704 leu1-32*	This work
PG22	*h^+ trr1::NatMX6 trx1::kanMX6*	This work
SG72.C30S	*h^- trx1.C30S ura4-D18 leu1-32*	This work
SG72.C33S	*h^- trx1.C33S ura4-D18 leu1-32*	This work
IC139	*h^+ trr1::kanMX6 tpx1::NatMX6 tpx1.C48S::$leu1^+$ ura4-D18 ade6-704*	This work
IC140	*h^+ trr1::kanMX6 tpx1::NatMX6 tpx1.C169S::$leu1^+$ ura4-D18 ade6-704*	This work
EHH14	*h^- his2 ura4 pap1::$ura4^-$ leu1–32 nmt::GFP-pap1::$leu1^+$*	(3)
IC67	*h^- trx1::kanMX6 ura4 cys nmt::GFP-pap1::$leu1^+$*	This work
IC40	*h^+ trx1::NatMX6 tpx1::kanMX6*	This work
IC123	*h^+ txl1::kanMX6 trx1::NatMX6 tpx1::Higro ura4-D18 leu1-32*	This work
SG69	*h^- pap1::kanMX6 trx1::NatMX6*	This work
IC1	*h^- pap1::$ura4^+$ ura4-D18 leu1-32*	(1)
IC75	*h^- pap1.C278A trx1::NatMX6 ura4-D18 leu1-32*	This work
IC82	*h^- pap1.C285S trx1::NatMX6 ura4-D18 leu1-32*	This work
IC48	*h^- trx2::kanMX6 trx1::NatMX6*	This work
IC44	*h^- grx1::kanMX6 trx1::NatMX6*	This work
SG5	*h^+ tpx1::NatMX6 leu1-32*	This work

REFERENCES

1. Calvo, I.A., Garcia, P., Ayte, J. and Hidalgo, E. (2012) The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H2O2. *Nucleic Acids Res*, **40**, 4816-4824.

2. Jimenez, A., Mateos, L., Pedrajas, J.R., Miranda-Vizuete, A. and Revuelta, J.L. (2007) The txl1+ gene from Schizosaccharomyces pombe encodes a new thioredoxin-like 1 protein that participates in the antioxidant defence against tert-butyl hydroperoxide. *Yeast*, **24**, 481-490.

3. Castillo, E.A., Ayte, J., Chiva, C., Moldon, A., Carrascal, M., Abian, J., Jones, N. and Hidalgo, E. (2002) Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. *Mol.Microbiol.*, **45**, 243-254.

4. CHARACTERIZATION OF Pap1 AS A TRANSCRIPTION FACTOR IN RESPONSE TO H_2O_2

Some of the results of this section have been published in (Calvo, Garcia et al. 2012) where I participated as a first co-author.
We will summarize below (sections 4.1- 4.3) the main aims and results of our study.

4.1. CHARACTERIZATION OF DIFFERENT MUTANT STRAINS EXPRESSING CONSTITUTIVELY NUCLEAR Pap1

Several genetic interventions trigger constitutive nuclear accumulation of Pap1: (i) induction of its oxidation by deletion of the thioredoxin reductase-coding gene can render a constitutively oxidized and nuclear Pap1; (ii), defects in the Crm1-dependent export machinery (i.e. cells lacking the Crm1 cofactor Hba1) or (iii) alteration of the NES in Pap1 (i.e. Cys-to-Asp mutation of residue 523 of Pap1) can block Pap1 in the nucleus in the absence of stress. In all three cases, constitutively nuclear Pap1 significantly enhances *S. pombe* resistance to caffeine. Unexpectedly, while cells lacking Trr1 display some sensitivity to peroxides probably due to the lack of an active thioredoxin reducing system, cells lacking Hba1 or expressing constitutively nuclear Pap1.C523D are not resistant to peroxides, and, in fact, display more sensitivity than wild-type cells.

These results prompted us to analyze the constitutively nuclear Pap1 mutants. The first observation was that while in Δ*trr1* cells Pap1 is not only nuclear but also oxidized while the other genetic interventions accumulate reduced Pap1 in the nucleus. Furthermore, the nuclear compartmentalization of these mutants (Δ*hba1* and *pap1.C523D* strains) inhibits H_2O_2-dependent oxidation of the transcription factor, probably due to the fact that Tpx1, the H_2O_2 sensor that initiates the redox relay towards Pap1, has cytoplasmic localization. That would explain why cells expressing constitutively reduced Pap1 have more sensitivity to H_2O_2 than wild-type cells.

4.2. CONSTITUTIVELY NUCLEAR REDUCED Pap1 CAN BIND AND ACTIVATE ONLY A SUBSET OF Pap1-DEPENDENT GENES

Analysis of the transcriptome of these constitutively nuclear-expressing cell types indicated that they only express a subset of Pap1-dependent genes: those encoding for proteins promoting drug tolerance, such as Caf5 and Hba2. On the contrary, the antioxidant genes such as *ctt1, trr1* and *srx1* were not expressed in these mutants, even after H_2O_2 stress. Using these different strain backgrounds, we demonstrated that the expression of some Pap1-induced genes such as *ctt1, trr1, srx1, obr1, SPCC663.08c* and *caf5* have different requirements regarding Pap1 activity/subcellular localization/oxidation state. The drug resistance genes only require nuclear Pap1 for its activation, whereas another subset of genes, those coding for the antioxidants catalase, sulfiredoxin or thioredoxin reductase, do need not only nuclear but also oxidized Pap1.

4.3. THE ROLE OF Prr1 IN THE Pap1-DEPENDENT ANTIOXIDANT RESPONSE

We decided to search for other transcription factors, which could mediate this different pattern of gene induction. The response regulator Prr1 has been shown to be important for the oxidative stress response in *S. pombe*, since a strain lacking this transcription factor is very sensitive to H_2O_2 and in its absence, induction of some Pap1-dependent genes is severely compromised (Ohmiya, Kato et al. 1999). We then tested the pattern of the Pap1-dependent gene expression program in the absence of Prr1, and we saw that mild H_2O_2 stress induced transcription of *caf5*, *obr1* and SPCC663.08c, but not of the antioxidant genes. Our experiments demonstrate that only oxidized nuclear Pap1 interacts with the response regulator Prr1 and both are necessary for the antioxidant response. Moreover, the ability of Pap1 to bind and activate drug tolerance promoters is independent on Prr1, whereas its ability to bind to the antioxidant promoters (*ctt1, trr1, srx1)* is significantly enhanced upon association with Prr1. But Prr1 is only recruited to all six promoters after mild oxidative stress in an oxidized Pap1-dependent manner.

In conclusion, while oxidation of Pap1 contributes to the activation of two distinct sets of genes, induction of the drug resistance ones can be accomplished when non-oxidized Pap1 is accumulated in the nucleus (see Figure 25). We have reported that drugs such as diethylmaleate (cysteine alkylating agent) or leptomycin B (Crm1 inhibitor) can also induce Pap1 nuclear accumulation without its oxidation (Castillo *et al.,* 2002; (Calvo, Garcia et al. 2012), and that triggers only the drug tolerance genes, what suggests that defense against these drugs may only require a partial and smaller gene expression program for adaptation. In conclusion, the distinct regulation of these two subsets of genes, drug-resistance and antioxidant, may reflect an evolutionary merge of previous and independent oxidative stress and multidrug resistance responses, using a common transcription factor, Pap1, and different effectors.

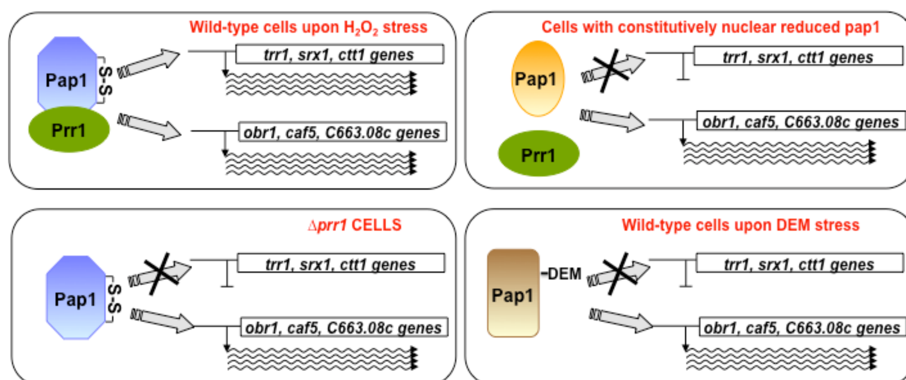


Figure 25. Association of oxidized Pap1 and Prr1 is required for the activation of the antioxidant, but not the drug resistance, genes.

Calvo IA, García P, Ayté J, Hidalgo E. The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H2O2. Nucleic Acids Res. 2012 Jun; 40 (11): 4816-24.

CHAPTER 3:DISCUSSION

"In the middle of every difficulty lies
the opportunity"

Albert Einstein

In our laboratory we work to try to understand signaling processes mediated by ROS. Thus, when we add H_2O_2 to the cells, two signal transduction pathways are induced in response to moderate doses (transcription factor pathway Pap1) or high (toxic) (Sty1 MAP kinase) peroxides.

1. The Pap1 and Sty1 pathways are essential for wild-type tolerance to oxidative stress and to caffeine

We carried out different screenings with an *S. pombe* deletion collection to isolate genes required for survival upon both exogenous and endogenous oxidative stress, and to elucidate the importance of some oxidative stress pathway on wild-type tolerance to caffeine.

Culture media most frequently used to grow fission yeast are minimal medium (MM) and rich medium (YE), which can vary the degrees of respiration and fermentation. The respiration rate of cells grown in MM (2% glucose) is higher than in YE (3% glucose) leading to an increase of production of ROS when cells are grown in MM (Zuin, Gabrielli et al. 2008). Using a double screening approach, we determined that defects in components of the electron transport chain lead to an accumulation of ROS, even though oxygen consumption is impaired. Thus is, electron leakage occurs in all the electron transport chain mutants present in the deletion collection.

Moreover, with our caffeine-sensitive genome-wide screen in parallel to our H_2O_2-sensitivity screen, we could demonstrate that some, but not all of the caffeine-sensitive mutants also display defects in the presence of H_2O_2. The cells with impaired growth on YE plates containing caffeine were involved in a number of cellular roles (Jacoby, Nilius et al. 1998; Kucharczyk, Gromadka et al. 1999) including the H_2O_2-induced Pap1 and Sty1 stress pathways. We believe that any of the toxic effects of caffeine is not mediated through direct generation of ROS because the sensitive Pap1 pathway is not induced at any concentration of the drug.

However, up-regulation of Pap1 induces resistance to caffeine due to an increase of the expression of efflux pumps proteins such as Hba2 or

Caf5 (Chen, Wilkinson et al. 2008). This phenomenum is similar to the Pap1 homologue in *S. cerevisiae*, Yap1, which over-activation also confers resistance to several drugs (Wemmie, Szczypka et al. 1994) and also is dependent on the presence of two YAP1-dependent efflux pumps, FLR1 and YCF1 (Jungwirth, Wendler et al. 2000). In contrast to Pap1, strains lacking Yap1 do not change the basal levels of efflux pumps, so it is not sensitive to drugs (Jungwirth, Wendler et al. 2000; (Wu, Wemmie et al. 1993).

A main conclusion from the genome-wide screening we have performed is that both the Pap1 and the Sty1 pathways are among the most important players in wild-type tolerance to oxidative stress and to multiple drugs.

2. Identification of four cysteines residues in Pap1 involved in the response to H_2O_2

The main aims of my PhD project has been the characterization of the redox signaling cascade leading to Pap1 activation, and its characterization as a transcriptor factor.

Among all the reversible oxidative cysteine modifications reported, disulfide bond formation has been hypothesized to play an essential role in H_2O_2 signaling and to activate several stress sensors including among others the bacterial OxyR (Zheng, Aslund et al. 1998) or the *S. cerevisiae* YAP1 (Delaunay, Pflieger et al. 2002; Vivancos, Castillo et al. 2005). In fission yeast, it has been described that Tpx1 is the initial sensor of H_2O_2 and once activated by the oxidant transduces a redox signal to Pap1 through its cysteine residues (Vivancos, Castillo et al. 2005). We have presented here, using biochemical, genetic and proteomic approaches, evidences for the role of four out of seven cysteine residues in Pap1, C278, C285, C501 and C532, each one being essential to sense and transduce the H_2O_2 signal. In the strains where these cysteine residues are mutated, Pap1 activation is abolished, and consequently there is no binding of these cytoplasmic mutant proteins to Pap1-dependent promoters. Pap1 appears to undergo signal-mediated activation via the formation of two intramolecular disulfide bonds between cysteine residues

278 with 501 (consistent with our previous genetic data (Vivancos, Castillo et al. 2004) and Cys285 with Cys532 (one mixed peptide was isolated by MS/MS with the expected molecular weight; our own unpublished data). Once Pap1 is activated, goes to the nucleus and it is recruited to different promoters inducing the antioxidant response after H_2O_2 treatment. Similarly, it has been described for YAP1, four multistep formations of disulfide bonds are required for its activity after H_2O_2 treatment (Okazaki, Tachibana et al. 2007). But it is worth to point out that in three of our mutants, with the substitutions in Cys278, Cys285 or Cys532, render cells as depleted in Pap1 activity as cells lacking the transcription factor. Therefore, these Cys residues do not modulate Pap1 activity, but are essential for its oxidation/activation.

3. The study of thioredoxin system in Pap1 activation

Although the role of Tpx1 in Pap1 activation is very clear, the mechanisms that regulate the sensitivity and selectivity of Pap1 activation and inactivation by peroxides are not fully understood. We have unambiguously demonstrated that Tpx1 is the direct inducer of Pap1. Our experiments have not clarified yet which form of oxidized Tpx1 (Tpx1 with one or two internal disulfides, Tpx1-SOH with or without one disulfide, mixed disulfide of Tpx1 with Trx1) is transducing the signal towards Pap1. But we strongly believe that a detailed characterization of the Tpx1 cycle is required to understand downstream signalling events.

The most complicated issue during the course of my project has been to study the role of Trx1 in Pap1 activation. The oxidation of Pap1 in cells lacking Trx1 is clearly impaired using TCA and non-reducing electrophoresis. However, the nuclear accumulation of Pap1 and the activation of its gene expression program is quite maintained. We have shown that Trx1 is the principal electron donor for the recycling of oxidized Tpx1, and Trx3 or Pap1 can act as alternative electron donors to Tpx1 when Trx1 is not present.

We are still left with three putative models that could explain the mechanism of activation of Pap1. Firstly, H_2O_2 oxidizes Tpx1, which then oxidizes the pool of Trx1; oxidized Trx1 would be the direct activator of

Pap1. We suspect that this is not a likely option, since we can see activity by NB and ChIP assays of Pap1 in cells lacking Trx1 protein. Secondly, oxidized Tpx1 directly activates Pap1, although to a lower pace than it oxidizes Trx1. Clearly, Tpx1 is fully required for Pap1 activation, as demonstrated by TCAs, NB and ChIP assays of cells lacking Tpx1. Only massive over-expression pf another thioredoxin peroxidase, Gpx1, can bypass the requirement of Tpx1 for Pap1 activation. The third and last model is based on the possibility that a mixed Trx1-Tpx1 intermediate in the Tpx1 cycle would be the inducer of Pap1 oxidation; we suspect that this option is not correct because in cells expressing the mutant Trx1.C33S the mixed Trx1-Tpx1 complex accumulates, and Pap1 is not oxidized.

In conclusion, Trx1 modulates, but is not essential for, the H_2O_2–dependent activation of Pap1.

4. Characterization of Pap1-dependent gene expression program

In response to non-toxic doses of H_2O_2 (extracellular 70–200 mM), Pap1 triggers >2-fold the transcription of 50 genes (Chen, Wilkinson et al. 2008). Many Pap1-dependent gene products are meant to scavenge ROS, and others are efflux pumps involved in the cellular defense against multiple drugs. Components of oxidative stress signaling pathways have often been isolated in screens of general drug resistance, since several of these regulons include genes reported to contribute to drug export or detoxification. In fact, Pap1 was first isolated as conferring resistance to multiple drugs, such as brefeldin A, staurosporine or caffeine, when constitutively activated or over-expressed (Toda, Shimanuki et al. 1991; Benko, Fenyvesvolgyi et al. 2004).

Through the detailed characterization of different mutants of Pap1, which confers resistant to caffeine due to its nuclear localization, we could see that they do not enhance tolerance to oxidative stress. So, we determined the distinct regulation of Pap1-dependent gene expression programs. We classified them into two different and not overlapping subsets of genes. The first subset of genes codes for activities

responsible for multidrug resistance, such as Caf5, Hba2, Obr1 and SPCC663.08c which are expressed upon nuclear Pap1 accumulation, irrespective of its oxidation state and neither the presence of the response regulator Prr1. The second one, which includes traditional antioxidant genes, such as *trr1*, *srx1* and *ctt1* is only engaged by oxidized Pap1, which then binds to Prr1 and the heterodimer will activate these antioxidant genes. We suspect that the access/affinity of Pap1 alone for the drug resistance promoters is high enough in the absence of Prr1 to allow Pol II transcription, but the low affinity of the reduced/ nuclear GFP-Pap1.C523D mutant is not sufficient for the activation of the antioxidant set of genes. However, this can be compensated with a 10-fold increase in the endogenous concentration of Pap1 leading to its occupancy on antioxidant promoters.

Our findings suggests that the activation of both antioxidant and drug resistance genes in response to oxidative stress share a common inducer, H_2O_2, but alternative effectors. The distinct regulation of these two subsets of genes may reflect an evolutionary merge of previous and independent oxidative stress and multidrug resistance responses.

.

CONCLUSIONS

1. **A deficient electron transfer chain leads to basal oxidative stress and shorter life span in *S. pombe.***

2. **Pap1 is essential for normal tolerance to caffeine and its overexpression enhances the resistance to caffeine.**

3. **H_2O_2 induces the reversible oxidation of four Cys residues in Pap1: at least one disulfide bridge has been detected between Cys285 of the n-CRD and Cys532 of the c-CRD.**

4. **Lack of Trr1 leads to constitutive activation of Pap1 irrespective of the presence of Tpx1 or Trx1.**

5. **Oxidation of Pap1 is dependent on Tpx1, even in the absence of thioredoxins.**

6. **Trx1 modulates, but is not essential for, the H_2O_2–dependent activation of Pap1.**

7. **Trx1 is the main electron donor of Tpx1; and Trx3 and Pap1 can also partially reduce Tpx1 disulfides in the absence of Trx1.**

8. **Constitutively nuclear Pap1 mutants display different gene expression patterns. While some genes such as *ctt1, trr1* and *srx1* need nuclear and oxidized Pap1, other genes like *obr1, caf5* and *663.08c* only require nuclear localization of Pap1.**

9. ***In vivo*, Prr1 form a heterodimer with Pap1 only when nuclear Pap1 is oxidized; the antioxidant response is Pap1 and Prr1-dependent.**

APPENDICES

This section included four manuscripts. The first and the second ones describe the results of our genetic screenings of a deletion collection, as described in Results (sections 1 and 2). I have experimentally contribuited to other published work in the laboratory (Appendix 3 and 4).

1. Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast.

2. Genome-wide screen of genes required for caffeine tolerance in fission yeast.

3. The peroxiredoxin Tpx1 is essential as a H_2O_2 scavenger during aerobic growth in fission yeast.

4. Yox1 links MBF-dependent transcription to completion of DNA synthesis

A.1 Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast.

Zuin A[1], Gabrielli N[1], **Calvo IA[1]**, Garía-Santamarina S, Hoe KL, Kim DU, Park HO, Hayles J, Ayté J, Hidalgo E. PLoS One. 2008 Jul 30;3(7):e2842. [1] These authors contributed equally to this work.

A.2 Genome-wide screen of genes required for caffeine tolerance in fission yeast.

Calvo IA[1], Gabrielli N[1], Iglesias-Baena I[1], García-Santamarina S, Hoe KL, Kim DU, Sansó M, Zuin A, Pérez P, Ayté J, Hidalgo E. PLoS One. 2009 Aug 12;4 (8):e6619.

[1] These authors contributed equally to this work.

A.3. The peroxiredoxin Tpx1 is essential as a H_2O_2 scavenger during aerobic growth in fission yeast.

Jara M, Vivancos AP, **Calvo IA**, Moldón A, Sansó M, Hidalgo E. Mol Biol Cell. 2007 Jun;18(6):2288-95. Epub 2007 Apr 4.

A.4. Yox1 links MBF-dependent transcription to completion of DNA synthesis.

Gómez-Escoda B, Ivanova T, **Calvo IA**, Alves-Rodrigues I, Hidalgo E, Ayté J. EMBO Rep. 2011 Jan;12(1):84-9. Epub 2010 Dec 3.

REFERENCES

REFERENCES

Alfa, C., P. Fantes, et al. (1993). Experiments with Fission Yeast: A Laboratory Course Manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory.

Andersen, K. M., C. Jensen, et al. (2011). "Txl1 and Txc1 are co-factors of the 26S proteasome in fission yeast." Antioxid Redox Signal 14(9): 1601-1608.

Anfinsen, C. B., E. Haber, et al. (1961). "The kinetics of formation of native ribonuclease during oxidation of the reduced polypeptide chain." Proceedings of the National Academy of Sciences of the United States of America 47: 1309-1314.

Aoyama, K., H. Aiba, et al. (2001). "Genetic analysis of the His-to-Asp phosphorelay implicated in mitotic cell cycle control: involvement of histidine-kinase genes of Schizosaccharomyces pombe." Bioscience, biotechnology, and biochemistry 65(10): 2347-2352.

Aoyama, K., Y. Mitsubayashi, et al. (2000). "Spy1, a histidine-containing phosphotransfer signaling protein, regulates the fission yeast cell cycle through the Mcs4 response regulator." Journal of bacteriology 182(17): 4868-4874.

Arner, E. S. and A. Holmgren (2000). "Physiological functions of thioredoxin and thioredoxin reductase." European journal of biochemistry / FEBS 267(20): 6102-6109.

Aslund, F. and J. Beckwith (1999). "The thioredoxin superfamily: redundancy, specificity, and gray-area genomics." Journal of bacteriology 181(5): 1375-1379.

Aslund, F., M. Zheng, et al. (1999). "Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status." Proceedings of the National Academy of Sciences of the United States of America 96(11): 6161-6165.

Benko, Z., C. Fenyvesvolgyi, et al. (2004). "The transcription factor Pap1/Caf3 plays a central role in the determination of caffeine resistance in Schizosaccharomyces pombe." Molecular genetics and genomics : MGG 271(2): 161-170.

Benko, Z. and M. Sipiczki (1993). "Caffeine tolerance in Schizosaccharomyces pombe: physiological adaptation and interaction with theophylline." Canadian journal of microbiology 39(5): 551-554.

Benko, Z., M. Sipiczki, et al. (1998). "Cloning of caf1+, caf2+ and caf4+ from Schizosaccharomyces pombe: their involvement in multidrug resistance, UV and pH sensitivity." Molecular & general genetics : MGG 260(5): 434-443.

Benko, Z., M. Sipiczki, et al. (1998). "Cloning of caf1+, caf2+ and caf4+ from Schizosaccharomyces pombe: their involvement in multidrug resistance, UV and pH sensitivity." Mol.Gen.Genet. 260(5): 434-443.

Berlett, B. S. and E. R. Stadtman (1997). "Protein oxidation in aging, disease, and oxidative stress." The Journal of biological chemistry 272(33): 20313-20316.

Biteau, B., J. Labarre, et al. (2003). "ATP-dependent reduction of cysteine-sulphinic acid by S. cerevisiae sulphiredoxin." Nature 425(6961): 980-984.

Bjornstedt, M., M. Hamberg, et al. (1995). "Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocystine strongly stimulates the reaction via catalytically generated selenols." The Journal of biological chemistry 270(20): 11761-11764.

Bora, P. S., C. A. Spilburg, et al. (1989). "Metabolism of ethanol and carcinogens by glutathione transferases." Proceedings of the National Academy of Sciences of the United States of America 86(12): 4470-4473.

Bozonet, S. M., V. J. Findlay, et al. (2005). "Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide." The Journal of biological chemistry 280(24): 23319-23327.

Bozonet, S. M., V. J. Findlay, et al. (2005). "Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide." J Biol Chem 280(24): 23319-23327.

Bradley, T. M., E. Hidalgo, et al. (1997). "Cysteine-to-alanine replacements in the Escherichia coli SoxR protein and the role of the [2Fe-2S] centers in transcriptional activation." Nucleic acids research 25(8): 1469-1475.

Brandes, H. K., F. W. Larimer, et al. (1993). "Direct identification of the primary nucleophile of thioredoxin f." The Journal of biological chemistry 268(25): 18411-18414.

Brown, J. L., H. Bussey, et al. (1994). "Yeast Skn7p functions in a eukaryotic two-component regulatory pathway." The EMBO journal 13(21): 5186-5194.

Brown, J. L., S. North, et al. (1993). "SKN7, a yeast multicopy suppressor of a mutation affecting cell wall beta-glucan assembly, encodes a product with domains homologous to prokaryotic two-component regulators and to heat shock transcription factors." Journal of bacteriology 175(21): 6908-6915.

Bryk, R., P. Griffin, et al. (2000). "Peroxynitrite reductase activity of bacterial peroxiredoxins." Nature 407(6801): 211-215.

Buchanan, B. B. (1991). "Regulation of CO2 assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. Perspective on its discovery, present status, and future development." Archives of biochemistry and biophysics 288(1): 1-9.

Buck, V., J. Quinn, et al. (2001). "Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway." Molecular biology of the cell 12(2): 407-419.

Bulleid, N. J. and L. Ellgaard (2011). "Multiple ways to make disulfides." Trends in biochemical sciences 36(9): 485-492.

Burney, S., J. C. Niles, et al. (1999). "DNA damage in deoxynucleosides and oligonucleotides treated with peroxynitrite." Chemical research in toxicology 12(6): 513-520.

Bushweller, J. H., M. Billeter, et al. (1994). "The nuclear magnetic resonance solution structure of the mixed disulfide between Escherichia coli glutaredoxin(C14S) and glutathione." Journal of molecular biology 235(5): 1585-1597.

Butcher, R. W. and E. W. Sutherland (1962). "Adenosine 3',5'-phosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine." The Journal of biological chemistry 237: 1244-1250.

Calvo, I. A., N. Gabrielli, et al. (2009). "Genome-wide screen of genes required for caffeine tolerance in fission yeast." PloS one 4(8): e6619.

Calvo, I. A., P. Garcia, et al. (2012). "The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H2O2." Nucleic acids research 40(11): 4816-4824.

Calvo, I. A., P. Garcia, et al. (2012). "The transcription factors Pap1 and Prr1 collaborate to activate antioxidant, but not drug tolerance, genes in response to H2O2." Nucleic Acids Res 40(11): 4816-4824.

Carmel-Harel, O. and G. Storz (2000). "Roles of the glutathione- and thioredoxin-dependent reduction systems in the Escherichia coli and saccharomyces cerevisiae responses to oxidative stress." Annual review of microbiology 54: 439-461.

Casso, D. and D. Beach (1996). "A mutation in a thioredoxin reductase homolog suppresses p53-induced growth inhibition in the fission yeast Schizosaccharomyces pombe." Molecular & general genetics : MGG 252(5): 518-529.

Castillo, E. A., J. Ayte, et al. (2002). "Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues." Molecular microbiology 45(1): 243-254.

Castillo, E. A., J. Ayte, et al. (2002). "Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues." Mol.Microbiol. 45(1): 243-254.

Castillo, E. A., A. P. Vivancos, et al. (2003). "Schizosaccharomyces pombe cells lacking the Ran-binding protein Hba1 show a multidrug resistance phenotype due to constitutive nuclear accumulation of Pap1." The Journal of biological chemistry 278(42): 40565-40572.

Castillo, E. A., A. P. Vivancos, et al. (2003). "Schizosaccharomyces pombe cells lacking the Ran-binding protein Hba1 show a multidrug resistance phenotype due to constitutive nuclear accumulation of Pap1." J Biol Chem 278(42): 40565-40572.

Chae, H. Z., H. J. Kim, et al. (1999). "Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin." Diabetes research and clinical practice 45(2-3): 101-112.

Chae, H. Z., I. H. Kim, et al. (1993). "Cloning, sequencing, and mutation of thiol-specific antioxidant gene of Saccharomyces cerevisiae." The Journal of biological chemistry 268(22): 16815-16821.

Chae, H. Z. and S. G. Rhee (1994). "A thiol-specific antioxidant and sequence homology to various proteins of unknown function." BioFactors 4(3-4): 177-180.

Chae, H. Z., T. B. Uhm, et al. (1994). "Dimerization of thiol-specific antioxidant and the essential role of cysteine 47." Proceedings of the National Academy of Sciences of the United States of America 91(15): 7022-7026.

Chauhan, R. and S. C. Mande (2001). "Characterization of the Mycobacterium tuberculosis H37Rv alkyl hydroperoxidase AhpC points to the importance of ionic interactions in oligomerization and activity." The Biochemical journal 354(Pt 1): 209-215.

Chen, D., W. M. Toone, et al. (2003). "Global transcriptional responses of fission yeast to environmental stress." Mol.Biol.Cell 14(1): 214-229.

Chen, D., W. M. Toone, et al. (2003). "Global transcriptional responses of fission yeast to environmental stress." Molecular biology of the cell 14(1): 214-229.

Chen, D., C. R. Wilkinson, et al. (2008). "Multiple pathways differentially regulate global oxidative stress responses in fission yeast." Mol Biol Cell 19(1): 308-317.

Chen, D., C. R. Wilkinson, et al. (2008). "Multiple pathways differentially regulate global oxidative stress responses in fission yeast." Molecular biology of the cell 19(1): 308-317.

Chen, Y., V. Kamat, et al. (2002). "Ratio statistics of gene expression levels and applications to microarray data analysis." Bioinformatics 18(9): 1207-1215.

Chevallet, M., E. Wagner, et al. (2003). "Regeneration of peroxiredoxins during recovery after oxidative stress: only some overoxidized peroxiredoxins can be reduced during recovery after oxidative stress." The Journal of biological chemistry 278(39): 37146-37153.

Chiang, S. M. and H. E. Schellhorn (2012). "Regulators of oxidative stress response genes in Escherichia coli and their functional conservation in bacteria." Archives of biochemistry and biophysics.

Cho, Y. W., E. H. Park, et al. (2002). "A second stress-inducible glutathione S-transferase gene from Schizosaccharomyces pombe." Biochimica et biophysica acta 1574(3): 399-402.

Choi, H., S. Kim, et al. (2001). "Structural basis of the redox switch in the OxyR transcription factor." Cell 105(1): 103-113.

Choi, J. H., W. Lou, et al. (1998). "A novel membrane-bound glutathione S-transferase functions in the stationary phase of the yeast Saccharomyces cerevisiae." The Journal of biological chemistry 273(45): 29915-29922.

Christman, M. F., R. W. Morgan, et al. (1985). "Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium." Cell 41(3): 753-762.

Chuang, M. H., M. S. Wu, et al. (2006). "The antioxidant protein alkylhydroperoxide reductase of Helicobacter pylori switches from a peroxide reductase to a molecular chaperone function." Proceedings of the National Academy of Sciences of the United States of America 103(8): 2552-2557.

Chung, W. H., K. D. Kim, et al. (2004). "Differential expression and role of two dithiol glutaredoxins Grx1 and Grx2 in Schizosaccharomyces pombe." Biochemical and biophysical research communications 321(4): 922-929.

Chung, W. H., K. D. Kim, et al. (2005). "Localization and function of three monothiol glutaredoxins in Schizosaccharomyces pombe." Biochemical and biophysical research communications 330(2): 604-610.

Collet, J. F. and J. Messens (2010). "Structure, function, and mechanism of thioredoxin proteins." Antioxid Redox Signal 13(8): 1205-1216.

Collet, J. F., J. Riemer, et al. (2002). "Reconstitution of a disulfide isomerization system." The Journal of biological chemistry 277(30): 26886-26892.

Collinson, E. J. and C. M. Grant (2003). "Role of yeast glutaredoxins as glutathione S-transferases." The Journal of biological chemistry 278(25): 22492-22497.

Collinson, E. J., G. L. Wheeler, et al. (2002). "The yeast glutaredoxins are active as glutathione peroxidases." The Journal of biological chemistry 277(19): 16712-16717.

Coyle, J. T. and P. Puttfarcken (1993). "Oxidative stress, glutamate, and neurodegenerative disorders." Science 262(5134): 689-695.

Dawson, T. M. and V. L. Dawson (2003). "Molecular pathways of neurodegeneration in Parkinson's disease." Science 302(5646): 819-822.

Debarbieux, L. and J. Beckwith (1998). "The reductive enzyme thioredoxin 1 acts as an oxidant when it is exported to the Escherichia coli periplasm." Proceedings of the National Academy of Sciences of the United States of America 95(18): 10751-10756.

Degols, G. and P. Russell (1997). "Discrete roles of the Spc1 kinase and the Atf1 transcription factor in the UV response of Schizosaccharomyces pombe." Molecular and cellular biology 17(6): 3356-3363.

Delaunay, A., A. D. Isnard, et al. (2000). "H2O2 sensing through oxidation of the Yap1 transcription factor." The EMBO journal 19(19): 5157-5166.

Delaunay, A., A. D. Isnard, et al. (2000). "H(2)O(2) sensing through oxidation of the Yap1 transcription factor." EMBO J. 19(19): 5157-5166.

Delaunay, A., D. Pflieger, et al. (2002). "A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation." Cell 111(4): 471-481.

Delaunay, A., D. Pflieger, et al. (2002). "A thiol peroxidase is an h(2)o(2) receptor and redox-transducer in gene activation." Cell 111(4): 471-481.

Dietz, K. J. (2003). "Plant peroxiredoxins." Annual review of plant biology 54: 93-107.

Drakulic, T., M. D. Temple, et al. (2005). "Involvement of oxidative stress response genes in redox homeostasis, the level of reactive oxygen species, and ageing in Saccharomyces cerevisiae." FEMS yeast research 5(12): 1215-1228.

Eckers, E., M. Bien, et al. (2009). "Biochemical characterization of dithiol glutaredoxin 8 from Saccharomyces cerevisiae: the catalytic redox mechanism redux." Biochemistry 48(6): 1410-1423.

Ellis, H. R. and L. B. Poole (1997). "Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase." Biochemistry 36(48): 15013-15018.

Ellis, H. R. and L. B. Poole (1997). "Roles for the two cysteine residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from Salmonella typhimurium." Biochemistry 36(43): 13349-13356.

Endo, T., K. Yamano, et al. (2010). "Structural basis for the disulfide relay system in the mitochondrial intermembrane space." Antioxidants & redox signaling 13(9): 1359-1373.

Esterbauer, H. and K. H. Cheeseman (1990). "Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal." Methods in enzymology 186: 407-421.

Esterbauer, H., K. H. Cheeseman, et al. (1982). "Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP-Fe2+ in rat liver microsomes." The Biochemical journal 208(1): 129-140.

Fabre, F. (1972). "Relation between repair mechanisms and induced mitotic recombination after UV irradiation, in the yeast Schizosaccharomyces pombe. Effects of caffeine." Molecular & general genetics : MGG 117(2): 153-166.

Fernandes, A. P., M. Fladvad, et al. (2005). "A novel monothiol glutaredoxin (Grx4) from Escherichia coli can serve as a substrate for thioredoxin reductase." The Journal of biological chemistry 280(26): 24544-24552.

Fernandes, A. P. and A. Holmgren (2004). "Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system." Antioxidants & redox signaling 6(1): 63-74.

Fernandes, L., C. Rodrigues-Pousada, et al. (1997). "Yap, a novel family of eight bZIP proteins in Saccharomyces cerevisiae with distinct biological functions." Molecular and cellular biology 17(12): 6982-6993.

Finkel, T. and N. J. Holbrook (2000). "Oxidants, oxidative stress and the biology of ageing." Nature 408(6809): 239-247.

Floyd, R. A., M. S. West, et al. (1990). "Mediation of 8-hydroxy-guanine formation in DNA by thiazin dyes plus light." Free radical biology & medicine 8(4): 327-330.

Forsburg, S. L. (2001). "The art and design of genetic screens: yeast." Nature reviews. Genetics 2(9): 659-668.

Fridovich, I. (2004). "Mitochondria: are they the seat of senescence?" Aging cell 3(1): 13-16.

Furukawa, Y., A. S. Torres, et al. (2004). "Oxygen-induced maturation of SOD1: a key role for disulfide formation by the copper chaperone CCS." The EMBO journal 23(14): 2872-2881.

Gabriel, K., D. Milenkovic, et al. (2007). "Novel mitochondrial intermembrane space proteins as substrates of the MIA import pathway." Journal of molecular biology 365(3): 612-620.

Gan, Z. R. (1991). "Yeast thioredoxin genes." The Journal of biological chemistry 266(3): 1692-1696.

Gan, Z. R. and W. W. Wells (1987). "Identification and reactivity of the catalytic site of pig liver thioltransferase." The Journal of biological chemistry 262(14): 6704-6707.

Garcia-Santamarina, S., S. Boronat, et al. (2011). "The oxidized thiol proteome in fission yeast--optimization of an ICAT-based method to identify H2O2-oxidized proteins." J Proteomics 74(11): 2476-2486.

Garcia-Santamarina, S., S. Boronat, et al. (2011). "The oxidized thiol proteome in fission yeast--optimization of an ICAT-based method to identify H2O2-oxidized proteins." Journal of proteomics 74(11): 2476-2486.

Gasdaska, P. Y., M. M. Berggren, et al. (1999). "Cloning, sequencing and functional expression of a novel human thioredoxin reductase." FEBS letters 442(1): 105-111.

Gentner, N. E. and M. M. Werner (1975). "Repair in Schizosaccharomyces pombe as measured by recovery from caffeine enhancement of radiation-induced lethality." Molecular & general genetics : MGG 142(3): 171-183.

Giles, N. M., A. B. Watts, et al. (2003). "Metal and redox modulation of cysteine protein function." Chemistry & biology 10(8): 677-693.

Giorgio, M., M. Trinei, et al. (2007). "Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals?" Nature reviews. Molecular cell biology 8(9): 722-728.

Gonzalez-Flecha, B. and B. Demple (1997). "Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing Escherichia coli." Journal of bacteriology 179(2): 382-388.

Gorlatov, S. N. and T. C. Stadtman (1998). "Human thioredoxin reductase from HeLa cells: selective alkylation of selenocysteine in the protein inhibits enzyme activity and reduction with NADPH influences affinity to heparin." Proceedings of the National Academy of Sciences of the United States of America 95(15): 8520-8525.

Grant, C. M. (2001). "Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions." Molecular microbiology 39(3): 533-541.

Grant, C. M., L. P. Collinson, et al. (1996). "Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation." Molecular microbiology 21(1): 171-179.

Grant, C. M., S. Luikenhuis, et al. (2000). "Differential regulation of glutaredoxin gene expression in response to stress conditions in the yeast Saccharomyces cerevisiae." Biochimica et biophysica acta 1490(1-2): 33-42.

Grant, C. M., F. H. MacIver, et al. (1996). "Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast Saccharomyces cerevisiae." Current genetics 29(6): 511-515.

Grant, C. M., G. Perrone, et al. (1998). "Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast Saccharomyces cerevisiae." Biochemical and biophysical research communications 253(3): 893-898.

Grauschopf, U., J. R. Winther, et al. (1995). "Why is DsbA such an oxidizing disulfide catalyst?" Cell 83(6): 947-955.

Gravina, S. A. and J. J. Mieyal (1993). "Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase." Biochemistry 32(13): 3368-3376.

Greenberg, J. T., P. Monach, et al. (1990). "Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in Escherichia coli." Proceedings of the National Academy of Sciences of the United States of America 87(16): 6181-6185.

Gruber, C. W., M. Cemazar, et al. (2006). "Protein disulfide isomerase: the structure of oxidative folding." Trends in biochemical sciences 31(8): 455-464.

Gulshan, K. and W. S. Moye-Rowley (2011). "Vacuolar import of phosphatidylcholine requires the ATP-binding cassette transporter Ybt1." Traffic 12(9): 1257-1268.

Gulshan, K., S. A. Rovinsky, et al. (2004). "YBP1 and its homologue YBP2/YBH1 influence oxidative-stress tolerance by nonidentical mechanisms in Saccharomyces cerevisiae." Eukaryotic cell 3(2): 318-330.

Gutteridge, J. M. and B. Halliwell (2000). "Free radicals and antioxidants in the year 2000. A historical look to the future." Annals of the New York Academy of Sciences 899: 136-147.

Hall, A., K. Nelson, et al. (2011). "Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins." Antioxidants & redox signaling 15(3): 795-815.

Halliwell, B. (2006). "Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life." Plant physiology 141(2): 312-322.

Halliwell, B. and J. M. Gutteridge (1986). "Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts." Archives of biochemistry and biophysics 246(2): 501-514.

Halliwell, B. and J. M. Gutteridge (1988). "Free radicals and antioxidant protection: mechanisms and significance in toxicology and disease." Human toxicology 7(1): 7-13.

Harshman, L. G. and A. A. James (1998). "Differential gene expression in insects: transcriptional control." Annual review of entomology 43: 671-700.

Hassan, H. M. and I. Fridovich (1977). "Regulation of the synthesis of superoxide dismutase in Escherichia coli. Induction by methyl viologen." The Journal of biological chemistry 252(21): 7667-7672.

Hayes, J. D. and L. I. McLellan (1999). "Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress." Free radical research 31(4): 273-300.

He, X. J. and J. S. Fassler (2005). "Identification of novel Yap1p and Skn7p binding sites involved in the oxidative stress response of Saccharomyces cerevisiae." Molecular microbiology 58(5): 1454-1467.

He, X. J., K. E. Mulford, et al. (2009). "Oxidative stress function of the Saccharomyces cerevisiae Skn7 receiver domain." Eukaryotic cell 8(5): 768-778.

Hekimi, S., J. Lapointe, et al. (2011). "Taking a "good" look at free radicals in the aging process." Trends in cell biology 21(10): 569-576.

Held, J. M. and B. W. Gibson (2012). "Regulatory control or oxidative damage? Proteomic approaches to interrogate the role of cysteine oxidation status in biological processes." Molecular & cellular proteomics : MCP 11(4): R111 013037.

Hidalgo, E., J. M. Bollinger, Jr., et al. (1995). "Binuclear [2Fe-2S] clusters in the Escherichia coli SoxR protein and role of the metal centers in transcription." The Journal of biological chemistry 270(36): 20908-20914.

Hidalgo, E. and B. Demple (1994). "An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein." The EMBO journal 13(1): 138-146.

Hidalgo, E. and B. Demple (1997). "Spacing of promoter elements regulates the basal expression of the soxS gene and converts SoxR from a transcriptional activator into a repressor." The EMBO journal 16(5): 1056-1065.

Hiltunen, J. K., A. M. Mursula, et al. (2003). "The biochemistry of peroxisomal beta-oxidation in the yeast Saccharomyces cerevisiae." FEMS microbiology reviews 27(1): 35-64.

Hirota, K., M. Matsui, et al. (1997). "AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1." Proceedings of the National Academy of Sciences of the United States of America 94(8): 3633-3638.

Hofmann, B., H. J. Hecht, et al. (2002). "Peroxiredoxins." Biological chemistry 383(3-4): 347-364.

Holmgren, A. (1984). "Enzymatic reduction-oxidation of protein disulfides by thioredoxin." Methods in enzymology 107: 295-300.

Holmgren, A. (1985). "Thioredoxin." Annual review of biochemistry 54: 237-271.

Holmgren, A. (1989). "Thioredoxin and glutaredoxin systems." The Journal of biological chemistry 264(24): 13963-13966.

Holmgren, A. (1995). "Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide." Structure 3(3): 239-243.

Holmgren, A. (2000). "Redox regulation by thioredoxin and thioredoxin reductase." BioFactors 11(1-2): 63-64.

Horling, F., P. Lamkemeyer, et al. (2003). "Divergent light-, ascorbate-, and oxidative stress-dependent regulation of expression of the peroxiredoxin gene family in Arabidopsis." Plant physiology 131(1): 317-325.

Hu, M. L. (1994). "Measurement of protein thiol groups and glutathione in plasma." Methods in enzymology 233: 380-385.

Hussain, M. and J. Lenard (1991). "Characterization of PDR4, a Saccharomyces cerevisiae gene that confers pleiotropic drug resistance in high-copy number: identity with YAP1, encoding a transcriptional activator [corrected]." Gene 101(1): 149-152.

Inoue, Y., T. Matsuda, et al. (1999). "Genetic analysis of glutathione peroxidase in oxidative stress response of Saccharomyces cerevisiae." The Journal of biological chemistry 274(38): 27002-27009.

Izawa, S., Y. Inoue, et al. (1995). "Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in Saccharomyces cerevisiae." FEBS letters 368(1): 73-76.

Izawa, S., Y. Inoue, et al. (1996). "Importance of catalase in the adaptive response to hydrogen peroxide: analysis of acatalasaemic Saccharomyces cerevisiae." The Biochemical journal 320 (Pt 1): 61-67.

Izquierdo, A., C. Casas, et al. (2008). "Saccharomyces cerevisiae Grx6 and Grx7 are monothiol glutaredoxins associated with the early secretory pathway." Eukaryotic cell 7(8): 1415-1426.

Jacoby, J. J., S. M. Nilius, et al. (1998). "A screen for upstream components of the yeast protein kinase C signal transduction pathway identifies the product of the SLG1 gene." Molecular & general genetics : MGG 258(1-2): 148-155.

Jang, H. H., K. O. Lee, et al. (2004). "Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function." Cell 117(5): 625-635.

Jara, M., A. P. Vivancos, et al. (2007). "The peroxiredoxin Tpx1 is essential as a H2O2 scavenger during aerobic growth in fission yeast." Molecular biology of the cell 18(6): 2288-2295.

Jara, M., A. P. Vivancos, et al. (2007). "The peroxiredoxin Tpx1 is essential as a H2O2 scavenger during aerobic growth in fission yeast." Mol Biol Cell 18(6): 2288-2295.

Jara, M., A. P. Vivancos, et al. (2008). "C-terminal truncation of the peroxiredoxin Tpx1 decreases its sensitivity for hydrogen peroxide without compromising its role in signal transduction." Genes to cells : devoted to molecular & cellular mechanisms 13(2): 171-179.

Jbel, M., A. Mercier, et al. (2011). "Grx4 monothiol glutaredoxin is required for iron limitation-dependent inhibition of Fep1." Eukaryotic cell 10(5): 629-645.

Jimenez, A., L. Mateos, et al. (2007). "The txl1+ gene from Schizosaccharomyces pombe encodes a new thioredoxin-like 1 protein that participates in the antioxidant defence against tert-butyl hydroperoxide." Yeast 24(6): 481-490.

Johnson, F. B., D. A. Sinclair, et al. (1999). "Molecular biology of aging." Cell 96(2): 291-302.

Jungwirth, H., F. Wendler, et al. (2000). "Diazaborine resistance in yeast involves the efflux pumps Ycf1p and Flr1p and is enhanced by a gain-of-function allele of gene YAP1." European journal of biochemistry / FEBS 267(15): 4809-4816.

Kadokura, H. (2003). "[Protein disulfide bond formation in vivo]." Tanpakushitsu kakusan koso. Protein, nucleic acid, enzyme 48(10): 1386-1393.

Kadokura, H., F. Katzen, et al. (2003). "Protein disulfide bond formation in prokaryotes." Annual review of biochemistry 72: 111-135.

Kalinina, E. V., N. N. Chernov, et al. (2008). "Involvement of thio-, peroxi-, and glutaredoxins in cellular redox-dependent processes." Biochemistry. Biokhimiia 73(13): 1493-1510.

Kalinina, E. V., N. N. Chernov, et al. (2007). "Expression of genes for redox-dependent glutathione S-transferase isoforms GSTP1-1 and GSTA4-4 in tumor cell during the development doxorubicin resistance." Bulletin of experimental biology and medicine 143(3): 328-330.

Kanoh, J., Y. Watanabe, et al. (1996). "Schizosaccharomyces pombe gad7+ encodes a phosphoprotein with a bZIP domain, which is required for proper G1 arrest and gene expression under nitrogen starvation." Genes to cells : devoted to molecular & cellular mechanisms 1(4): 391-408.

Ketela, T., J. L. Brown, et al. (1998). "Yeast Skn7p activity is modulated by the Sln1p-Ypd1p osmosensor and contributes to regulation of the HOG pathway." Molecu3smotic stresses." Molecules and cells 20(1): 43-50.

Kim, H. G., K. N. Park, et al. (2001). "Characterization and regulation of glutathione S-transferase gene from Schizosaccharomyces pombe." Biochimica et biophysica acta 1520(2): 179-185.

Kim, J. S., M. A. Bang, et al. (2010). "Distinct functional roles of peroxiredoxin isozymes and glutathione peroxidase from fission yeast, Schizosaccharomyces pombe." BMB Rep 43(3): 170-175.

Kim, S. J., E. M. Jung, et al. (2007). "Cellular functions and transcriptional regulation of a third thioredoxin from Schizosaccharomyces pombe." Can J Microbiol 53(6): 775-783.

Kim, S. J., E. M. Jung, et al. (2007). "Cellular functions and transcriptional regulation of a third thioredoxin from Schizosaccharomyces pombe." Canadian journal of microbiology 53(6): 775-783.

Kim, S. O., K. Merchant, et al. (2002). "OxyR: a molecular code for redox-related signaling." Cell 109(3): 383-396.

Kim, S. Y., E. J. Kim, et al. (2002). "Yeast thioredoxin peroxidase expression enhances the resistance of Escherichia coli to oxidative stress induced by singlet oxygen." Redox report : communications in free radical research 7(2): 79-84.

Koo, K. H., S. Lee, et al. (2002). "Regulation of thioredoxin peroxidase activity by C-terminal truncation." Archives of biochemistry and biophysics 397(2): 312-318.

Krems, B., C. Charizanis, et al. (1996). "The response regulator-like protein Pos9/Skn7 of Saccharomyces cerevisiae is involved in oxidative stress resistance." Current genetics 29(4): 327-334.

Kristal, B. S. and B. P. Yu (1992). "An emerging hypothesis: synergistic induction of aging by free radicals and Maillard reactions." Journal of gerontology 47(4): B107-114.

Kucharczyk, R., R. Gromadka, et al. (1999). "Disruption of six novel yeast genes located on chromosome II reveals one gene essential for vegetative growth and two required for sporulation and conferring hypersensitivity to various chemicals." Yeast 15(10B): 987-1000.

Kudo, N., H. Taoka, et al. (1999). "A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor Pap1." J.Biol.Chem. 274(21): 15151-15158.

Kuge, S. and N. Jones (1994). "YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides." The EMBO journal 13(3): 655-664.

Kuge, S., N. Jones, et al. (1997). "Regulation of yAP-1 nuclear localization in response to oxidative stress." The EMBO journal 16(7): 1710-1720.

Kuge, S., T. Toda, et al. (1998). "Crm1 (XpoI) dependent nuclear export of the budding yeast transcription factor yAP-1 is sensitive to oxidative stress." Genes to cells : devoted to molecular & cellular mechanisms 3(8): 521-532.

Kumada, K., M. Yanagida, et al. (1996). "Caffeine-resistance in fission yeast is caused by mutations in a single essential gene, crm1+." Molecular & general genetics : MGG 250(1): 59-68.

Kumar, C., A. Igbaria, et al. (2011). "Glutathione revisited: a vital function in iron metabolism and ancillary role in thiol-redox control." The EMBO journal 30(10): 2044-2056.

Kumar, S., M. Bjornstedt, et al. (1992). "Selenite is a substrate for calf thymus thioredoxin reductase and thioredoxin and elicits a large non-stoichiometric oxidation of NADPH in the presence of oxygen." European journal of biochemistry / FEBS 207(2): 435-439.

Laurent, T. C., E. C. Moore, et al. (1964). "Enzymatic Synthesis of Deoxyribonucleotides. Iv. Isolation and Characterization of Thioredoxin, the Hydrogen Donor from Escherichia Coli B." The Journal of biological chemistry 239: 3436-3444.

Lee, J., I. W. Dawes, et al. (1997). "Isolation, expression, and regulation of the pgr1(+) gene encoding glutathione reductase absolutely required for the growth of Schizosaccharomyces pombe." The Journal of biological chemistry 272(37): 23042-23049.

Lee, J., C. Godon, et al. (1999). "Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast." The Journal of biological chemistry 274(23): 16040-16046.

Lee, S. Y., J. Y. Song, et al. (2008). "Gpx1 is a stationary phase-specific thioredoxin peroxidase in fission yeast." Biochemical and biophysical research communications 367(1): 67-71.

Leppert, G., R. McDevitt, et al. (1990). "Cloning by gene amplification of two loci conferring multiple drug resistance in Saccharomyces." Genetics 125(1): 13-20.

Levine, R. L., J. A. Williams, et al. (1994). "Carbonyl assays for determination of oxidatively modified proteins." Methods in enzymology 233: 346-357.

Li, S., A. Ault, et al. (1998). "The yeast histidine protein kinase, Sln1p, mediates phosphotransfer to two response regulators, Ssk1p and Skn7p." The EMBO journal 17(23): 6952-6962.

Li, Z. S., R. G. Zhen, et al. (1995). "1-Chloro-2,4-Dinitrobenzene-Elicited Increase in Vacuolar Glutathione-S-Conjugate Transport Activity." Plant physiology 109(1): 177-185.
Lin, M. T. and M. F. Beal (2006). "Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases." Nature 443(7113): 787-795.
Llobell, A., A. Lopez-Ruiz, et al. (1988). "Glutathione reductase directly mediates the stimulation of yeast glucose-6-phosphate dehydrogenase by GSSG." The Biochemical journal 249(1): 293-296.
Longo, V. D., E. B. Gralla, et al. (1996). "Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Mitochondrial production of toxic oxygen species in vivo." The Journal of biological chemistry 271(21): 12275-12280.
Lopez-Aviles, S., M. Grande, et al. (2005). "Inactivation of the Cdc25 phosphatase by the stress-activated Srk1 kinase in fission yeast." Molecular cell 17(1): 49-59.
Loprieno, N., R. Barale, et al. (1974). "Genetic effects of caffeine." Mutation research 26(2): 83-87.
Low, F. M., M. B. Hampton, et al. (2008). "Peroxiredoxin 2 and peroxide metabolism in the erythrocyte." Antioxidants & redox signaling 10(9): 1621-1630.
Luikenhuis, S., G. Perrone, et al. (1998). "The yeast Saccharomyces cerevisiae contains two glutaredoxin genes that are required for protection against reactive oxygen species." Molecular biology of the cell 9(5): 1081-1091.
Lundberg, M., C. Johansson, et al. (2001). "Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms." The Journal of biological chemistry 276(28): 26269-26275.
Luthman, M. and A. Holmgren (1982). "Rat liver thioredoxin and thioredoxin reductase: purification and characterization." Biochemistry 21(26): 6628-6633.
Martin, J. L., J. C. Bardwell, et al. (1993). "Crystal structure of the DsbA protein required for disulphide bond formation in vivo." Nature 365(6445): 464-468.
Martinez-Pastor, M. T., G. Marchler, et al. (1996). "The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for

transcriptional induction through the stress response element (STRE)." The EMBO journal 15(9): 2227-2235.

Matthews, J. R., N. Wakasugi, et al. (1992). "Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62." Nucleic acids research 20(15): 3821-3830.

Maundrell, K. (1993). "Thiamine-repressible expression vectors pREP and pRIP for fission yeast." Gene 123(1): 127-130.

Meister, A. (1988). "On the discovery of glutathione." Trends in biochemical sciences 13(5): 185-188.

Mesecke, N., S. Mittler, et al. (2008). "Two novel monothiol glutaredoxins from Saccharomyces cerevisiae provide further insight into iron-sulfur cluster binding, oligomerization, and enzymatic activity of glutaredoxins." Biochemistry 47(5): 1452-1463.

Mesecke, N., N. Terziyska, et al. (2005). "A disulfide relay system in the intermembrane space of mitochondria that mediates protein import." Cell 121(7): 1059-1069.

Meyer, Y., B. B. Buchanan, et al. (2009). "Thioredoxins and glutaredoxins: unifying elements in redox biology." Annu Rev Genet 43: 335-367.

Michelet, L., M. Zaffagnini, et al. (2006). "Thioredoxins, glutaredoxins, and glutathionylation: new crosstalks to explore." Photosynthesis research 89(2-3): 225-245.

Millar, J. B., V. Buck, et al. (1995). "Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast." Genes & development 9(17): 2117-2130.

Miranda-Vizuete, A., A. E. Damdimopoulos, et al. (1999). "cDNA cloning, expression and chromosomal localization of the mouse mitochondrial thioredoxin reductase gene(1)." Biochimica et biophysica acta 1447(1): 113-118.

Moon, J. S., H. W. Lim, et al. (2005). "Characterization and regulation of the gene encoding monothiol glutaredoxin 3 in the fission yeast Schizosaccharomyces pombe." Molecules and cells 20(1): 74-82.

Morgan, B. A., G. R. Banks, et al. (1997). "The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast Saccharomyces cerevisiae." The EMBO journal 16(5): 1035-1044.

Morgan, B. A., N. Bouquin, et al. (1995). "A yeast transcription factor bypassing the requirement for SBF and DSC1/MBF in budding yeast has homology to bacterial signal transduction proteins." The EMBO journal 14(22): 5679-5689.

Moye-Rowley, W. S., K. D. Harshman, et al. (1989). "Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins." Genes & development 3(3): 283-292.

Muhlenhoff, U., S. Molik, et al. (2010). "Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster." Cell metabolism 12(4): 373-385.

Mulford, K. E. and J. S. Fassler (2011). "Association of the Skn7 and Yap1 transcription factors in the Saccharomyces cerevisiae oxidative stress response." Eukaryotic cell 10(6): 761-769.

Muller, E. G. (1996). "A glutathione reductase mutant of yeast accumulates high levels of oxidized glutathione and requires thioredoxin for growth." Molecular biology of the cell 7(11): 1805-1813.

Muller, M. R., K. A. Wright, et al. (1991). "Differential properties of cisplatin and tetraplatin with respect to cytotoxicity and perturbation of cellular glutathione levels." Cancer chemotherapy and pharmacology 28(4): 273-276.

Murphy, J. E. and R. K. Porter (2009). "The control of oxidative phosphorylation in the adrenal gland (Y1) cell line." Advances in experimental medicine and biology 645: 35-41.

Mustacich, D. and G. Powis (2000). "Thioredoxin reductase." The Biochemical journal 346 Pt 1: 1-8.

Mutoh, N., C. W. Nakagawa, et al. (1999). "The role of catalase in hydrogen peroxide resistance in fission yeast Schizosaccharomyces pombe." Canadian journal of microbiology 45(2): 125-129.

Nakagawa, C. W., K. Yamada, et al. (2000). "Role of Atf1 and Pap1 in the induction of the catalase gene of fission yeast schizosaccharomyces pombe." Journal of biochemistry 127(2): 233-238.

Naoe, M., Y. Ohwa, et al. (2004). "Identification of Tim40 that mediates protein sorting to the mitochondrial intermembrane space." The Journal of biological chemistry 279(46): 47815-47821.

Nguyen, A. N. and K. Shiozaki (1999). "Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases." Genes & development 13(13): 1653-1663.

Nogoceke, E., D. U. Gommel, et al. (1997). "A unique cascade of oxidoreductases catalyses trypanothione-mediated peroxide metabolism in Crithidia fasciculata." Biological chemistry 378(8): 827-836.

Nordstrand, K., F. Aslund, et al. (1999). "Direct NMR observation of the Cys-14 thiol proton of reduced Escherichia coli glutaredoxin-3 supports the presence of an active site thiol-thiolate hydrogen bond." FEBS letters 449(2-3): 196-200.

Nordstrand, K., F. slund, et al. (1999). "NMR structure of Escherichia coli glutaredoxin 3-glutathione mixed disulfide complex: implications for the enzymatic mechanism." Journal of molecular biology 286(2): 541-552.

Novogrodsky, A., A. Ravid, et al. (1982). "Hydroxyl radical scavengers inhibit lymphocyte mitogenesis." Proceedings of the National Academy of Sciences of the United States of America 79(4): 1171-1174.

Ohmiya, R., C. Kato, et al. (1999). "A fission yeast gene (prr1(+)) that encodes a response regulator implicated in oxidative stress response." Journal of biochemistry 125(6): 1061-1066.

Ohmiya, R., H. Yamada, et al. (2000). "The Prr1 response regulator is essential for transcription of ste11+ and for sexual development in fission yeast." Molecular & general genetics : MGG 264(4): 441-451.

Okazaki, S., A. Naganuma, et al. (2005). "Peroxiredoxin-mediated redox regulation of the nuclear localization of Yap1, a transcription factor in budding yeast." Antioxidants & redox signaling 7(3-4): 327-334.

Okazaki, S., T. Tachibana, et al. (2007). "Multistep disulfide bond formation in Yap1 is required for sensing and transduction of H2O2 stress signal." Mol Cell 27(4): 675-688.

Okazaki, S., T. Tachibana, et al. (2007). "Multistep disulfide bond formation in Yap1 is required for sensing and transduction of H2O2 stress signal." Molecular cell 27(4): 675-688.

Osman, F. and S. McCready (1998). "Differential effects of caffeine on DNA damage and replication cell cycle checkpoints in the fission

yeast Schizosaccharomyces pombe." Molecular & general genetics : MGG 260(4): 319-334.

Palabiyik, B., C. Kig, et al. (2012). "Investigation of the relationship between oxidative stress and glucose signaling in Schizosaccharomyces pombe." Biochemical genetics 50(5-6): 336-349.

Paulsen, C. E. and K. S. Carroll (2009). "Chemical dissection of an essential redox switch in yeast." Chemistry & biology 16(2): 217-225.

Pedrajas, J. R., E. Kosmidou, et al. (1999). "Identification and functional characterization of a novel mitochondrial thioredoxin system in Saccharomyces cerevisiae." The Journal of biological chemistry 274(10): 6366-6373.

Pollard, M. G., K. J. Travers, et al. (1998). "Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum." Molecular cell 1(2): 171-182.

Pollegioni, L., L. Piubelli, et al. (2007). "Physiological functions of D-amino acid oxidases: from yeast to humans." Cellular and molecular life sciences : CMLS 64(11): 1373-1394.

Pomposiello, P. J. and B. Demple (2001). "Redox-operated genetic switches: the SoxR and OxyR transcription factors." Trends in biotechnology 19(3): 109-114.

Poole, L. B., P. A. Karplus, et al. (2004). "Protein sulfenic acids in redox signaling." Annual review of pharmacology and toxicology 44: 325-347.

Porras, P., C. A. Padilla, et al. (2006). "One single in-frame AUG codon is responsible for a diversity of subcellular localizations of glutaredoxin 2 in Saccharomyces cerevisiae." The Journal of biological chemistry 281(24): 16551-16562.

Powis, G. and W. R. Montfort (2001). "Properties and biological activities of thioredoxins." Annual review of biophysics and biomolecular structure 30: 421-455.

Quinn, J., V. J. Findlay, et al. (2002). "Distinct regulatory proteins control the graded transcriptional response to increasing H(2)O(2) levels in fission yeast Schizosaccharomyces pombe." Molecular biology of the cell 13(3): 805-816.

Quinn, J., P. Malakasi, et al. (2011). "Two-component mediated peroxide sensing and signal transduction in fission yeast." Antioxidants & redox signaling 15(1): 153-165.

Radi, R., J. S. Beckman, et al. (1991). "Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide." Archives of biochemistry and biophysics 288(2): 481-487.

Requejo, R., T. R. Hurd, et al. (2010). "Cysteine residues exposed on protein surfaces are the dominant intramitochondrial thiol and may protect against oxidative damage." The FEBS journal 277(6): 1465-1480.

Rietsch, A. and J. Beckwith (1998). "The genetics of disulfide bond metabolism." Annual review of genetics 32: 163-184.

Rietsch, A., D. Belin, et al. (1996). "An in vivo pathway for disulfide bond isomerization in Escherichia coli." Proceedings of the National Academy of Sciences of the United States of America 93(23): 13048-13053.

Rodriguez-Gabriel, M. A., G. Burns, et al. (2003). "RNA-binding protein Csx1 mediates global control of gene expression in response to oxidative stress." The EMBO journal 22(23): 6256-6266.

Rodriguez-Manzaneque, M. T., J. Ros, et al. (1999). "Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in Saccharomyces cerevisiae." Molecular and cellular biology 19(12): 8180-8190.

Rodriguez-Manzaneque, M. T., J. Tamarit, et al. (2002). "Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes." Molecular biology of the cell 13(4): 1109-1121.

Roos, G. and J. Messens (2011). "Protein sulfenic acid formation: from cellular damage to redox regulation." Free radical biology & medicine 51(2): 314-326.

Rundlof, A. K., M. Carlsten, et al. (2000). "Prominent expression of the selenoprotein thioredoxin reductase in the medullary rays of the rat kidney and thioredoxin reductase mRNA variants differing at the 5' untranslated region." The Biochemical journal 347 Pt 3: 661-668.

Saitoh, M., H. Nishitoh, et al. (1998). "Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1." The EMBO journal 17(9): 2596-2606.

Salinas, A. E. and M. G. Wong (1999). "Glutathione S-transferases--a review." Current medicinal chemistry 6(4): 279-309.

Samejima, I., S. Mackie, et al. (1997). "Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast." The EMBO journal 16(20): 6162-6170.

Sanchez-Piris, M., F. Posas, et al. (2002). "The serine/threonine kinase Cmk2 is required for oxidative stress response in fission yeast." The Journal of biological chemistry 277(20): 17722-17727.

Santos, J. L. and K. Shiozaki (2001). "Fungal histidine kinases." Science's STKE : signal transduction knowledge environment 2001(98): re1.

Schmidt, P. J., C. Kunst, et al. (2000). "Copper activation of superoxide dismutase 1 (SOD1) in vivo. Role for protein-protein interactions with the copper chaperone for SOD1." The Journal of biological chemistry 275(43): 33771-33776.

Schnell, N. and K. D. Entian (1991). "Identification and characterization of a Saccharomyces cerevisiae gene (PAR1) conferring resistance to iron chelators." European journal of biochemistry / FEBS 200(2): 487-493.

Seo, M. S., S. W. Kang, et al. (2000). "Identification of a new type of mammalian peroxiredoxin that forms an intramolecular disulfide as a reaction intermediate." The Journal of biological chemistry 275(27): 20346-20354.

Sevier, C. S., J. W. Cuozzo, et al. (2001). "A flavoprotein oxidase defines a new endoplasmic reticulum pathway for biosynthetic disulphide bond formation." Nature cell biology 3(10): 874-882.

Shacter, E. (2000). "Quantification and significance of protein oxidation in biological samples." Drug metabolism reviews 32(3-4): 307-326.

Shieh, J. C., M. G. Wilkinson, et al. (1997). "The Mcs4 response regulator coordinately controls the stress-activated Wak1-Wis1-Sty1 MAP kinase pathway and fission yeast cell cycle." Genes & development 11(8): 1008-1022.

Shin, Y. H., E. H. Park, et al. (2002). "Characterization, expression and regulation of a third gene encoding glutathione S-transferase from the fission yeast." Biochimica et biophysica acta 1577(1): 164-170.

Shiozaki, K. (1998). "[Negative regulation of the stress-activated protein kinase pathway by protein phosphatases]." Tanpakushitsu

kakusan koso. Protein, nucleic acid, enzyme 43(8 Suppl): 1055-1061.

Shiozaki, K. and P. Russell (1995). "Counteractive roles of protein phosphatase 2C (PP2C) and a MAP kinase kinase homolog in the osmoregulation of fission yeast." The EMBO journal 14(3): 492-502.

Shiozaki, K. and P. Russell (1996). "Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast." Genes & development 10(18): 2276-2288.

Shiozaki, K. and P. Russell (1997). "Stress-activated protein kinase pathway in cell cycle control of fission yeast." Methods in enzymology 283: 506-520.

Sideris, D. P. and K. Tokatlidis (2010). "Oxidative protein folding in the mitochondrial intermembrane space." Antioxidants & redox signaling 13(8): 1189-1204.

Sideris, D. P. and K. Tokatlidis (2010). "Trapping oxidative folding intermediates during translocation to the intermembrane space of mitochondria: in vivo and in vitro studies." Methods in molecular biology 619: 411-423.

Slekar, K. H., D. J. Kosman, et al. (1996). "The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection." The Journal of biological chemistry 271(46): 28831-28836.

Smart, D. K., K. L. Ortiz, et al. (2004). "Thioredoxin reductase as a potential molecular target for anticancer agents that induce oxidative stress." Cancer research 64(18): 6716-6724.

Song, J. Y., K. D. Kim, et al. (2008). "Thiol-independent action of mitochondrial thioredoxin to support the urea cycle of arginine biosynthesis in Schizosaccharomyces pombe." Eukaryot Cell 7(12): 2160-2167.

Song, J. Y. and J. H. Roe (2008). "The role and regulation of Trxl, a cytosolic thioredoxin in Schizosaccharomyces pombe." J Microbiol 46(4): 408-414.

Song, J. Y. and J. H. Roe (2008). "The role and regulation of Trxl, a cytosolic thioredoxin in Schizosaccharomyces pombe." Journal of microbiology 46(4): 408-414.

Soto, T., F. F. Beltran, et al. (2002). "Cold induces stress-activated protein kinase-mediated response in the fission yeast Schizosaccharomyces pombe." European journal of biochemistry / FEBS 269(20): 5056-5065.

Stephen, D. W. and D. J. Jamieson (1996). "Glutathione is an important antioxidant molecule in the yeast Saccharomyces cerevisiae." FEMS microbiology letters 141(2-3): 207-212.

Stewart, E. J., F. Aslund, et al. (1998). "Disulfide bond formation in the Escherichia coli cytoplasm: an in vivo role reversal for the thioredoxins." The EMBO journal 17(19): 5543-5550.

Stroher, E. and A. H. Millar (2012). "The biological roles of glutaredoxins." Biochem J 446(3): 333-348.

Sturtz, L. A., K. Diekert, et al. (2001). "A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage." The Journal of biological chemistry 276(41): 38084-38089.

Sun, Q. A., L. Kirnarsky, et al. (2001). "Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems." Proceedings of the National Academy of Sciences of the United States of America 98(7): 3673-3678.

Tachibana, T., S. Okazaki, et al. (2009). "A major peroxiredoxin-induced activation of Yap1 transcription factor is mediated by reduction-sensitive disulfide bonds and reveals a low level of transcriptional activation." The Journal of biological chemistry 284(7): 4464-4472.

Takeda, T., T. Toda, et al. (1995). "Schizosaccharomyces pombe atf1+ encodes a transcription factor required for sexual development and entry into stationary phase." The EMBO journal 14(24): 6193-6208.

Tamarit, J., G. Belli, et al. (2003). "Biochemical characterization of yeast mitochondrial Grx5 monothiol glutaredoxin." The Journal of biological chemistry 278(28): 25745-25751.

Tan, S. X., D. Greetham, et al. (2010). "The thioredoxin-thioredoxin reductase system can function in vivo as an alternative system to reduce oxidized glutathione in Saccharomyces cerevisiae." The Journal of biological chemistry 285(9): 6118-6126.

Tao, K., N. Fujita, et al. (1993). "Involvement of the RNA polymerase alpha subunit C-terminal region in co-operative interaction and transcriptional activation with OxyR protein." Molecular microbiology 7(6): 859-864.

Toda, T., M. Shimanuki, et al. (1991). "Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases." Genes & development 5(1): 60-73.

Toda, T., M. Shimanuki, et al. (1991). "Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases." Genes Dev. 5(1): 60-73.

Toledano, M. B., A. Delaunay, et al. (2004). "Microbial H2O2 sensors as archetypical redox signaling modules." Trends in biochemical sciences 29(7): 351-357.

Toledano, M. B., I. Kullik, et al. (1994). "Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection." Cell 78(5): 897-909.

Toledano, M. B., C. Kumar, et al. (2007). "The system biology of thiol redox system in Escherichia coli and yeast: differential functions in oxidative stress, iron metabolism and DNA synthesis." FEBS letters 581(19): 3598-3607.

Toone, W. M. and N. Jones (1999). "AP-1 transcription factors in yeast." Current opinion in genetics & development 9(1): 55-61.

Toone, W. M., S. Kuge, et al. (1998). "Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1." Genes & development 12(10): 1453-1463.

Toone, W. M., S. Kuge, et al. (1998). "Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1 [published erratum appears in Genes Dev 1998 Aug 15;12(16):2650] [see comments]." Genes Dev. 12(10): 1453-1463.

Trotter, E. W. and C. M. Grant (2003). "Non-reciprocal regulation of the redox state of the glutathione-glutaredoxin and thioredoxin systems." EMBO reports 4(2): 184-188.

Tsaneva, I. R. and B. Weiss (1990). "soxR, a locus governing a superoxide response regulon in Escherichia coli K-12." Journal of bacteriology 172(8): 4197-4205.

Tu, B. P. and J. S. Weissman (2004). "Oxidative protein folding in eukaryotes: mechanisms and consequences." The Journal of cell biology 164(3): 341-346.

Uchida, K. and E. R. Stadtman (1993). "Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase. A possible involvement of intra- and intermolecular cross-linking reaction." The Journal of biological chemistry 268(9): 6388-6393.

Ueno, M., H. Masutani, et al. (1999). "Thioredoxin-dependent redox regulation of p53-mediated p21 activation." The Journal of biological chemistry 274(50): 35809-35815.

Umeda, M., S. Izaddoost, et al. (2005). "The fission yeast Schizosaccharomyces pombe has two importin-alpha proteins, Imp1p and Cut15p, which have common and unique functions in nucleocytoplasmic transport and cell cycle progression." Genetics 171(1): 7-21.

Urao, T., K. Yamaguchi-Shinozaki, et al. (2001). "Plant histidine kinases: an emerging picture of two-component signal transduction in hormone and environmental responses." Science's STKE : signal transduction knowledge environment 2001(109): re18.

Vaca, C. E., J. Wilhelm, et al. (1988). "Studies on lipid peroxidation in rat liver nuclei and isolated nuclear membranes." Biochimica et biophysica acta 958(3): 375-387.

van Loon, A. P., B. Pesold-Hurt, et al. (1986). "A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen." Proceedings of the National Academy of Sciences of the United States of America 83(11): 3820-3824.

Veal, E. A., V. J. Findlay, et al. (2004). "A 2-Cys peroxiredoxin regulates peroxide-induced oxidation and activation of a stress-activated MAP kinase." Molecular cell 15(1): 129-139.

Veal, E. A., S. J. Ross, et al. (2003). "Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor." The Journal of biological chemistry 278(33): 30896-30904.

Veal, E. A., W. M. Toone, et al. (2002). "Distinct roles for glutathione S-transferases in the oxidative stress response in Schizosaccharomyces pombe." The Journal of biological chemistry 277(38): 35523-35531.

Vivancos, A. P., E. A. Castillo, et al. (2005). "A cysteine-sulfinic acid in peroxiredoxin regulates H2O2-sensing by the antioxidant Pap1 pathway." Proceedings of the National Academy of Sciences of the United States of America 102(25): 8875-8880.

Vivancos, A. P., E. A. Castillo, et al. (2005). "A cysteine-sulfinic acid in peroxiredoxin regulates H2O2-sensing by the antioxidant Pap1 pathway." Proc Natl Acad Sci U S A 102(25): 8875-8880.

Vivancos, A. P., E. A. Castillo, et al. (2004). "Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration." Mol Microbiol 52(5): 1427-1435.

Vivancos, A. P., E. A. Castillo, et al. (2004). "Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration." Molecular microbiology 52(5): 1427-1435.

Vogt, W. (1995). "Oxidation of methionyl residues in proteins: tools, targets, and reversal." Free radical biology & medicine 18(1): 93-105.

Wahls, W. P. and G. R. Smith (1994). "A heteromeric protein that binds to a meiotic homologous recombination hot spot: correlation of binding and hot spot activity." Genes & development 8(14): 1693-1702.

Wallace, M. A., L. L. Liou, et al. (2004). "Superoxide inhibits 4Fe-4S cluster enzymes involved in amino acid biosynthesis. Cross-compartment protection by CuZn-superoxide dismutase." The Journal of biological chemistry 279(31): 32055-32062.

Wemmie, J. A., M. S. Szczypka, et al. (1994). "Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1." The Journal of biological chemistry 269(51): 32592-32597.

Wemmie, J. A., A. L. Wu, et al. (1994). "Transcriptional activation mediated by the yeast AP-1 protein is required for normal cadmium tolerance." The Journal of biological chemistry 269(20): 14690-14697.

Wheeler, G. L., K. A. Quinn, et al. (2002). "Glutathione regulates the expression of gamma-glutamylcysteine synthetase via the Met4 transcription factor." Molecular microbiology 46(2): 545-556.

Wheeler, G. L., E. W. Trotter, et al. (2003). "Coupling of the transcriptional regulation of glutathione biosynthesis to the availability of glutathione and methionine via the Met4 and Yap1 transcription factors." The Journal of biological chemistry 278(50): 49920-49928.

Wilkinson, M. G., M. Samuels, et al. (1996). "The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast." Genes & development 10(18): 2289-2301.

Wingert, R. A., J. L. Galloway, et al. (2005). "Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis." Nature 436(7053): 1035-1039.

Winkler, P., W. Lindner, et al. (1984). "Detection of 4-hydroxynonenal as a product of lipid peroxidation in native Ehrlich ascites tumor cells." Biochimica et biophysica acta 796(3): 232-237.

Winterbourn, C. C. (2008). "Reconciling the chemistry and biology of reactive oxygen species." Nature chemical biology 4(5): 278-286.

Winterbourn, C. C. (2008). "Reconciling the chemistry and biology of reactive oxygen species." Nat Chem Biol 4(5): 278-286.

Winterbourn, C. C. and M. B. Hampton (2008). "Thiol chemistry and specificity in redox signaling." Free radical biology & medicine 45(5): 549-561.

Winterbourn, C. C. and M. B. Hampton (2008). "Thiol chemistry and specificity in redox signaling." Free Radic Biol Med 45(5): 549-561.

Woo, H. A., H. Z. Chae, et al. (2003). "Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation." Science 300(5619): 653-656.

Woo, H. A., W. Jeong, et al. (2005). "Reduction of cysteine sulfinic acid by sulfiredoxin is specific to 2-cys peroxiredoxins." The Journal of biological chemistry 280(5): 3125-3128.

Woo, H. A., S. W. Kang, et al. (2003). "Reversible oxidation of the active site cysteine of peroxiredoxins to cysteine sulfinic acid. Immunoblot detection with antibodies specific for the hyperoxidized cysteine-containing sequence." The Journal of biological chemistry 278(48): 47361-47364.

Wood, V., R. Gwilliam, et al. (2002). "The genome sequence of Schizosaccharomyces pombe." Nature 415(6874): 871-880.

Wood, Z. A., L. B. Poole, et al. (2002). "Dimers to doughnuts: redox-sensitive oligomerization of 2-cysteine peroxiredoxins." Biochemistry 41(17): 5493-5504.

Wood, Z. A., L. B. Poole, et al. (2003). "Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling." Science 300(5619): 650-653.

Wood, Z. A., E. Schroder, et al. (2003). "Structure, mechanism and regulation of peroxiredoxins." Trends in biochemical sciences 28(1): 32-40.

Wu, A., J. A. Wemmie, et al. (1993). "Yeast bZip proteins mediate pleiotropic drug and metal resistance." The Journal of biological chemistry 268(25): 18850-18858.

Wu, A. L. and W. S. Moye-Rowley (1994). "GSH1, which encodes gamma-glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation." Molecular and cellular biology 14(9): 5832-5839.

Wu, J., W. R. Dunham, et al. (1995). "Overproduction and physical characterization of SoxR, a [2Fe-2S] protein that governs an oxidative response regulon in Escherichia coli." The Journal of biological chemistry 270(17): 10323-10327.

Yamawaki, H., J. Haendeler, et al. (2003). "Thioredoxin: a key regulator of cardiovascular homeostasis." Circulation research 93(11): 1029-1033.

Yan, C., L. H. Lee, et al. (1998). "Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor." The EMBO journal 17(24): 7416-7429.

Yang, K. S., S. W. Kang, et al. (2002). "Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid." The Journal of biological chemistry 277(41): 38029-38036.

Yang, K. S., S. W. Kang, et al. (2002). "Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid." J Biol Chem 277(41): 38029-38036.

Yang, M., P. A. Cobine, et al. (2006). "The effects of mitochondrial iron homeostasis on cofactor specificity of superoxide dismutase 2." The EMBO journal 25(8): 1775-1783.

Yoon, S. J., J. E. Park, et al. (2002). "OxyR regulon controls lipid peroxidation-mediated oxidative stress in Escherichia coli." Journal of biochemistry and molecular biology 35(3): 297-301.

Yuan, H., X. Meng, et al. (2011). "The characterization of two peroxiredoxin genes in Dunaliella viridis provides insights into antioxidative response to salt stress." Plant cell reports 30(8): 1503-1512.

Zhao, T., S. S. Singhal, et al. (1999). "The role of human glutathione S-transferases hGSTA1-1 and hGSTA2-2 in protection against oxidative stress." Archives of biochemistry and biophysics 367(2): 216-224.

Zheng, M., F. Aslund, et al. (1998). "Activation of the OxyR transcription factor by reversible disulfide bond formation." Science 279(5357): 1718-1721.

Zheng, M., F. Aslund, et al. (1998). "Activation of the OxyR transcription factor by reversible disulfide bond formation [see comments]." Science 279(5357): 1718-1721.

Zheng, M., B. Doan, et al. (1999). "OxyR and SoxRS regulation of fur." Journal of bacteriology 181(15): 4639-4643.

Zheng, M. and G. Storz (2000). "Redox sensing by prokaryotic transcription factors." Biochemical pharmacology 59(1): 1-6.

Zhong, L., E. S. Arner, et al. (1998). "Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue." The Journal of biological chemistry 273(15): 8581-8591.

Zhong, L. and A. Holmgren (2000). "Essential role of selenium in the catalytic activities of mammalian thioredoxin reductase revealed by characterization of recombinant enzymes with selenocysteine mutations." The Journal of biological chemistry 275(24): 18121-18128.

Zuin, A., N. Gabrielli, et al. (2008). "Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast." PloS one 3(7): e2842.