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Nrf2 regulation by Hsp90, oxidation, and in breast cancer

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Abstract

To cope with the dynamic range of stressful stimuli that a cell experiences within its lifetime, a host of adaptive cell survival and cell stress response pathways have evolved. The antioxidant and heat shock responses are two key cell stress response pathways primarily involved in the detoxification and elimination of oxidative stress and the maintenance of protein integrity, respectively. Traditionally, these responses are regarded and studied as two independent pathways. In this exploratory work, we hypothesize that oxidative damage to Nrf2 and Keap1 and their interactions with Hsp90 alter their function within the cellular antioxidant stress response. By establishing and characterizing a novel yeast model for human Nrf2, the transcriptional master regulator of the antioxidant response, a previously unexplored interaction was found between Nrf2 and the major heat shock response protein, Hsp90. Further investigation into this interaction using mammalian and breast cancer cells reveals the co-involvement of these proteins in key aspects of protein oxidation, protein misfolding, and cellular responses to cancer therapy. Additionally, Nrf2 and its regulating protein Keap1 were observed to misfold and form protein inclusions upon exposure to oxidative stress, which might implicate a previously unknown mechanism of Nrf2 regulation by inclusion formation. These findings suggest that investigating the antioxidant and heat shock responses in parallel may provide an additional layer of knowledge that is relevant to both basic science and clinical research.

Keywords

Nrf2, Keap1, antioxidant response, oxidative stress, oxidation, yeast model, protein misfolding, Hsp90, heat shock response, molecular chaperones, HER2, cancer, breast cancer, cell stress.

Summary for Lay Audience

All living things experience stress from the environment that can be harmful to cells within the body. As a means of protection, cells have evolved numerous cell stress response pathways to eliminate these insults. This includes the antioxidant response, which protects against harmful free radicals, and the heat shock response, which protects cells from protein-damaging stress. Traditionally, these responses are regarded and studied as two independent and separate pathways. In this exploratory work, we hypothesize that oxidative damage to Nrf2 and Keap1 and their interactions with Hsp90 alter their function within the cellular antioxidant stress response. Using laboratory yeast, a new binding interaction was discovered between the key cell stress protein, Nrf2, which regulates the antioxidant response, and Hsp90, which is a key player in the heat shock response. Further investigation into these interactions using human cells (including cancer cells) shows their co-involvement in important aspects of protein folding and cellular responses to cancer therapy. Additionally, Nrf2 and its regulating protein Keap1 were observed to misfold and form clusters known as inclusions inside the cell under certain stress conditions which could function as a previously unknown “on/off switch” for their cellular activity. Since these two cell stress response pathways overlap, these findings suggest that studying the antioxidant and heat shock responses in parallel may provide important information that is relevant to both basic science and clinical research.

Co-Authorships

Vy Ngo is the main author and contributor to the experimental work presented in this dissertation. The following authors contributed to the chapters highlighted below:

Chapter 2 is adapted from the following manuscript that has been submitted for publication: Ngo, V., Brickenden, A., Liu, H., Yeung, C., Choy, W. Y., Duennwald, M. L. (2020). A novel yeast model detects Nrf2 and Keap1 interactions with Hsp90. VN and MLD conceived the ideas, associated experiments, and interpreted the results. VN conducted the experiments. AB, HL, and CY cloned certain plasmid constructs and assisted with some growth assays. VN drafted the manuscript with support and feedback from MLD. WYC provided critical feedback for the manuscript.

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Dedication

To my parents,

Luận án này con dành tặng cho ba mạ.

Cảm ơn ba mạ đã lo cho con học đến nơi đến chốn.

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List of Abbreviations

5FOA	5-fluorouracil
AD	Alzheimer's disease
AD	activating domain
ADP	adenosine diphosphate
Aha1	activator of hsp90 ATPase protein 1
ALS	amyotrophic lateral sclerosis
AP-1	activator protein 1
ARE	antioxidant response element
ATF6	activating transcription factor 6
Atg8	autophagy-related protein 8
ATP	adenosine triphosphate
Bach1	BTB domain and CNC homolog 1
Bach2	BTB domain and CNC homolog 2
BCA assay	bicinchoninic acid assay
BRCA1	breast cancer 1
BRCA2	breast cancer 2
BSA	bovine serum albumin
BSO	buthionine sulfoximine
BTB	broad complex, tramtrack, and bric à brac
bZIP	basic leucine zipper domain
CBP	CREB-binding protein
Cdc37	cell division cycle protein 37
Cdk2	cyclin-dependent kinase 2
CHD6	DNA-binding protein 6
CLIPS	chaperones linked to protein synthesis
CMA	chaperone-mediated autophagy
CNC	cap 'n' collar
CRL	Cullin-RING E3 ubiquitin ligases
CRU	Cub-R-*URA3*

CTR	C-terminal region
Cub	C-ubiquitin
Cul3	Cullin 3
DBD	DNA-binding domain
DJ-1	protein deglycase DJ-1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DPP3	dipeptidyl-peptidase 3
DsRed	Discosoma red fluorescent protein
eIF2α	eukaryotic initiation factor 2 alpha
eNOS	endothelial isoform of nitric oxide synthase
EpRE	electrophile response element
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated protein degradation
FANCN	Fanconi anemia complementation group N
FBS	fetal bovine serum
GEO	Gene Expression Omnibus
GFP	green fluorescent protein
GKS-3	glycogen synthase kinase-3
GCL	glutamate-cysteine ligase
GCLC	glutamate-cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase modifier subunit
GOF	gain of function
GPx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HD	Huntington’s disease

HER2	human epidermal growth factor receptor 2
HIF1α	hypoxia-inducible factor 1α
HO-1/HMOX1	heme oxygenase 1
Hop	Hsp70/Hsp90 organizing protein
Hsf1	heat shock factor 1
Hsp	heat shock protein
Hsp70	heat shock protein 70
Hsp90	heat shock protein 90
ICGC	International Cancer Genome Consortium
IDP	intrinsically disordered protein
IDR	intrinsically disordered region
IHC	immunohistochemistry
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IPOD	insoluble protein deposit
ITC	isothermal titration calorimetry
IVR	intervening region
JUNQ	juxtanuclear quality control
Keap1	Kelch-like ECH-associated protein 1
KIR	Keap1-interacting region
KPNA6	karyopherin α6
LOF	loss of function
MG132	carbobenzoxy-l-leucyl-l-leucyl-l-leucinal
MTOC	microtubule-organizing centre
Miro2	mitochondrial Rho GTPase 2
Msr	methionine sulfoxide reductase
MS	multiple sclerosis
NAC	N-acetylcysteine
Neh	Nrf2-ECH homology
NF-κB	nuclear factor kappa B
NLS	nuclear localization signal

NMR	nuclear magnetic resonance
NQO1	NAD(P)H:quinone dehydrogenase 1
Nrf2/NFE2L2	nuclear factor (erythroid-derived 2)-like 2
NTR	N-terminal region
Nub	N-ubiquitin
OD_{600}	optical density at 600 nm
OE	overexpression
PALB2	partner and localizer of BRCA2
PBST	phosphate-buffered saline with tween
PCR	polymerase chain reaction
PD	Parkinson's disease
PEG	polyethylene glycol
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PGAM5	phosphoglycerate mutase family member 5
Pgk1	phosphoglycerate kinase 1
PI	propidium iodide
PKC	protein kinase C
PPI	protein-protein interaction
ProTα/PTMA	prothymosin alpha
Rb	retinoblastoma
Rbx1	RING box protein 1
RNAseq	RNA sequencing
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640
RT-qPCR	quantitative reverse transcription-polymerase chain reaction
RXRα	retinoid X receptor α
SD	selective dextrose
SDD-AGE	semi-denaturing detergent agarose gel electrophoresis
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

sHsp	small heat shock protein
sMaf	small musculoaponeurotic fibrosarcoma
SOD	superoxide dismutase
SQSTM1	sequestosome-1
Srx	sulfiredoxin
Sti1	stress-inducible protein 1
tBHQ	tertiary butylhydroquinone
TCGA	The Cancer Genome Atlas
TE	tris-ethylenediaminetetraacetic acid
TF	transcription factor
TPR	tetratricopeptide repeat
Trx	thioredoxin
TrxR	thioredoxin reductase
UAS	upstream activating sequence
UGT	uridine diphosphate (UDP)-glucuronosyltransferase
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
v/v	volume/volume
WT	wild-type
Y2H	yeast two-hybrid
YFP	yellow fluorescent protein
YPD	yeast extract peptone dextrose
αMEM	alpha Minimum Essential Medium
βME	beta-mercaptoethanol
βTrCP	beta-transducin repeat-containing protein
γ-GCS	gamma-glutamylcysteine synthetase

Chapter 1

1 Introduction

1.1 Oxidative Stress and Antioxidant Enzymes

Organisms are continually exposed to exogenous and endogenous sources of reactive oxygen species (ROS) and other oxidants that have both beneficial and deleterious effects on the cell. ROS have important roles in a wide range of biological processes; however, high levels are associated with oxidative stress and disease progression. Antioxidant defence systems have thus been evolved as a means of protection against oxidative stress.

1.1.1 Reactive Oxygen Species

Free radicals are unstable atoms, ions, or molecules containing one or more unpaired electrons in the outermost electron shell. An unpaired valence electron is unstable and highly reactive. To attain stability, free radicals attack and acquire electrons from other compounds or molecules within their proximity. The attacked entity loses an electron to become oxidized and becomes a free radical itself, thereby initiating a chain reaction cascade that can result in cellular damage (Halliwell & Gutteridge, 2015). ROS and reactive nitrogen species (RNS) are unstable molecules containing oxygen and/or nitrogen and include both free radical and non-radical species. The oxygen molecule (O_2••) is a weak free radical itself due to the presence of two unpaired electrons in its valence shell; however, it is less reactive than other oxygen species due to the parallel spin of its electrons (Apel & Hirt, 2004). Major ROS and RNS are outlined in **Table 1.1**.

Table 1.1: Major Reactive Oxygen and Reactive Nitrogen Species.

Molecule Type	Radical Status	Name	Symbol
ROS	Radical	Molecular oxygen	O_2••
		Superoxide	O_2•$^-$
		Hydroxyl	•OH
		Alkoxyl	RO•
		Peroxyl	ROO•
		Hydroperoxyl	HO_2•
	Non-radical	Hydrogen peroxide	H_2O_2
		Peroxide	ROOR
		Singlet oxygen	O_2
		Ozone	O_3
		Hydroxyl ion	OH^-
		Peroxynitrite	$ONOO^-$
RNS	Radical	Nitric oxide	•NO
		Nitrogen dioxide	•NO_2
	Non-radical	Peroxynitrite	$ONOO^-$
		Alkyl peroxynitrites	ROONO
		Nitronium cation	NO^{2+}
		Nitroxyl cation	NO^+
		Nitroxyl anion	NO^-
		Nitrogen oxides	N_xO_x

RNS is a family of nitrogen moieties associated with oxygen. They are produced when nitric oxide (•NO) reacts with oxygen species. For example, nitric oxide can react with superoxide (O_2•$^-$) to form peroxynitrite ($ONOO^-$):

$$\bullet NO + O_2\bullet^- \longrightarrow ONOO^-$$

Peroxynitrite is very reactive and readily attacks lipid molecules, resulting in lipid peroxidation and lipoprotein oxidation (Radi, 2018). However, like ROS, low levels of RNS have important roles in cellular processes. For example, nitric oxide produced by nitric oxide synthase (NOS) regulates blood vessel dilation and is involved in synaptic transmission in the brain (O'Dell et al., 1991; Schuman & Madison, 1991). On the other hand, high levels of RNS results in nitrosative stress, macromolecule damage, and activation of transcription factors NF-$_K$B and activator protein 1 (AP-1) involved in inflammation and other pathological pathways (Kröncke, 2003; Martínez & Andriantsitohaina, 2009). RNS and ROS often act together to cause cellular damage (Valko et al., 2006).

ROS are oxidants (i.e., a molecule that removes electrons from other molecules) predominantly produced as byproducts of normal cellular metabolism and biochemical processes within the cell. Mitochondria are a primary source of ROS produced by aerobic respiration (Muller, 2000; Turrens, 2003; Andreyev et al., 2005; Adam-Vizi & Chinopoulos, 2006), where the reduction of molecular oxygen in the electron transport chain results in the leaking of superoxide radicals which are readily detoxified to hydrogen peroxide (H_2O_2) by antioxidant enzymes such as catalase and glutathione peroxidase. Hydrogen peroxide may react with transition metals such as iron (Fe^{2+}) to produce hydroxyl radicals via the Fenton reaction to further produce hydroxyl radicals (•OH) which are highly reactive towards all components of DNA molecules as well as lipids (Imlay et al., 1988). Peroxisomes also generate ROS from aerobic metabolism (Fransen et al., 2012), and phagocytic neutrophils and macrophages produce ROS to eliminate invading pathogens (Roos et al., 2003). At low to moderate levels, ROS plays an important role in normal cell physiology, serving as secondary messengers in intracellular signalling cascades that mediate cell growth, autophagy, inflammatory and immune function, and contribute to overall redox regulation (Bae et al., 2011; Finkel, 2011). However, both radical and non-radical ROS can be powerful oxidants that are detrimental to the cell upon high or chronic exposure. Toxic exogenous sources of ROS include pollution, tobacco smoke, alcohol, ozone, environmental and industrial toxins, and radiation. Due to their reactive nature, ROS production and elimination must be strictly regulated by the cell. **Figure 1.1** summarizes the major sources of exogenous and endogenous ROS.

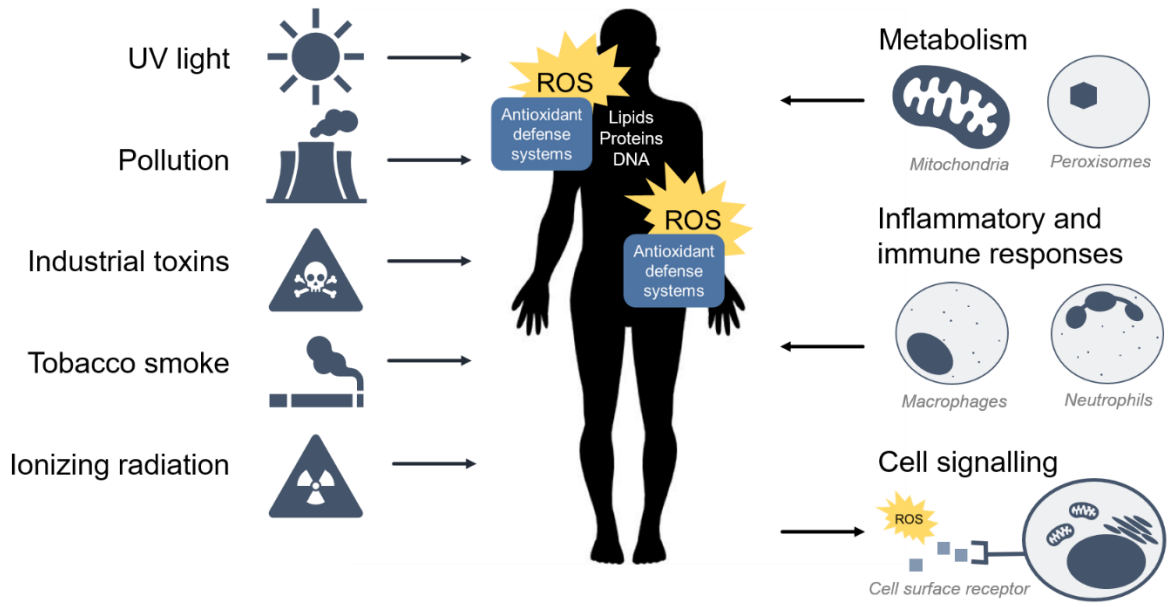

Figure 1.1: Sources of exogenous and endogenous ROS. ROS can come from toxic exogenous sources in the environment, or be produced as by-products of normal cell metabolism, inflammation, and immunity. ROS may also function as secondary messengers within cell signalling pathways.

1.1.2 Oxidative Stress

Extensive or prolonged exposure to ROS results in oxidative stress, a deleterious process that damages lipids, proteins, and nucleic acids in the cell, thereby inhibiting their normal function (Apel & Hirt, 2004). In this scenario, there is an imbalance between the production of ROS and cellular defence mechanisms against oxidative stress, i.e., the antioxidant defence systems. Chronic oxidative stress and the resultant oxidative damage have been implicated in many human diseases including cardiovascular disease, neurodegenerative diseases, diabetes, cancer, and the ageing process (Barnham et al., 2004; Reuter et al., 2010; Alfadda & Sallam, 2012; Asmat et al., 2016; Liguori et al., 2018) (**Figure 1.2**).

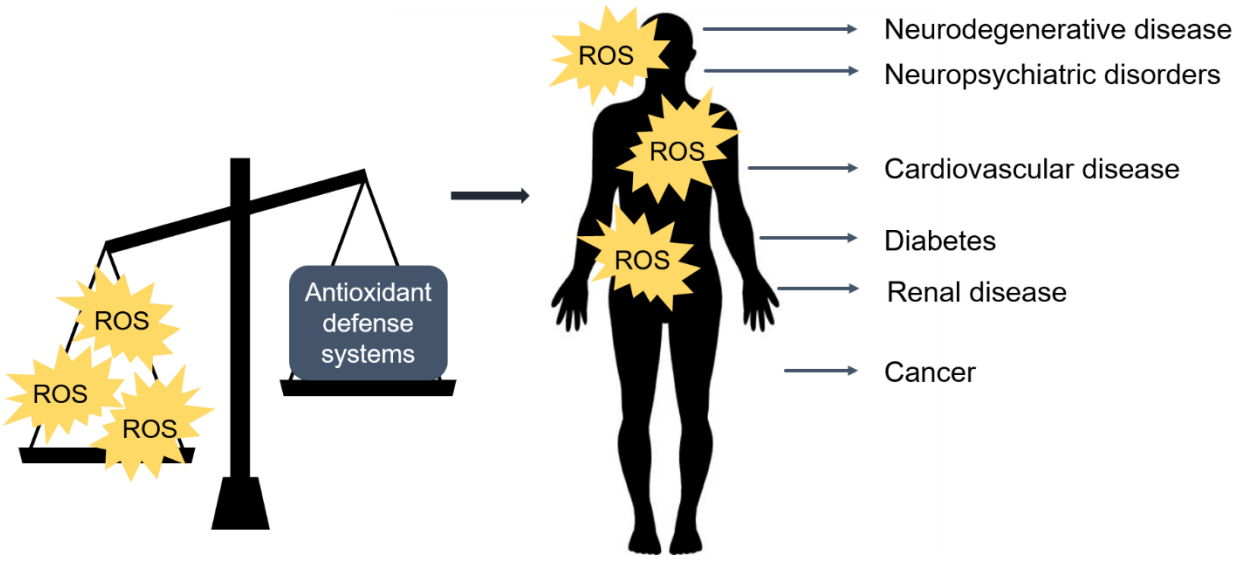

Figure 1.2: Oxidative stress in human disease. A prolonged imbalance between ROS production and cellular antioxidant defence systems leads to oxidative stress. Oxidative stress damages cellular macromolecules and has been implicated in many human diseases.

The consequence of ROS or oxidants and the extent of oxidative stress is dependent on the strength, duration, and context of exposure. In response to oxidative stress, cells typically undergo cell cycle arrest and enter the G_0 phase (i.e., a quiescent, non-dividing stage) due to activation of the p53-regulated cyclin-dependent kinase inhibitor p21, which halts cell cycle progression and inhibits DNA synthesis (Xiong et al., 1993; Gartel & Radhakrishnan, 2005). ROS can also trigger the p53 and p21-mediated dephosphorylation and activation of the tumour-suppressor retinoblastoma protein (Rb) resulting in further inhibition of cell cycle progression (Brugarolas et al., 1999). It is interesting to note that p21 is also involved in the regulation of the antioxidant response through its binding to the antioxidant transcription factor, Nrf2 (Chen et al., 2009b) (to be discussed in Section 1.2.5). Depending on the nature of the exposure, cells can activate cell survival pathways; however, chronic exposure or excessively high levels of ROS may result in the induction of autophagic or apoptotic pathways (Chen et al., 2008; Redza-Dutordoir & Averill-Bates, 2016).

To preserve the delicate balance between the beneficial and harmful effects of ROS, living organisms have evolved cellular defence mechanisms against oxidative stress to maintain redox homeostasis. Alterations in redox status can lead to the transcriptional activation of pathways and enzymes involved in the detoxification, transport, and elimination of ROS.

1.1.3 Antioxidant Response Enzymes

Complex antioxidant defence systems have been evolved to protect cells and tissue against oxidative stress. Halliwell and Gutteridge have defined antioxidants as "any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate" (Halliwell & Gutteridge, 1995). Key antioxidant defences include (1) antioxidants that directly scavenge ROS, such as glutathione, vitamin C, and vitamin E, and (2) antioxidant enzymes including superoxide dismutase, catalase, and glutathione peroxidase.

Superoxide dismutases (SOD) are a class of enzymes found within the cytosol and mitochondria of nearly all aerobic cells and contain metal ion cofactors such as copper, zinc, manganese, or iron. SOD isoenzymes include Cu,Zn SOD (SOD1), Mn SOD (SOD2), and extracellular (EC) SOD (SOD3) (Zelko et al., 2002; Abreu & Cabelli, 2010). SODs are responsible for the dismutation (simultaneous oxidation and reduction) and breakdown of superoxide radicals into molecular oxygen and hydrogen peroxide:

$$2O_2\bullet^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

Molecular oxygen and hydrogen peroxide are weak oxidants that are relatively stable; however, hydrogen peroxide can be converted into extremely reactive hydroxyl radicals and must therefore be targeted for further breakdown. Two enzymes responsible for the decomposition of hydrogen peroxide are catalase and glutathione peroxidase.

Catalase is found in nearly all living organisms and exists primarily within peroxisomes as well as in the mitochondria and nucleus (Chelikani et al., 2004). Catalases catalyze the breakdown of hydrogen peroxide to molecular oxygen and water:

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O$$

Glutathione peroxidases (GPx) are a class of enzymes that also break down hydrogen peroxide but do so specifically through the oxidation of a glutathione (GSH) cofactor:

$$2GSH + H_2O_2 \xrightarrow{\text{GPx}} GSSG + 2H_2O$$

GSH is a tripeptide comprised of three amino acids (cysteine, glutamic acid, and glycine) and is the most abundant and important low molecular weight antioxidant synthesized in cells. GSH plays a critical role in protecting cells from oxidative damage through direct antioxidant activity or coupled to GPx enzymatic activity (Pompella et al., 2003; Forman et al., 2009). Enzymes in the GPx family include GPx1 through 8, each with different expression patterns within the body (Brigelius-Flohé & Maiorino, 2013). GPx1 is the most abundant isoform and is ubiquitously expressed in the cytosol and mitochondria. GPx2 is an intestinal extracellular enzyme, while GPx3 is extracellular, and GPx4 prefers lipid peroxides. Four additional isoforms of GPx (GPx5-8) have been identified in humans but are not well studied. GPx enzymes are part of a family of critical proteins known as the phase II enzymes responsible for the conjugation of xenobiotics with peptides and sugars for detoxification.

Xenobiotic metabolism consists of phase I, phase II, and phase III enzymes involved in oxidation, conjugation/detoxification, and elimination, respectively (Xu et al., 2005; Nakata et al., 2006). Phase II enzymes are particularly important in cellular responses to oxidative stress and include GPx, glutathione S-transferase (GST), and UDP-glucuronosyltransferase (UGT). Other important antioxidant enzymes include sulfiredoxin (Srx), thioredoxin (Trx), thioredoxin reductase (TrxR), heme oxygenase 1 (HO-1), and NAD(P)H:quinone oxidoreductase 1 (NQO1). Activation of these enzymes leads to robust xenobiotic detoxification and/or antioxidant effects. Early mechanistic studies on the induction of the rat glutathione S-transferase subunit genes, *GSTA1* and *GSTA2*, led to the discovery of a specific enhancer sequence within their promoter region termed the antioxidant response element (ARE) (Rushmore et al., 1991). Since then, AREs have been found in many other antioxidant genes including, among others, *NQO1* and *HMOX1* (Rushmore et al., 1991; Nioi et al., 2003).

1.1.4 Antioxidant Response Element (ARE)

The ARE (Rushmore et al., 1991), also referred to as the electrophile response element (EpRE), is a cis-acting enhancer sequence found within the promoter region of many cytoprotective antioxidant and phase II enzyme genes. It has a core sequence of 5'-TGACnnnGC-3' and is involved in inducible gene expression in response to oxidative stress (Rushmore et al., 1991). The ARE is also responsible for low-level basal gene expression to mitigate the ROS produced by cellular respiration. Thus, the ARE is important for redox regulation under both stressed and non-stressed conditions. Using *in vivo* studies in mice, Itoh et al. discovered that the induction of phase II enzymes through the ARE is mediated by a protein transcription factor called Nrf2 (Itoh et al., 1997) (**Figure 1.3**). Nrf2-deficient mice showed marked reductions in the expression of the phase II enzyme GST α_1 subunit and the antioxidant enzyme NQO1 (Itoh et al., 1997), and ensuing studies demonstrated increased sensitivity to carcinogens and impaired detoxification of acetaminophen in Nrf2$^{-/-}$ mice (Chan et al., 2001; Enomoto et al., 2001; Ramos-Gomez et al., 2001). This illustrates the important role of Nrf2 in the activation of ARE-regulated antioxidant and phase II enzyme genes.

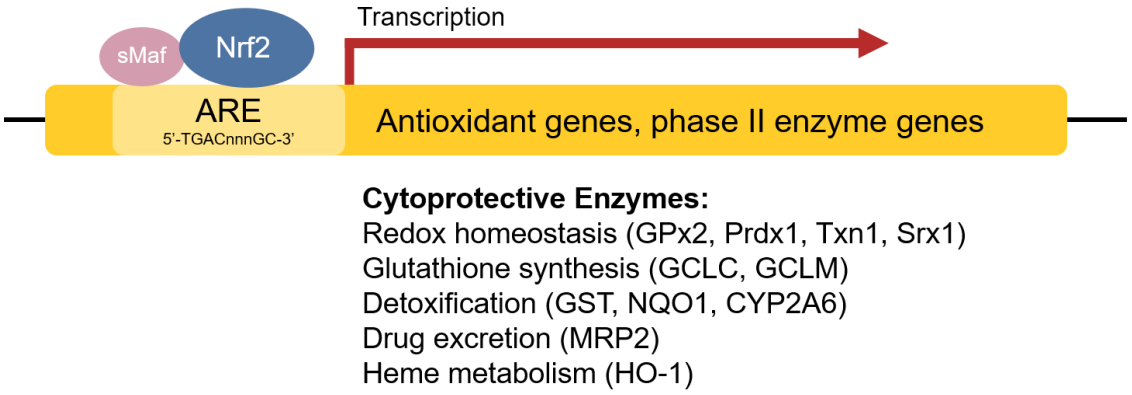

Figure 1.3: Activation of the ARE by Nrf2. Nrf2 heterodimerizes with sMaf proteins and binds to the ARE found within the promoter region of antioxidant and phase II enzyme genes to activate their transcription.

1.2 Keap1-Nrf2 Antioxidant Pathway

Nuclear factor erythroid 2-related factor 2 (Nrf2) (Moi et al., 1994) is the transcriptional master regulator of cellular responses against oxidative stress. Nrf2 regulates the expression of a multitude of antioxidant and phase II enzyme genes and is negatively regulated by Kelch-like ECH-associated protein (Keap1) (Itoh et al., 1999), a substrate adaptor protein that binds to Nrf2 in the cytosol to facilitate its polyubiquitination by the Cullin 3 (Cul3) E3 ubiquitin ligase for proteasomal degradation (McMahon et al., 2003; Nguyen et al., 2003; Kobayashi et al., 2004). Constitutive Nrf2 degradation allows for low basal expression under non-stressed conditions. Upon oxidative stress, specific stress-sensing cysteine residues in Keap1 are modified (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003; Wakabayashi et al., 2004), leading to a conformational change that prevents Keap1 from mediating the ubiquitination of Nrf2 by Cul3 (Kobayashi et al., 2006). This results in Nrf2 stabilization, accumulation, and nuclear translocation where Nrf2 heterodimerizes with sMaf proteins and binds to the ARE for the robust induction of cytoprotective genes for enzymes involved in the detoxication of ROS and other oxidants (Itoh et al., 1997) (**Figure 1.4**).

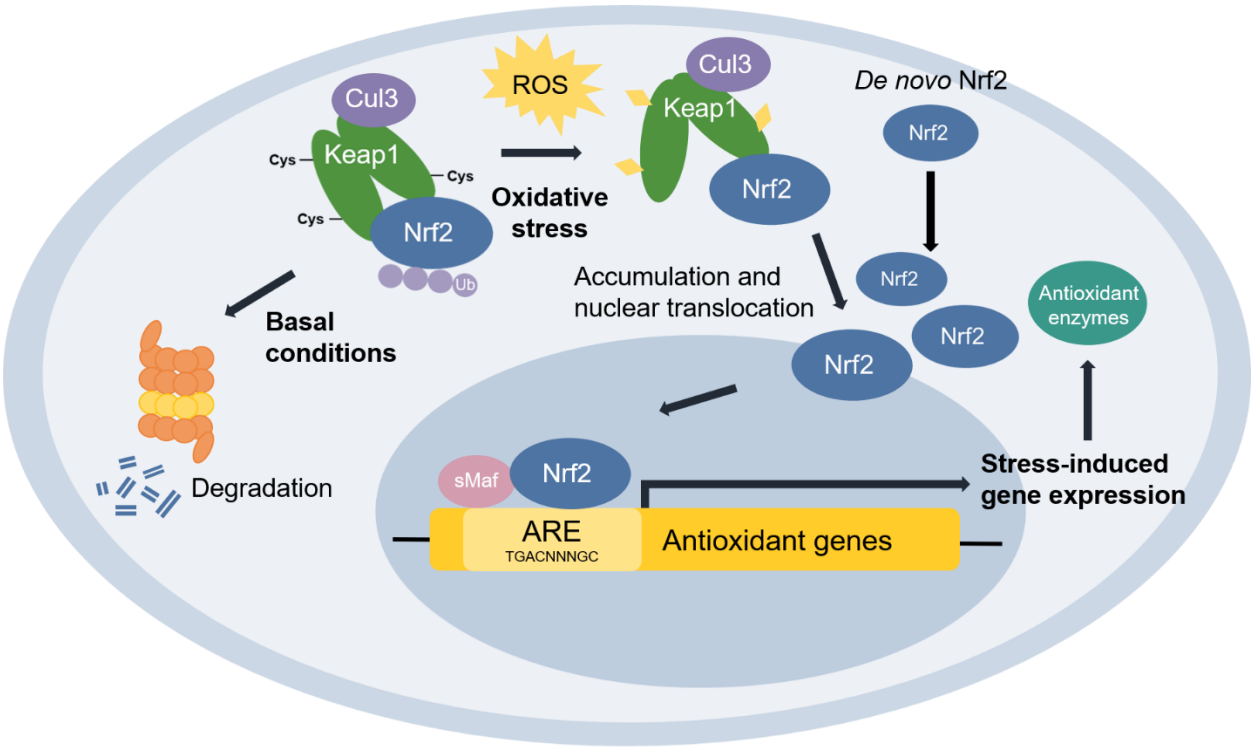

Figure 1.4: The Keap1-Nrf2 pathway. Under basal conditions, Keap1 is bound to Nrf2 and Nrf2 is ubiquitinated by the Cul3 E3 ubiquitin ligase for degradation by the proteasome. Upon oxidative stress, sensor cysteines in Keap1 are modified by ROS, leading to Nrf2 stabilization, accumulation, and translocation to the nucleus where Nrf2 heterodimerizes with sMaf and binds to the ARE to activate the transcription of antioxidant genes.

1.2.1 Nuclear factor erythroid 2-related factor 2 (Nrf2)

Nrf2 (Moi et al., 1994) belongs to the cap 'n' collar (CNC) subfamily of basic leucine zipper (bZIP) transcription factors together with NF-E2 p45-related factors 1 and 3 (Nrf1 and Nrf3), NF-E2 p45, and transcriptional repressors BTB Domain and CNC homolog 1 and 2 (Bach1 and Bach2) (Sykiotis & Bohmann, 2010). Nrf2 contains seven conserved regions that are referred to as the Nrf2-ECH homology (Neh) domains, designated Neh1 through 7 (**Figure 1.5**). The key function of each domain is summarized in **Table 1.2**.

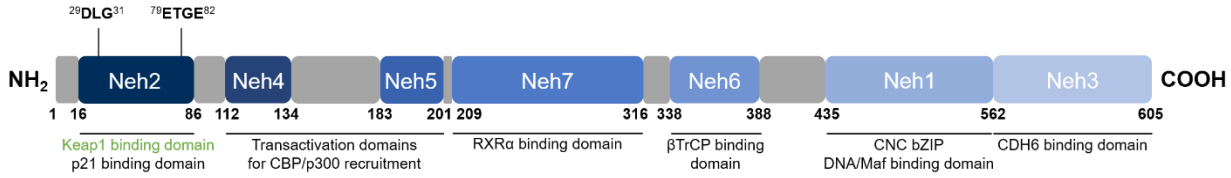

Figure 1.5: Domain structure of human Nrf2. Nrf2 contains seven conserved Neh domains. The Neh2 domain contains two motifs (^{29}DLG^{31} and ^{79}ETGE^{82}) wherein Keap1 binds as a substrate adaptor for the Cul3-mediated ubiquitination and degradation of Nrf2.

Table 1.2: Summary of Nrf2's functional domains and their key binding proteins.

Domain	Key Associated Function	Binds to	Reference(s)
Neh1	DNA-binding via the ARE; dimerization with sMaf proteins	sMaf, ARE	(Moi et al., 1994; Itoh et al., 1997)
Neh2	Keap1-binding for negative regulation	Keap1	(Itoh et al., 1999; Tong et al., 2006a)
Neh3	Transactivation	CHD6	(Nioi et al., 2005)
Neh4, Neh5	Transactivation	CBP	(Katoh et al., 2001; Kim et al., 2013a)
Neh6	βTrCP-binding for negative regulation	βTrCP	(Rada et al., 2011; Chowdhry et al., 2013)
Neh7	RXRα-binding for suppressed transactivation	RXRα	(Wang et al., 2013a)

Neh1 is the DNA-binding domain that contains the CNC-bZIP region important for Nrf2's association with sMafs, binding to the ARE, and transcription factor activity (Moi et al., 1994; Itoh et al., 1997). The N-terminal Neh2 domain is a redox-sensitive degron that negatively regulates Nrf2 activity and contains two highly conserved ^{29}DLG^{31} and ^{79}ETGE^{82} motifs to which Keap1 binds, as well as seven lysine residues that are targets for ubiquitination by the Cul3 E3 ubiquitin ligase (Itoh et al., 1999; Tong et al., 2006b). The C-terminal Neh3 domain is a transactivation domain responsible for the transcriptional activation (transactivation) of Nrf2 and has been shown

to interact with chromodomain helicase DNA-binding protein 6 (CHD6) which plays a role in chromatin remodelling (Nioi et al., 2005). Neh4 and Neh5 are also transactivation domains where the binding of the CREB-binding protein (CBP) (Katoh et al., 2001) or various other cofactors (Kim et al., 2013a) increases the rate of Nrf2 transcriptional activity. The Neh6 domain is a redox-insensitive degron that provides Keap1-independent negative Nrf2 regulation. Similar to Neh2, Neh6 contains two highly conserved ^{334}DSGIS^{338} and ^{373}DSAPGS^{378} motifs to which the β-transducin repeat-containing protein (βTrCP) binds, and within the DSGIS motif, a phosphorylation site for glycogen synthase kinase-3 (GSK3) that enhances βTrCP activity upon GSK3-mediated phosphorylation of Nrf2 (Rada et al., 2011; Chowdhry et al., 2013). Neh7 is the binding domain for retinoid X receptor α (RXRα), which upon RXRα binding impairs the recruitment of cofactors to Neh4 and Neh5 necessary for transactivation, thereby suppressing transcriptional activation (Wang et al., 2013a).

1.2.2 Kelch-like ECH-associated protein (Keap1)

Keap1 (Itoh et al., 1999) belongs to the BTB-Kelch family of proteins which includes about 50 members, all of which assemble with the Cul3 E3 ubiquitin ligase and RING box protein-1 (Rbx1) to form the Cullin-RING E3 ubiquitin ligases (CRLs) involved in the ubiquitination of BTB-Kelch proteins, such as Keap1 (Kobayashi et al., 2004; Zhang et al., 2004). Cul3 assembly requires a "3-box" motif that is characteristic of BTB-Kelch proteins (Canning et al., 2013). Accordingly, Keap1 contains three functional domains (**Figure 1.6**). The N-terminal BTB (broad complex, tramtrack, and bric à brac) domain mediates Keap1 homodimerization and contributes to its interaction with Cul3 (Cleasby et al., 2014). Additional Cul3 interaction is provided by a 3-box motif found within the proximal part of the intervening region (IVR) (Canning et al., 2013). The IVR contains key reactive cysteine residues through which Nrf2 activity is regulated, including Cys226, Cys257, Cys273, and Cys288 (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003; Wakabayashi et al., 2004; McMahon et al., 2010). The C-terminal Kelch domain, also known as the double glycine repeat (DGR) domain, is important for Nrf2 binding (Lo et al., 2006; Tong et al., 2006a).

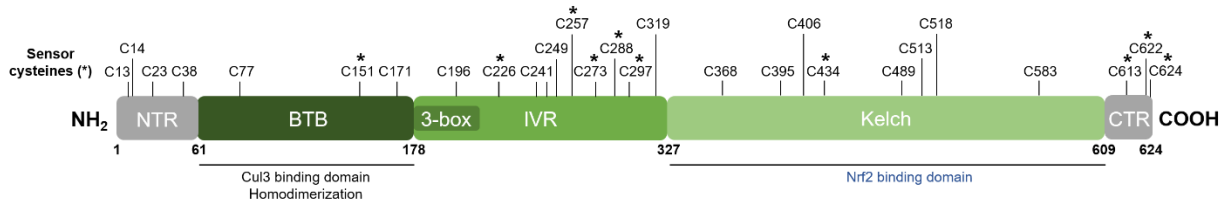

Figure 1.6: Domain structure of human Keap1. Keap1 contains 3 functional domains and a 3-box motif within the proximal part of the IVR domain. The location of all cysteine (C) residues in Keap1 is shown, and key stress-sensing cysteines are marked with an asterisk (*).

Dissociation of Nrf2 from Keap1 occurs through the oxidative modification of specific stress-sensing cysteine residues of Keap1 (**Figure 1.7**) (Wakabayashi et al., 2004). Intriguingly, Keap1 contains a very high content of cysteines, with the 27 cysteine residues in human Keap1 accounting for approximately 4% of its total amino acid content, which is notably greater than the 2% average for the human proteome (Miseta & Csutora, 2000). Cys273 and Cys288 are required for sensing oxidative stress under both basal and stress conditions, whereas Cys151 may be required only during oxidative stress conditions (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003). These three key cysteines may function independently or collaboratively depending on the class of Nrf2-inducing compounds, characterized by Yamamoto et al. (Saito et al., 2016), who also found some inducers to function independently of these three specific cysteines. Correspondingly, Cys226, Cys613, Cys622 and Cys624 are specifically involved in sensing hydrogen peroxide through a mechanism that is distinct from that used for sensing electrophilic Nrf2 inducers (Suzuki et al., 2019). Additional cysteine residues that respond to redox-active agents include the Cys288 alkenal sensor, the zinc sensor comprised of His225, Cys226, and Cys613, and the nitric oxide sensor comprised of a cluster of basic amino acids (His129, Lys131, Arg135, Lys150, and His154) that facilitate the *S*-nitrosylation of Cys151 within Keap1 (McMahon et al., 2010).

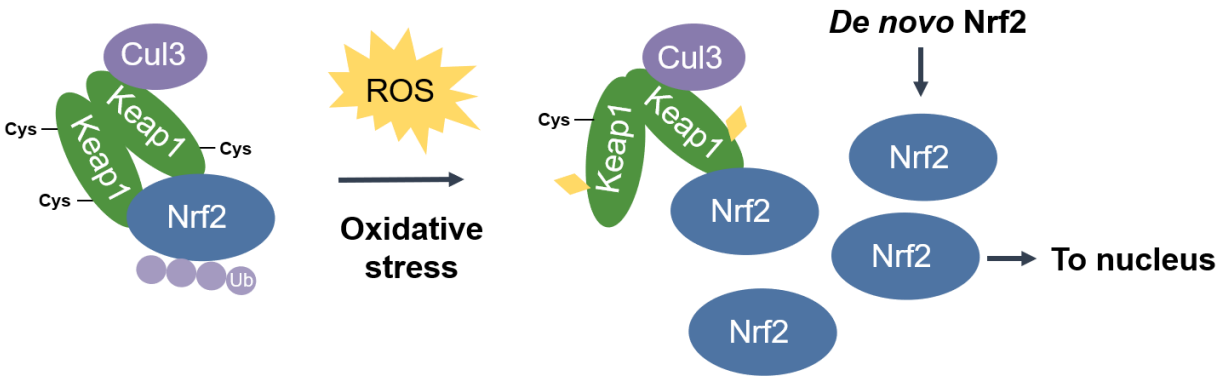

Figure 1.7: Stress-induced cysteine modification of Keap1. Under oxidative stress conditions, specific stress-sensing cysteine residues in Keap1 are modified, leading to a conformational change in Keap1 that results in Nrf2 stabilization, accumulation, and nuclear translocation for the induction of ARE-containing cytoprotective genes.

1.2.3 Keap1-Dependent Nrf2 Regulation

As previously mentioned, Nrf2-regulated genes contain an ARE in their regulatory region and encode numerous antioxidant and phase II enzymes (Itoh et al., 1997). Transcriptional activation of the ARE is primarily dependent on Nrf2 stabilization, accumulation, and nuclear translocation through its dissociation from the cytoskeleton-associated Keap1 (Itoh et al., 1999). Thus, Nrf2 activity is tightly regulated by its interaction with Keap1.

Nrf2 association requires the homodimerization of Keap1 (Zipper & Mulcahy, 2002). Keap1 recruits Nrf2 firstly through the binding of one Keap1 molecule to the high-affinity ETGE motif within the Nrf2's Neh2 domain. Subsequent binding of the other Keap1 molecule at the low-affinity DLG motif locks Nrf2 in place by orienting the lysine residues within Neh2 in the correct position for ubiquitination by Cul3 and degradation by the 26S proteasome (Tong et al., 2006a; Tong et al., 2006b). This is known as the two-site substrate recognition model and has been accepted as the primary mechanism of Nrf2 regulation (**Figure 1.8**).

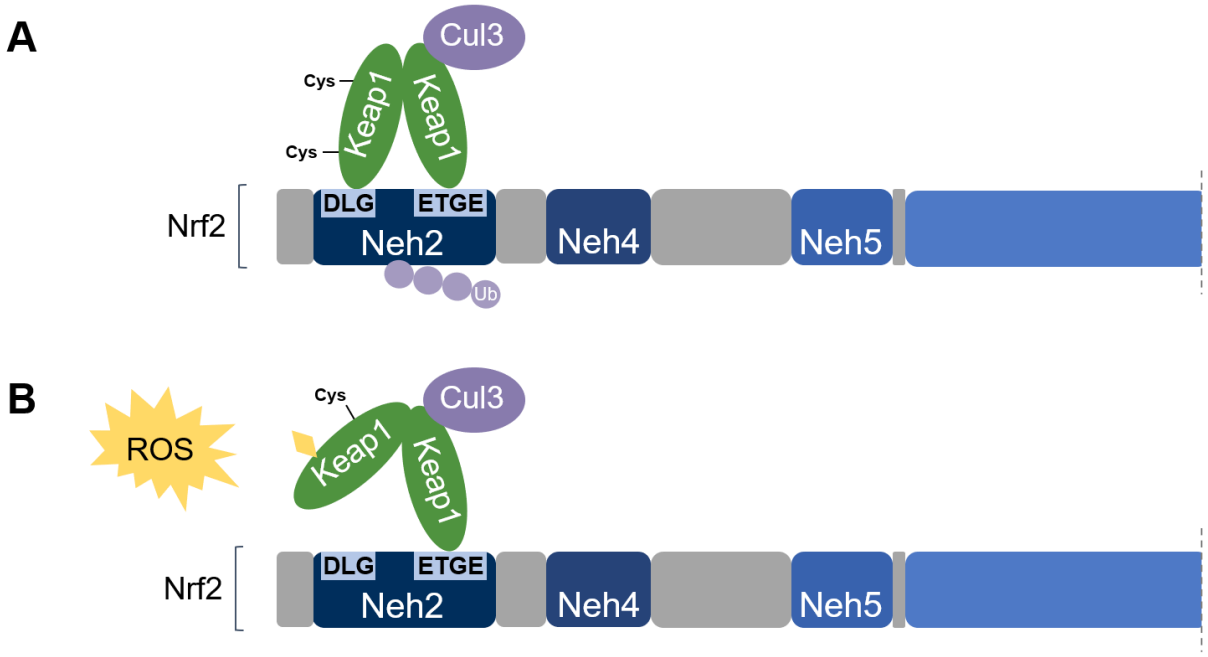

Figure 1.8: Two-site substrate recognition model for Keap1-dependent Nrf2 regulation. **(A)** A Keap1 homodimer binds to the Neh2 domain of Nrf2 at the DLG and ETGE motifs, allowing for the ubiquitination of Nrf2 by Cul3. **(B)** Stress-sensing cysteine residue(s) in Keap1 are modified by oxidative stress (ROS) causing a conformational change in Keap1 that impairs Nrf2-binding. Nrf2 is stabilized and no ubiquitination occurs.

Notably, the ETGE motif has a binding affinity that is two orders of magnitude higher than that of the DLG motif due to the presence of more electrostatic interactions (Tong et al., 2007). The DLG motif utilizes hydrogen bonding whereas the ETGE motif utilizes both hydrophobic interactions and hydrogen bonding (Fukutomi et al., 2014). Accordingly, stress-induced cysteine modifications that alter the structural conformation of Keap1 result in the prompt dissociation of Keap1 from the weak-binding DLG motif, thereby impairing Nrf2 ubiquitination. On the other hand, the Keap1-Nrf2 association may remain intact via the tight-binding ETGE motif even though ubiquitination is impaired without DLG binding (Kobayashi et al., 2006; Tong et al., 2006a). Taken together, the DLG motif is particularly important in Keap1-dependent degradation of Nrf2 by functioning as an "on/off switch" for Nrf2 ubiquitination. Under basal conditions, Nrf2 has a short half-life of only 10-30 minutes (Nguyen et al., 2003; Stewart et al., 2003).

When Keap1-Nrf2 binding is impaired, Nrf2 may be stabilized and for accumulation and nuclear translocation. Within the nucleus, Nrf2 cannot bind to the ARE as a monomer and must heterodimerize with the small Maf protein (sMaf) family (MafF, MafG, MafK) for transcriptional activation (Itoh et al., 1997). The Nrf2-sMaf complex binds, in a sequence-specific manner, to the ARE present within the promoter region of antioxidant and phase II enzyme genes, leading to their robust activation. **Table 1.3** lists key examples of Nrf2 regulated genes and their associated protein function.

Table 1.3: Examples of cytoprotective genes regulated by Nrf2.

Primary Role	Gene	Protein	Function
Redox homeostasis	*GPX2*	Glutathione peroxidase 2 (GPx2)	Reduces hydrogen peroxide and lipid hydroperoxides at the expense of glutathione
	PRDX1	Peroxiredoxin 1 (Prdx1)	Reduces hydrogen peroxide and alkyl hydroperoxides
	TXN1	Thioredoxin 1 (Trx1)	Reduces oxidized protein thiols
	SRXN1	Sulfiredoxin 1 (Srx1)	Contributes to the thioredoxin system by reducing sulfinic acid to thiols
Glutathione biosynthesis	*GCLC*	Glutamate-cysteine ligase catalytic subunit (GCLC)	The first rate-limiting enzyme of glutathione synthesis (heavy subunit)
	GCLM	Glutamate-cysteine ligase modifier subunit (GCLM)	The first rate-limiting enzyme of glutathione synthesis (light subunit)
Detoxification	*GST*	Glutathione S-transferase (GST)	Catalyzes the conjugation of glutathione to electrophilic compounds
	NQO1	NAD(P)H:quinone oxidoreductase-1 (NQO1)	Reduces quinone to hydroquinone
	CYP2A6	Cytochrome P450 2A6 (CYP2A6)	Involved in the hydroxylation of some anti-cancer drugs
Drug Excretion	*ABCC2*	Multidrug resistance protein 2 (MRP2)	Mediates hepatobiliary excretion; implicated in multidrug resistance
Heme metabolism	*HMOX1*	Heme oxygenase 1 (HO-1)	Cleaves heme to form biliverdin during heme catabolism

1.2.4 Non-Canonical Nrf2 Regulation

Apart from its regulation by Keap1, Nrf2 is subject to further non-canonical forms of regulation by a series of other proteins, summarized in **Table 1.4**. Direct interaction of these proteins with either Nrf2 or Keap1 results in competitive inhibition that disrupts the Keap1-Nrf2 complex, decreases Nrf2 ubiquitination, and increases Nrf2 stabilization and stress-induced ARE activation. Some of these non-canonical forms of Nrf2 regulation are discussed in further detail.

Table 1.4: Non-canonical Nrf2 regulation by direct protein interaction.

	Interacting Protein	Known Interaction Motif(s)	Nrf2 Domain	+ or – Nrf2 Regulation	Reference(s)
Nrf2	βTrCP	^{334}DSGIS^{338} (Nrf2) ^{373}DSAPGS^{378} (Nrf2)	Neh6	– ; Nrf2 degradation	(Rada et al., 2011; Chowdhry et al., 2013)
	RXRα	^{209}ETT…NGP^{316} (Nrf2)	Neh7	– ; ↓ transactivation	(Wang et al., 2013a)
	p21	^{29}DLG^{31} (Nrf2) ^{79}ETGE^{82} (Nrf2) ^{154}KRR^{156} (p21)	Neh2	+ ; Nrf2 stabilization	(Chen et al., 2009b)
	DJ-1	Currently unknown	---	+ ; Nrf2 stabilization	(Clements et al., 2006)
	BRCA1	^{79}ETGE^{82} (Nrf2) BRCT domain $^{(1591\text{-}1784)}$ (BRCA1)	Neh2	+ ; Nrf2 stabilization	(Gorrini et al., 2013; Xu et al., 2018)
	Interacting Protein	**Interaction Motif(s)**	**Keap1 Domain**	**+ or – Nrf2 Regulation**	**Reference(s)**
Keap1	p62 / SQSTM1	^{349}DPSTGE^{354} (p62)	Kelch	+ ; Keap1 inhibition	(Copple et al., 2010; Fan et al., 2010; Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010)
	ProTα / PTMA	^{38}NANEENGE^{45} (ProTα)	Kelch	+ ; Keap1 inhibition	(Karapetian et al., 2005)
	DPP3	^{480}ETGE^{483} (DPP3)	Kelch	+ ; Keap1 inhibition	(Hast et al., 2013)
	WTX	^{286}SPETGE^{291} (WTX)	Kelch	+ ; Keap1 inhibition	(Camp et al., 2012)
	PALB2 / FANCN	^{91}ETGE^{94} (PALB2)	BTB	+ ; Keap1 inhibition	(Ma et al., 2012)
	KPNA6 / Importin α7	ARM domain $^{(108\text{-}563)}$ (KPNA6)	Kelch	- ; Nrf2 degradation	(Sun et al., 2011)

1.2.5 Nrf2-Interacting Proteins

β-transducin repeat-containing protein (βTrCP) is involved in the negative regulation of Nrf2 at the Neh6 domain in a similar manner to Keap1 at the Neh2 domain. βTrCP interacts with Neh6 at two conserved sites, ^{334}DSGIS^{338} and ^{373}DSAPGS^{378}, and acts as a substrate receptor for degradation by the Skp1-Cul1-Rbx1/Roc1 E3 ubiquitin ligase complex (Rada et al., 2011; Chowdhry et al., 2013). Deletion of either motif results in the loss of βTrCP-mediated ubiquitination (Rada et al., 2011). Additionally, the DSGIS motif in Neh6 overlaps with a phosphorylation site for GSK3, wherein phosphorylation of Nrf2 at this motif by GSK3 enhances βTrCP activity (Rada et al., 2011; Chowdhry et al., 2013). Accordingly, when Keap1 activity is impaired in Keap1$^{-/-}$ mouse embryonic fibroblasts or in an Nrf2 ETGE deletion mutant that cannot bind to Keap1, treatment with GSK3 inhibitors leads to impaired βTrCP-regulation and results in Nrf2 stabilization and accumulation (Rada et al., 2011). On the other hand, activation of GSK3 in Keap1$^{-/-}$ mouse embryonic fibroblasts or human lung A549 cells reduces Nrf2 protein levels and mRNA levels for Nrf2-regulated enzymes (Chowdhry et al., 2013).

Retinoid X receptor alpha (RXRα) is involved in numerous developmental and physiological pathways and in mediating the biological effects of retinoids (Szanto et al., 2004). RXRα directly interacts with the Neh7 domain of Nrf2 which impairs the recruitment of cofactors to Neh4 and Neh5 that are required for transactivation (Wang et al., 2013a). Accordingly, RNAi-mediated knockout of RXRα increases the induction of Nrf2-regulated antioxidant gene expression, and overexpression of RXRα in non-small cell lung cancer A549 cells leads to Nrf2 downregulation and increases sensitivity to therapeutic drugs (Wang et al., 2013a).

p21 (or p21$^{CIP1/WAF1}$) is a p53-regulated cyclin-dependent kinase inhibitor involved in inhibiting the activity of cyclin/cyclin-dependent kinase (Cdk) complexes for the negative regulation of cell cycle progression (Xiong et al., 1993). The ^{154}KRR^{156} motif within p21 directly binds to the DLG and ETGE motifs in Nrf2, thereby competing with Keap1 for Nrf2 binding (Chen et al., 2009b); but instead of Nrf2 degradation, p21-Nrf2 binding leads to Nrf2 stabilization and increased response to oxidative stress (Chen et al., 2009b). Accordingly, p21$^{-/-}$ mice show reduced levels of Nrf2 protein and Nrf2 target genes (Chen et al., 2009b). Importantly, p21-dependent protection from oxidative stress requires Nrf2, as colorectal cancer HCT116 cells overexpressing p21

demonstrate enhanced survival in response to hydrogen peroxide in $Nrf2^{+/+}$ but not $Nrf2^{-/-}$ cells (Chen et al., 2009b).

Protein deglycase DJ-1 (DJ-1) (also known as Parkinson disease protein 7, Park7) is a redox-dependent molecular chaperone that mediates protein folding and prevents the misfolding and inclusion formation of neuronal proteins such as α-Synuclein (Zondler et al., 2014). DJ-1 inhibits Keap1-mediated Nrf2 degradation by competitively binding to Nrf2 (Clements et al., 2006). In both primary human cells and mice, loss of DJ-1 leads to deficits in the expression of Nrf2-mediated stress response enzymes, particularly the detoxification enzyme NQO1, suggesting that DJ-1 is required for Nrf2 stability and Nrf2-mediated transcription (Clements et al., 2006). Notably, a mutation in the DJ-1 gene has been strongly implicated in early-onset Parkinson's disease (PD) (Bonifati et al., 2003), suggesting the role of impaired oxidative stress regulation in neurodegenerative diseases, such as PD.

Breast cancer type 1 susceptibility protein (BRCA1) is a tumour suppressor protein primarily responsible for DNA damage repair in cells of the breast and other tissue (Deng & Wang, 2003). The BRCT domain of BRCA1 interacts with the ETGE motif in the Neh2 domain of Nrf2, which inhibits Keap1-mediated ubiquitination and increases the response to oxidative stress (Gorrini et al., 2013; Xu et al., 2018). Expression of BRCA1 in neurons confers protection from ischemia/reperfusion injury through activation of the Nrf2-mediated antioxidant pathway (Xu et al., 2018), and $BRCA1^{-/-}$ mouse primary mammary epithelial cells demonstrate low expression of Nrf2 target genes and increased ROS levels associated with decreased survival (Gorrini et al., 2013). Intriguingly, BRCA1 contains an ARE sequence in its promoter region and is thereby regulated by Nrf2, creating a positive feedback loop (Wang et al., 2013b).

1.2.6 Keap1-Interacting Proteins

p62 (also known as sequestosome-1, SQSTM1) is a stress-inducible scaffold protein involved in numerous signalling pathways, including the targeting of proteins for selective autophagy (Lin et al., 2013; Bitto et al., 2014). In 2010, five independent groups discovered the interaction between p62 and Keap1 (Copple et al., 2010; Fan et al., 2010; Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010). This interaction is mediated by a ^{349}DPSTGE^{354} motif in p62's Keap1-interacting region (KIR) that resembles the ETGE motif in the Keap1-binding domain of Nrf2 (Jain et al.,

2010; Komatsu et al., 2010; Lau et al., 2010). p62 sequesters Keap1 into inclusion bodies for autophagy-mediated degradation, thereby disrupting the Keap1-Nrf2 interaction and inhibiting Nrf2 ubiquitination. Additionally, the binding affinity between p62 and Keap1 is significantly increased when Ser351 in p62 is phosphorylated, leading to increased Nrf2 transcriptional activity (Ichimura et al., 2013). Notably, p62 contains ARE sequences in its promoter and is thereby regulated by Nrf2, indicating a positive feedback loop (Jain et al., 2010).

Prothymosin α (ProTα/PTMA) is a small, highly charged protein involved in cell proliferation and survival through chromatin remodelling and pro-apoptotic activity (Malicet et al., 2006; George & Brown, 2010). ProTα interacts with the Kelch domain of Keap1 and shuttles it into the nucleus, thereby preventing its association with Nrf2 (Karapetian et al., 2005). The ^{38}NANEENGE^{45} motif in ProTα is required for its interaction with the Kelch domain (Khan et al., 2013). HeLa cells overexpressing ProTα show increased Nrf2-mediated *HMOX1* gene expression; however, overexpression of a mutant variant of ProTα that impairs Keap1-binding fails to upregulate *HMOX1* (Karapetian et al., 2005), thereby demonstrating the role of ProTα in the expression of certain antioxidant genes.

Dipeptidyl-peptidase 3 (DPP3) is involved in the cleavage and degradation of bioactive peptides generated by the proteasome during protein degradation (Shimamori et al., 1988; Prajapati & Chauhan, 2011). DPP3, which contains an ^{480}ETGE^{483} motif, interacts with Keap1 by binding to the Kelch domain, thereby inhibiting the Keap1-Nrf2 interaction (Hast et al., 2013). Estrogen receptor-positive MCF7 breast cancer cells demonstrate overexpression of DPP3 that is associated with increased Nrf2 gene expression and poor prognosis (Lu et al., 2017).

WTX is a tumour suppressor and regulator in the canonical Wnt signalling pathway, which mediates critical aspects of embryonic development by promoting the ubiquitination and degradation of β-catenin (Major et al., 2007; Komiya & Habas, 2008). WTX is also involved in oxidative stress regulation through its competitive binding to the Keap1, which inhibits Nrf2 ubiquitination (Camp et al., 2012). siRNA knockdown of WTX in HEK293T cells reduces the activation of Nrf2 target genes in response to tBHQ, a potent Nrf2-activating compound (Camp et al., 2012). WTX contains a ^{286}SPETGE^{291} motif that is similar to the ETGE motif in Nrf2, which allows for interaction with the Kelch domain in Keap1; however, this interaction requires the

phosphorylation of Ser286 to attain a sufficient binding affinity between the two proteins (Camp et al., 2012).

Partner and localizer of BRCA2 (PALB2) (also known as Fanconi anemia complementation group N, FANCN), is a protein that co-localizes with the breast cancer 2 early onset protein (BRCA2) to regulate its stabilization, nuclear localization, and involvement in DNA repair (Xia et al., 2006). siRNA knockdown of PALB2 in bone-derived U2OS cells results in reduced Nrf2 activity and increased ROS levels (Xia et al., 2006). Like the WTX protein, PALB2 contains a ^{91}ETGE^{94} motif that permits its interaction with Keap1 through binding to the Kelch domain (Ma et al., 2012).

KPNA6 (also known as importin α7) is a nucleocytoplasmic transport adaptor involved in the nuclear import of proteins. Keap1 has been shown to shuttle between the nucleus and cytoplasm via KPNA6 which interacts with the Kelch domain of Keap1. Within the nucleus, Keap1 binds to Nrf2 to facilitate its nuclear export and subsequent ubiquitination in the cytosol, thus allowing for attenuation of Nrf2 activity during the postinduction phase (Sun et al., 2011). Knockdown of KPNA6 impairs Keap1 nuclear shuttling and attenuates the Keap1-mediated ubiquitination of Nrf2, whereas overexpression of KPNA6 facilitates Keap1 nuclear import and inhibits Nrf2 signalling (Sun et al., 2011).

1.2.7 Other Mechanisms of Nrf2 Regulation

The transcriptional activity of Nrf2 may also be inhibited by Bach1, a protein in the same CNC-bZIP family as Nrf2 that functions as a transcriptional repressor. Bach1 competes with Nrf2 in the nucleus for heterodimerization with the sMaf proteins which are required for Nrf2/ARE binding (Dhakshinamoorthy et al., 2005). Other forms of Nrf2 regulation include phosphorylation of Nrf2 at Ser40 by protein kinase C (PKC), which impairs Keap1 binding (Huang et al., 2002), and phosphorylation of Nrf2 by the MAPK/ERK pathway, which increases Nrf2 stability (Nguyen et al., 2003).

Finally, phosphoglycerate mutase family member 5 (PGAM5) is a protein phosphatase with various functions in mitochondrial homeostasis and mitophagy (Hammond et al., 2001). Interestingly, PGAM5 can recruit both Keap1 and Nrf2 to the outer mitochondrial matrix by binding to one molecule of a Keap1 dimer while simultaneously binding Nrf2 to form a ternary

Keap1-PGAM5-Nrf2 complex (Lo & Hannink, 2006; Lo & Hannink, 2008). Interestingly, this results in the stress-induced Keap1-mediated ubiquitination and degradation of *not* Nrf2, but of mitochondrial Rho GTPase 2 (Miro2), a mitochondrial GTPase involved in mitochondrial motility (Mealey et al., 2017). This demonstrates that Nrf2 function is not limited to stress-induced gene transcription but highlights Nrf2's involvement in other cellular processes.

1.3 Nrf2 in Cell Physiology

In addition to its primary role as the master transcriptional regulator of the antioxidant response, Nrf2 is involved in numerous other cellular processes including, among others, mitochondrial redox signalling, autophagy, inflammation, and endoplasmic reticulum stress. Each will be discussed very briefly.

1.3.1 Mitochondrial Redox Signaling

Mitochondria are powerhouses for ATP synthesis and are a major source of ROS (Muller, 2000; Turrens, 2003; Andreyev et al., 2005; Adam-Vizi & Chinopoulos, 2006). Mitochondria primarily traffic along microtubules (Leopold et al., 1992; Drubin et al., 1993; Lazzarino et al., 1994) which require adaptor proteins TRAK1/2 and Miro1/2 to link mitochondria to microtubules (Schwarz, 2013). Although the functional significance is not well understood, the retrograde microtubule-dependent movement of mitochondria towards the centrosome has been observed in response to various cell stress conditions including oxidative stress (Hallmann et al., 2004) and hypoxia (Al-Mehdi et al., 2012). As previously mentioned, binding of Nrf2 and Keap1 to PGAM5 results in a ternary complex that is recruited to the outer mitochondrial membrane (Lo & Hannink, 2006; Lo & Hannink, 2008). Importantly, retrograde microtubule-dependent mitochondrial movement requires an intact Keap1-PGAM5-Nrf2 complex, and disruption of this complex results in Keap1-Cul3 mediated degradation of mitochondrial Rho GTPase 2 (Miro2), a mitochondrial GTPase involved in mitochondrial motility, thereby impairing the association between mitochondria and microtubules (Mealey et al., 2017). This demonstrates the importance of Nrf2 in mitochondrial redox signalling during oxidative stress. Additionally, Nrf2 protects mitochondria from oxidative damage by regulating mitochondrial ROS levels through the Keap1-Nrf2 antioxidant pathway (Kovac et al., 2015) and possibly through direct interaction with mitochondrial components (Strom

et al., 2016). Cells lacking Nrf2 are more prone to mitochondrial damage and apoptosis (Piantadosi Claude et al., 2008). Additionally, Nrf2 is involved in mitochondrial biogenesis, the process by which cells increase their mitochondrial mass, through its ARE-driven activation Nrf1 which stimulates mitochondrial biogenesis for increased stress resistance (Piantadosi Claude et al., 2008).

1.3.2 Autophagy

Autophagy is a controlled catabolic process that results in the degradation and recycling of old, damaged, or excess cellular proteins and organelles and is vital to protein homeostasis and cellular and organelle health. Autophagy can be induced by oxidative stress, ER stress, or nutrient deprivation (Eskelinen & Saftig, 2009). The aforementioned p62 works as an autophagy adaptor protein that binds to ubiquitinated proteins and delivers them to autophagosomes for degradation (Komatsu et al., 2007; Pankiv et al., 2007; Ichimura et al., 2008). As previously discussed, the autophagy and oxidative stress pathways are linked by p62 which can sequester Keap1 into apoptotic inclusions to facilitate Nrf2 stabilization (Copple et al., 2010; Fan et al., 2010; Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010), implying a noncanonical mechanism of Nrf2 activation by autophagy. p62 overexpression decreases Keap1 levels, suggesting that Keap1 is a substrate for p62-mediated autophagy (Copple et al., 2010). Also recall that p62 is induced by oxidative stress through activation of the ARE within its promotor by Nrf2, thereby creating a positive feedback loop (Jain et al., 2010).

1.3.3 Inflammation

Inflammation is a biological defence mechanism that is triggered in response to harmful insults such as pathogens, toxins, injury, and damaged cells. Through cytokine production and the recruitment of inflammatory cells, inflammation aims to eliminate the insult, limit its spread, and clear the area for healing and repair (Turner et al., 2014; Bennett et al., 2018). Nrf2 plays a role in regulating the anti-inflammatory response through redox control and activation of ARE-mediated anti-inflammatory genes, including the expression of the antioxidant genes *NQO1*, *HO-1*, and *PRX1*, all of which exhibit anti-inflammatory effects (Braun et al., 2002; Itoh et al., 2004; Chen et al., 2006b; Rushworth et al., 2008). The anti-inflammatory role of Nrf2 also includes Nrf2-mediated inhibition of the pro-inflammatory NF-κB pathway and inhibition of pro-inflammatory

cytokines (Ma et al., 2003; Li et al., 2008; Freigang et al., 2011). Of note, the expression of pro-inflammatory cytokine genes in M1 macrophages is inhibited by Nrf2-ARE binding (Kobayashi et al., 2016); however, Nrf2 has also been found to block the transcriptional upregulation of pro-inflammatory cytokine genes including interleukin 6 (IL-6) and interleukin 1 beta (IL-1β) in an ARE-*independent* manner through direct binding to the proximity of pro-inflammatory genes to inhibit RNA polymerase II recruitment, suggesting that Nrf2's role in inflammation is not limited to just oxidative stress control (Kobayashi et al., 2016). Nrf2 plays numerous additional roles in inflammation that are nicely summarized in the following review (Ahmed et al., 2017).

1.3.4 ER Stress and the UPR

Endoplasmic reticulum (ER) stress is caused by the accumulation of unfolded, misfolded, or excess proteins in the ER lumen which triggers the unfolded protein response (UPR). The UPR is induced by three key signalling pathways in humans: PERK, IRE1α, and ATF6 (Ron & Walter, 2007; Hetz, 2012; Hetz et al., 2020). Activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) which leads to inhibition of protein translation and cell cycle arrest in response to protein stress in the ER (Harding et al., 1999). Notably, Nrf2 is a PERK substrate and PERK-dependent phosphorylation of Nrf2 results in dissociation of the Keap1/Nrf2 complex and induction of Nrf2-mediated antioxidant genes that promote increased glutathione levels and reduced ROS in the ER (Cullinan et al., 2003; Cullinan & Diehl, 2004). Cells with an Nrf2 deletion experience significantly higher levels of apoptotic cell death following exposure to ER stress compared to wild-type cells (Cullinan et al., 2003), thereby illustrating the important role of Nrf2 in ER stress.

1.4 Nrf2 in Human Disease

Due to its crucial role in oxidative stress regulation and additional roles in normal cell physiology, oxidative stress and aberrant Nrf2 expression have been associated with numerous disease pathologies. Three major human diseases, cardiovascular disease, neurodegenerative disease, and cancer are briefly discussed.

1.4.1 Cardiovascular Disease

Cardiovascular disease is a multifaceted disease with a variety of risk factors including hypercholesterolemia, hypertension, and atherosclerosis (Nabel, 2003). Oxidative stress may play a role in the development of vascular complications that promote cardiovascular disease by contributing to the pathogenesis of hypertension (Ceriello, 2008; Rodrigo et al., 2011) and atherosclerosis (Ruotsalainen et al., 2013). The endothelial isoform of nitric oxide synthase (eNOS) is responsible for the biosynthesis of NO in endothelial cells which mediates vascular relaxation (Vallance et al., 1989). The uncoupling of eNOS under pathogenic conditions (e.g., hypertension, atherosclerosis, or diabetes) results in both impaired NO production and increased superoxide production, which leads to hypertension and blood vessel damage, respectively (Santhanam et al., 2012). Additionally, increased oxidative stress has been found to promote the conversion of harmful low-density lipoprotein (LDL) cholesterol to the more atherogenic oxidized LDL form (oxLDL) (Itabe, 2012; Lara-Guzmán et al., 2018). Nrf2 has been shown to protect cardiomyocytes from ROS-induced damage through the expression of antioxidant enzymes (Cao et al., 2006; Ichikawa et al., 2009) while lack of Nrf2 promotes aggravation of vessel lesions towards atherosclerosis (Ruotsalainen et al., 2013). Nrf2 is thus a critical regulator of cardiovascular homeostasis with implications in the development of cardiovascular disease.

1.4.2 Neurodegeneration

The link between oxidative stress and the pathogenesis of neurodegenerative diseases is well established (Halliwell, 2001; Barnham et al., 2004). The brain consumes 20% of the body's oxygen relative to its small mass (2%) and is particularly susceptible to oxidative damage due to its high rate of metabolic activity, high rate of oxygen metabolite production, relatively low levels of antioxidants, low capacity for repair, and high composition of lipids which are prone to

peroxidation and oxidative modification by ROS (Butterfield et al., 2002; Niedzielska et al., 2016). Damaged mitochondria and activated microglia are major sources of ROS in the brain (Halliwell, 2001). Oxidative damage has been implicated in all major neurodegenerative diseases including Alzheimer's disease (AD) (Smith et al., 2000; Cioffi et al., 2019), Parkinson's disease (PD) (Dias et al., 2013; Blesa et al., 2015), Huntington's disease (HD) (Browne et al., 1999; Kumar & Ratan, 2016), amyotrophic lateral sclerosis (ALS) (Barber et al., 2006), and multiple sclerosis (MS) (Gilgun-Sherki et al., 2004). Except for MS, all are characterized by the loss and/or deterioration of neurons in a specific brain region due to hallmark protein misfolding and inclusion formation (Soto, 2003).

Oxidative damage has been observed in the post-mortem brain tissues of patients with neurodegenerative diseases (Halliwell, 2001), suggesting that oxidative stress plays a role in the formation and/or aggravation of these hallmark protein inclusions. Nrf2 is activated in response to oxidative stress but may be impaired or insufficient in neurodegenerative diseases. Significantly reduced levels of nuclear Nrf2 have been observed in the brain regions of AD patients (Ramsey et al., 2007). Conversely, while Nrf2 nuclear localization is observed in PD patient samples, the response may be insufficient to prevent neuronal cell death (Uttara et al., 2009). Additionally, studies have reported the protective role of Nrf2 in neurodegenerative diseases (Calkins et al., 2005; Dinkova-Kostova et al., 2018). For example, Nrf2 activation in astrocytes confers protection against neurodegeneration in mouse models of ALS (Vargas et al., 2008), and Nrf2-deficiency results in increased sensitivity to MPTP-induced PD-like lesions in mice which is improved by Nrf2 overexpression in astrocytes (Chen et al., 2009a). Nrf2 inducers have been shown to have protective effects in the development of neurogenerative disease-associated brain lesions (Calkins et al., 2005).

1.4.3 Cancer

Most cancers show elevated levels of ROS which cause DNA damage, impair protein function, and alter mechanisms of cellular proliferation to promote tumorigenesis (Liou & Storz, 2010). Traditionally, Nrf2 has been considered a tumour suppressor that confers protection against ROS and cancer progression. For instance, mice deficient in Nrf2 are prone to chemical-induced toxicity and tumorigenesis (Aoki et al., 2001). However, despite its beneficial role in cellular protection

and cancer prevention, Nrf2 also has a harmful "dark side" in cancer (Wang et al., 2008). Some somatic mutations give rise to hyperactive Nrf2 activity which allows for an enhanced antioxidant capacity and confers protection of cancer cells from ROS and cancer therapy, thereby leading to cancer progression and cancer therapy resistance (e.g., chemoresistance) (**Figure 1.9**) (Padmanabhan et al., 2006; Singh et al., 2006; Nioi & Nguyen, 2007; Shibata et al., 2008; Wang et al., 2008; Shibata et al., 2011; Ooi et al., 2013; Kerins & Ooi, 2018).

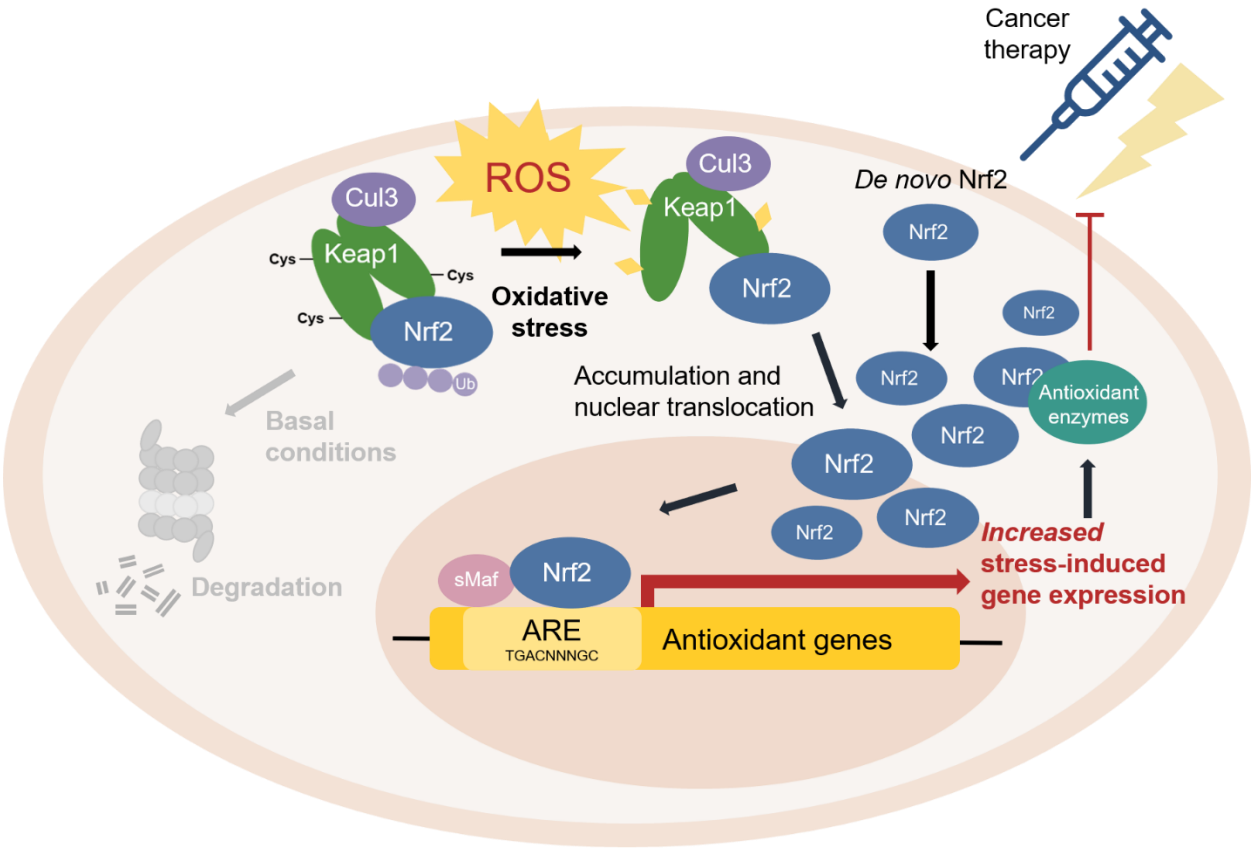

Figure 1.9: The aberrant Keap1-Nrf2 pathway in cancer. Some mutations are associated with Nrf2 hyperactivation, which protects cancer cells from ROS and chemotherapeutic agents by increased antioxidant activity.

Constitutive Nrf2 hyperactivation is common in cancer (Praslicka et al., 2016) and numerous studies have revealed aberrant Nrf2 expression and poor prognosis in a wide range of cancers, including, among others, lung, esophageal, breast, bladder, liver, prostate, and colorectal carcinomas (Ikeda et al., 2004; Shibata et al., 2008; Takahashi et al., 2010; Li et al., 2011; Shibata et al., 2011; Hartikainen et al., 2012; Liu et al., 2015), most of which have been attributed to loss-of-function mutations in the *KEAP1* gene and/or gain-of-function mutations in the *NFE2L2* gene encoding Nrf2 (Padmanabhan et al., 2006; Singh et al., 2006; Nioi & Nguyen, 2007; Shibata et al., 2008; Ooi et al., 2013). Mutations in *KEAP1* were first discovered in human lung adenocarcinoma cell lines, wherein a glycine-to-cysteine substitution within the Nrf2-binding domain of Keap1 reduces Keap1's affinity for Nrf2, resulting in loss of canonical Nrf2 regulation and constitutive Nrf2 hyperactivation (Padmanabhan et al., 2006). Similarly, mutations within the Keap1-binding domain of Nrf2 impairs Keap1 recognition, allowing Nrf2 to escape Keap1-mediated degradation and accumulate at high levels in cancer cells (Shibata et al., 2008). Genomic characterization of squamous cell lung cancers showed significant alterations in the Nrf2 pathway in 34% of all tumour specimens examined (The Cancer Genome Atlas Research, 2012). Mutation frequencies vary greatly across different cancer types, but interestingly, some cancers show high rates of Nrf2 pathway alterations but low rates of *KEAP1* or *NFE2L2* mutations. This suggests that aberrant Nrf2 regulation in certain cancers may also be due to Keap1-independent Nrf2 regulatory pathways, or impaired Keap1-Nrf2 interactions at the protein level.

Nrf2 hyperactivation creates an environment that protects normal but also malignant cells from oxidative stress and cancer therapy. The resultant upregulation of Nrf2-mediated antioxidant proteins renders cancer cells resistant to chemotherapeutic drugs (e.g., 5-fluorouracil, docetaxel, and bortezomib) and radiotherapy (Ramos-Gomez et al., 2001; Lee et al., 2004; Wang et al., 2008; Jiang et al., 2010; Rushworth et al., 2011; Shibata et al., 2011; Manandhar et al., 2012; Zhou et al., 2013). Cancer cells appear to hijack the Nrf2 antioxidant pathway as a means of protection against chemotherapeutics to promote chemoresistance and tumorigenesis (**Figure 1.10**).

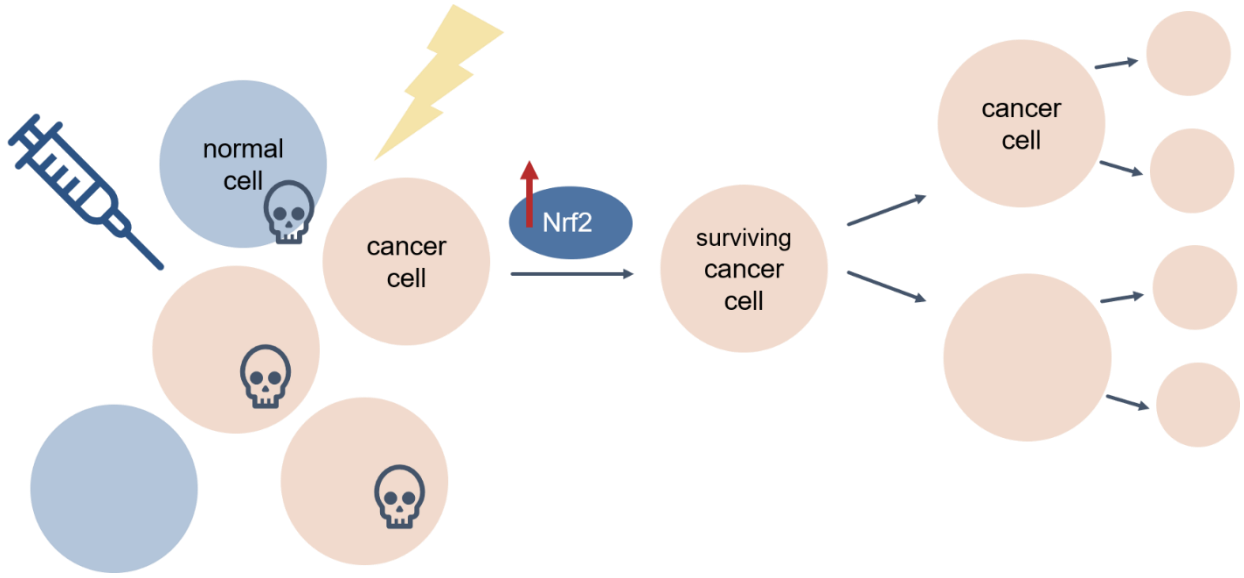

Figure 1.10: Nrf2 protects cancer cells from cancer therapy. Cancer cells hijack the Nrf2 pathway to confer protection against cancer therapies such as chemotherapy and radiation. Cancer cells that survive therapy develop resistance and proliferate, leading to chemoresistance and cancer progression.

1.4.4 Nrf2 as a Therapeutic Target

The importance of Nrf2 in the protection against human diseases is well established and much research has looked into the use of Nrf2 activators in the treatment of disease (Dinkova-Kostova & Talalay, 2008; Suzuki et al., 2013; Zhuang et al., 2017). Examples include dimethyl fumarate which has been approved for the treatment of multiple sclerosis (Linker et al., 2011), among numerous others currently in clinical trials (ClinicalTrials.gov; Robledinos-Antón et al., 2019). While some Nrf2 activators have shown promise, high levels of Nrf2 can have negative effects, as observed in chemotherapy-resistant cancer cells. Research has thus also looked into the use of Nrf2 inhibitors as adjuvants to cancer therapy (Zhu et al., 2016; Robledinos-Antón et al., 2019). For example, brusatol has been shown to enhance the efficacy of chemotherapy by inhibiting Nrf2 (Ren et al., 2011; Olayanju et al., 2015). Taken together, targeting Nrf2 for the treatment of human disease has shown promise, and increased understanding of the delicate balance between Nrf2's protective and deleterious effects will contribute to its value as a therapeutic target.

1.4.5 Ageing

Ageing is not a disease per se, but a predominant risk factor for the development of disease. Progressive and irreversible oxidative damage accumulates with age and diminishes critical aspects of cell physiology (Sykiotis & Bohmann, 2010; Jacinto et al., 2018; Luceri et al., 2018). For example, ageing is associated with impaired activity of the proteasome and mitochondrial Lon proteases (Bota & Davies, 2002; Bota et al., 2002; Morimoto & Cuervo, 2014) and reduced capacity for macromolecule repair (Jacinto et al., 2018; Luceri et al., 2018). The "oxidative damage theory of ageing" (Lin & Flint Beal, 2003) thus postulates that: (1) age-related functional losses are caused by the gradual accumulation of ROS and general oxidative damage to macromolecules, and that (2) ROS reduction and oxidative damage repair attenuate the rate of ageing and increases lifespan. In line with this hypothesis, Nrf2 signalling has been found to decrease with age (Zhang et al., 2015b) in a variety of model organisms including flies (Rahman et al., 2013), mice (Sachdeva et al., 2014), nonhuman primates (Ungvari et al., 2011a; Ungvari et al., 2011b), and humans (Suh et al., 2004; Valcarcel-Ares et al., 2012; Zhang et al., 2015b; Zhou et al., 2018).

Notably, experimental amplification of Nrf2-regulated antioxidant genes has been found to increase resistance to oxidative stress in some aged models but not others (Zhang et al., 2015b), indicating that increased steady-state levels of ROS and oxidized macromolecules may not be the only contributor to age-related functional losses. The alternative "redox stress hypothesis" (Sohal & Orr, 2012) instead proposes that impairments in physiologic function are due to an age-related "pro-oxidizing shift" in the redox state of cells that results in the over-oxidation of redox-sensitive thiol groups within the cysteine residues of proteins, resulting in the impairment of cellular signalling pathways. Much evidence suggests that oxidative damage to proteins is associated with ageing and is linked to protein misfolding (Beal, 2002; Santra et al., 2019).

1.5 Proteins and Oxidative Stress

Proteins must fold and maintain a specific three-dimensional conformation to carry out their normal functions within the cell. Factors that impair this proper protein folding, such as cell stress, genetic mutations, metabolic aberrations, or pathological stress can therefore have detrimental effects. The cell has thus evolved quality control mechanisms to maintain protein homeostasis, or "proteostasis".

1.5.1 Protein Folding and Quality Control

The amino acid sequence of a protein contains the information required for a protein to adopt its proper three-dimensional conformation. Proteins demonstrate a funnel-shaped energy landscape with many high-energy unfolded or partially folded conformations and few low-energy folded conformations (Onuchic et al., 1997; Dill & MacCallum, 2012). A newly synthesized unfolded polypeptide chain contains a high level of energy that is gradually reduced as it undergoes folding events and adopts intermediate states to ultimately achieve its thermodynamically stable, native conformation (**Figure 1.11**). From there, the protein may assemble with other proteins to form protein complexes or undergo further modifications that are crucial for protein function.

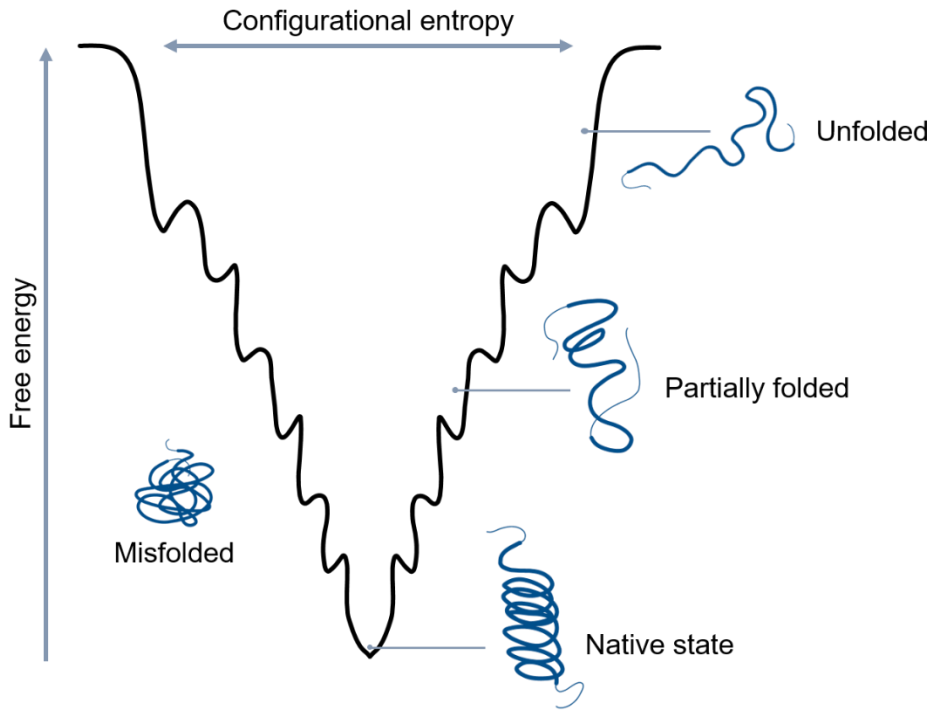

Figure 1.11: Thermodynamics of protein folding. Proteins fold into their native conformations by minimizing free energy. Adopted from Onuchic et al. (Onuchic et al., 1997).

Cells, however, are continually exposed to endogenous and exogenous sources of stress, and proteins are constantly being unfolded and misfolded in the cell. There thus exists a dynamic equilibrium between folded, partially unfolded, and misfolded proteins in the cell which favours the thermodynamically stable native conformation (Ptitsyn, 1994) (**Figure 1.12**).

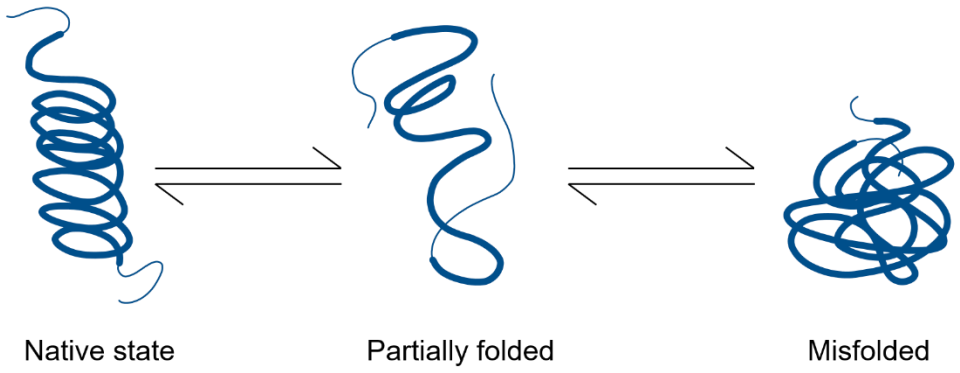

Figure 1.12: Equilibrium between native and non-native protein folding conformations. The continual unfolding and misfolding of proteins in the cell create a dynamic equilibrium that favours the thermodynamically favourable, folded native state.

A misfolded protein is a protein that has acquired a non-native, aberrant conformation. Misfolded proteins often lose their normal function (i.e., loss of protein function) and can aggregate and form inclusions that can have deleterious effects on the cell (i.e., toxic gain of function) (Dobson, 2003). The cellular accumulation of misfolded proteins is strongly associated with ageing (Morley et al., 2002; Morley & Morimoto, 2003) and is implicated in many diseases such as cancer (Dai et al., 2007) and neurodegenerative disease (Soto, 2003; Morimoto, 2008). Cancer cells typically have a higher load of misfolded proteins that is attributed to higher cell division and mutation rates (Whitesell & Lindquist, 2005), while the development of most neurodegenerative diseases arises as a result of protein misfolding and/or mislocalization that leads to neurotoxicity (Soto, 2003).

To prevent and resolve protein misfolding, the cell has evolved intricate protein quality control mechanisms, including the refolding of misfolded proteins by molecular chaperones, which is the primary form of proteostasis (Hartl & Hayer-Hartl, 2002; Kim et al., 2013b; Saibil, 2013), followed by the degradation of misfolded proteins by the ubiquitin-proteasome system (UPS) (Glickman & Ciechanover, 2002; Lecker et al., 2006; Amm et al., 2014) and the sequestration of misfolded proteins into specific quality control compartments (Johnston et al., 1998; García-Mata et al., 1999; Kaganovich et al., 2008). Misfolded proteins that cannot be refolded or degraded are eliminated by autophagy (Hyttinen et al., 2014; Ciechanover & Kwon, 2015). These systems function in parallel and cooperate to maintain the protein folding equilibrium and overall cellular proteostasis (**Figure 1.13**).

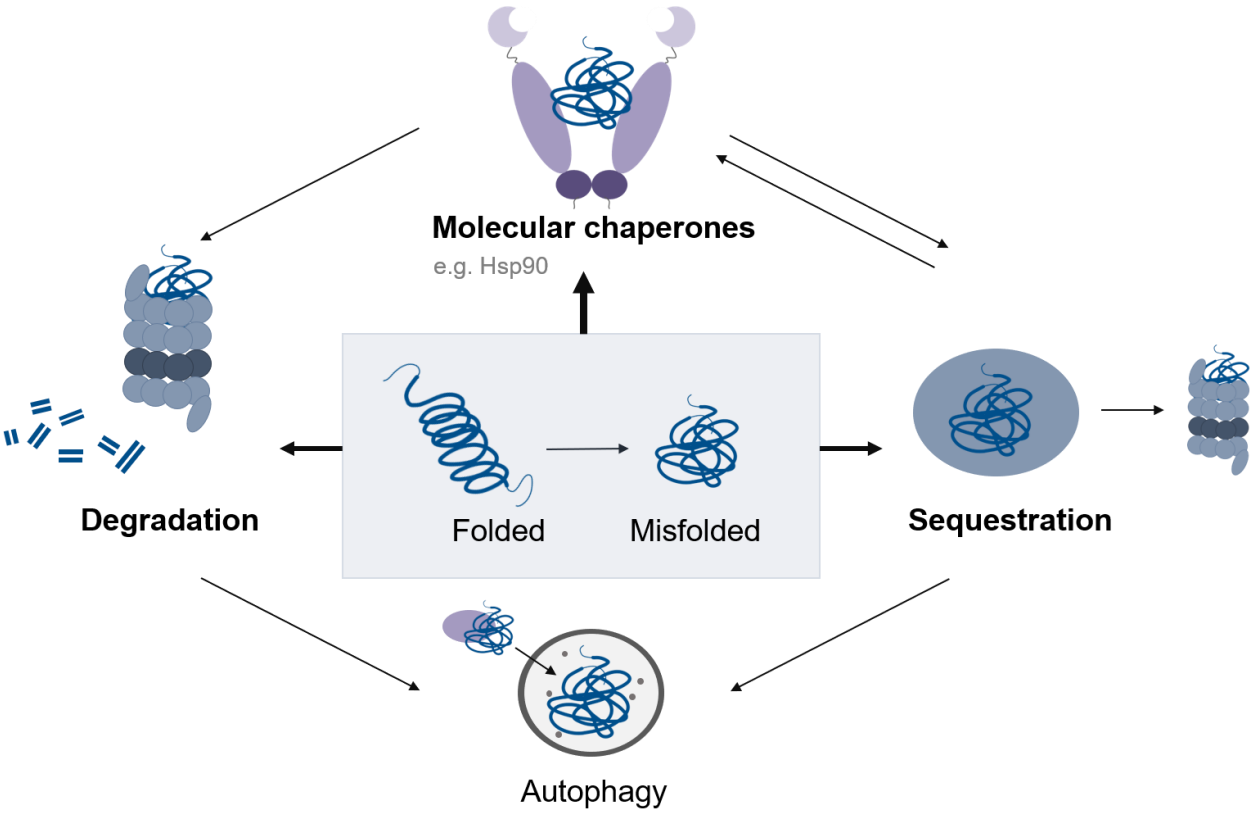

Figure 1.13: Cellular mechanisms of protein quality control. Misfolded proteins are primarily refolded by molecular chaperones. However, misfolded, or aggregated proteins that cannot be refolded are degraded by the proteasome or sequestered into spatial compartments. Sequestered proteins may be sent for refolding or targeted for degradation. If these mechanisms fail, clearance by autophagy occurs.

1.5.2 Molecular Chaperones

Proteostasis is primarily dependent on molecular chaperones (Frydman, 2001; Bukau et al., 2006) which assist in the folding and refolding of proteins. Chaperones bind to unfolded, partially folded, or exposed hydrophobic regions within protein substrates (known as 'clients') to stabilize them, prevent further misfolding, and provide a favourable environment for proper protein folding (Hartl et al., 2011). This chaperone/protein interaction is typically driven by transient cycles of binding and release in an ATP-dependent manner (Tapley et al., 2010). For that reason, molecular chaperones either contain ATPases or form interaction networks with ATPase-containing

chaperone counterparts (co-chaperones) to jointly facilitate protein folding (Tapley et al., 2010). Co-chaperones are proteins that assist chaperones by helping them bind to client proteins and/or providing ATPase activity (Mayer & Bukau, 2005). Besides their well-established roles in protein folding, chaperones cooperate with proteases to facilitate the degradation of misfolded proteins (Youker et al., 2004; Kundrat & Regan, 2010) and may function as disaggregases in certain species such as yeast (Warrick et al., 1999; Douglas et al., 2009).

Eukaryotic cells have two distinct molecular chaperone networks: chaperones linked to protein synthesis (CLIPS) which are localized to protein translation machinery and guide in the folding of newly synthesized proteins (Albanèse et al., 2010); and heat shock proteins (Hsps) which refold misfolded proteins into their native conformation, thereby restoring their function and reducing toxicity (Feder & Hofmann, 1999). Hsps are a family of molecular chaperones that are ubiquitously expressed and recruited under conditions of proteotoxic cell stress, first observed in response to heat stress but also induced upon cold stress, chemical stress, and exposure to UV radiation (Feder & Hofmann, 1999). Hsps are classified according to their molecular weight, which ranges from 10 to 200 kDa. The six major families of human Hsps are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsp (sHsp) (Kampinga et al., 2009). Each class of Hsp has a distinct structure and specific function in protein quality control. Hsp90 is of particular interest to this work and will be discussed in further detail.

1.5.3 Heat shock protein 90 (Hsp90)

Heat shock protein 90 (Hsp90) is a highly abundant molecular chaperone that facilitates the folding of over 200 client proteins under both basal and stressed conditions (Schopf et al., 2017). Hsp90 can adopt dynamic conformations to bind to its functionally and structurally diverse array of client proteins which include kinases, transcription factors, steroid hormone receptors, and E3 ubiquitin ligases, among many others (Schopf et al., 2017). Human Hsp90 contains three highly conserved domains (**Figure 1.14**): an N-terminal domain responsible for ATP-binding (Prodromou et al., 1997); a middle domain responsible for ATP hydrolysis and the binding of client proteins and co-chaperones (Meyer et al., 2003); and a C-terminal domain that is essential for Hsp90 dimerization (Harris et al., 2004). The N-terminal and middle domains are connected by a flexible charged linker sequence that regulates Hsp90 function (Hainzl et al., 2009; Tsutsumi et al., 2012). In

eukaryotes, a MEEVD polypeptide chain is attached to the end of the C-terminus and allows for binding to co-chaperones containing tetratricopeptide repeat (TPR) motifs (Taipale et al., 2010).

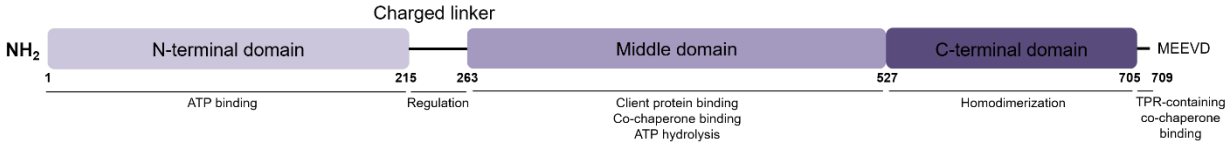

Figure 1.14: Domain structure of human Hsp90. Hsp90 contains three functional domains, a charged linker region between the N-terminal and middle domains, and a MEEVD polypeptide chain attached to the C-terminus for recognition of co-chaperones with TPR motifs.

Hsp90 is predominately dimeric under physiological conditions, as C-terminal dimerization is essential for Hsp90 function and contributes to the dynamic conformational changes that are coupled to N-terminal dimerization and ATPase activity (Prodromou et al., 2000; Wayne & Bolon, 2007). As outlined in **Figure 1.15**, in the absence of ATP, the C-terminal-mediated Hsp90 homodimer maintains an "open" 'V'-shaped conformation with no interaction between the N-terminal domains ('N'). The binding of ATP to the N-terminal domains results in N-terminal dimerization and Hsp90 acquires a "closed" conformation that allows for the binding of client proteins via the middle domain ('M'). ATP hydrolysis to ADP results in a return to the open conformation and the release of a properly folded protein (Li et al., 2012). Although Hsp90 is dependent on ATPase activity, the rate of ATP turnover is very slow and Hsp90 co-chaperones are required for the regulation of Hsp90 activity. These co-chaperones assist in Hsp90 binding to client proteins, support Hsp90-mediated folding, and can inhibit or activate ATPase activity, thereby regulating the rate of Hsp90 chaperoning activity (Li et al., 2012). Examples of Hsp90 co-chaperones in humans include Aha1, a potent activator of Hsp90 ATPase activity (Meyer et al., 2004); Cdc37, which suppresses ATP turnover and allows for the loading of kinase client proteins to Hsp90 (Siligardi et al., 2002); and Hop, which modulates the interaction between Hsp90 and Hsp70 and stimulates protein refolding (Johnson et al., 1998).

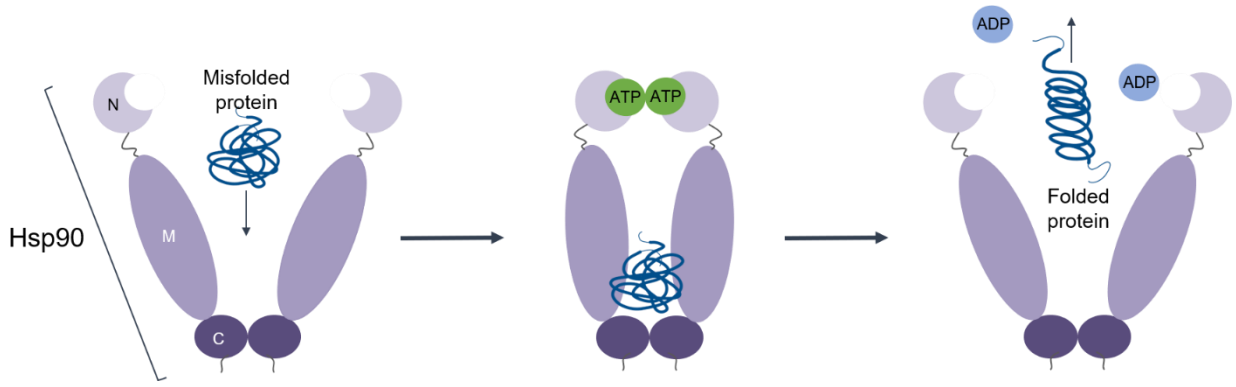

Figure 1.15: Hsp90 chaperone activity. In the absence of ATP, Hsp90 exhibits an "open" configuration with no chaperone activity. ATP-binding to the N-terminal domains leads to homodimer interaction and Hsp90 adopts a "closed" configuration that allows for the binding and refolding of the client protein. Hydrolysis of ATP to ADP results in a return to the open conformation and release of a newly folded protein.

Hsp90 is highly conserved across species (Chen et al., 2006a). In humans, there are five Hsp90 isoforms, including the cytosolic stress-induced Hsp90α (isoforms 1 and 2), the constitutively expressed Hsp90β, as well as two isoforms specific to the ER and mitochondria (Chen et al., 2006a) (**Table 1.5**). In the budding yeast *Saccharomyces cerevisiae*, there are two cytosolic isoforms: stress-inducible Hsp82 and constitutively expressed Hsc82 (**Table 1.5**). Constitutively expressed Hsp90 isoforms are expressed at high levels under basal conditions, whereas stress-inducible Hsp90 isoforms are expressed at low levels and strongly induced in response to protein-denaturing stressors (Sreedhar et al., 2004).

Table 1.5: Hsp90 isoforms in humans and yeast.

	Subcellular Localization	Gene	Protein
Human	Cytosolic	*HSP90AA1*	Hsp90α_1 *
		HSP90AA2	Hsp90α_2 *
		HSP90AB1	Hsp90β **
	ER	*HSP90B1*	Grp94
	Mitochondria	*TRAP1*	Trap1
Yeast	Cytosolic	*HSP82*	Hsp82 *
		HSC82	Hsc82 **

Stress-inducible *; constitutive **

Hsps such as Hsp90 are regulated by heat shock factor 1 (Hsf1), the master transcription factor responsible for the upregulation of *hsp* genes in response to proteotoxic stress (Anckar & Sistonen, 2011). In turn, Hsp90 regulates the transcriptional activation of Hsf1 (Ali et al., 1998) (**Figure 1.16**).

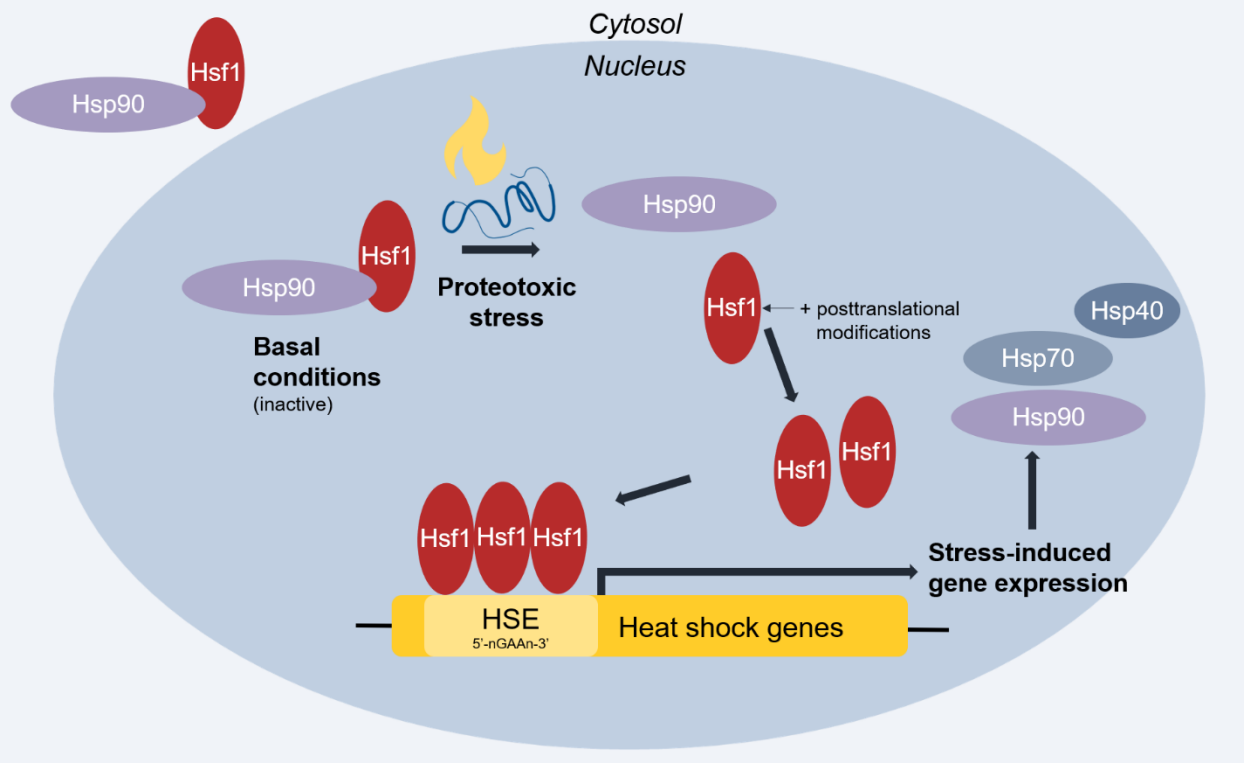

Figure 1.16: Hsps are regulated by Hsf1. Under basal conditions, Hsf1 is bound to Hsp90 and maintained in its inactive form. Upon proteotoxic stress, Hsf1 is released from Hsp90 and undergoes post-translational modification(s) that result in Hsf1 homotrimerization and binding to the heat shock element (HSE) in the promotor region of *hsp* genes to initiate the transcription of Hsps such as Hsp90.

In addition to its key role in protein folding, Hsp90 has central roles in cell growth, differentiation, and survival. Notably, during oncogenesis, these functions may be subverted to promote malignant transformation (Whitesell & Lindquist, 2005). Hsp90 expression is significantly increased in tumours, and high Hsp90 expression levels are associated with decreased survival in cancers including breast cancer (Pick et al., 2007). Somatic mutations in *HSP90* have not yet been identified; however, hyperactivation of Hsf1 in cancer enhances the expression of Hsps and may thus contribute to oncogenic Hsp90 overexpression. For example, loss of the tumour suppressor gene neurofibromatosis type 1 (*NF1*) activates Hsf1 to promote tumorigenesis in mouse embryonic fibroblasts (Dai et al., 2012). Many Hsp90 client proteins, including tumour suppressor p53, proto-oncogene Src, and hypoxia-inducible factor 1α (HIF1α) regulate processes that are critical to

tumour progression (Irby & Yeatman, 2000; Zilfou & Lowe, 2009; Jun et al., 2017) (**Figure 1.17**). Hsp90 inhibitors have thus been the focus of many anti-cancer regimens. Several well-known inhibitors of Hsp90 that inhibit ATP-binding are geldanamycin and radicicol (Roe et al., 1999; Soga et al., 2003). Although some clinical trials have shown promising results, others have been halted due to suboptimal therapeutic effects for reasons that remain to be investigated (Schopf et al., 2017). More work must be done to investigate the use of Hsp90 inhibitors in cancer therapy.

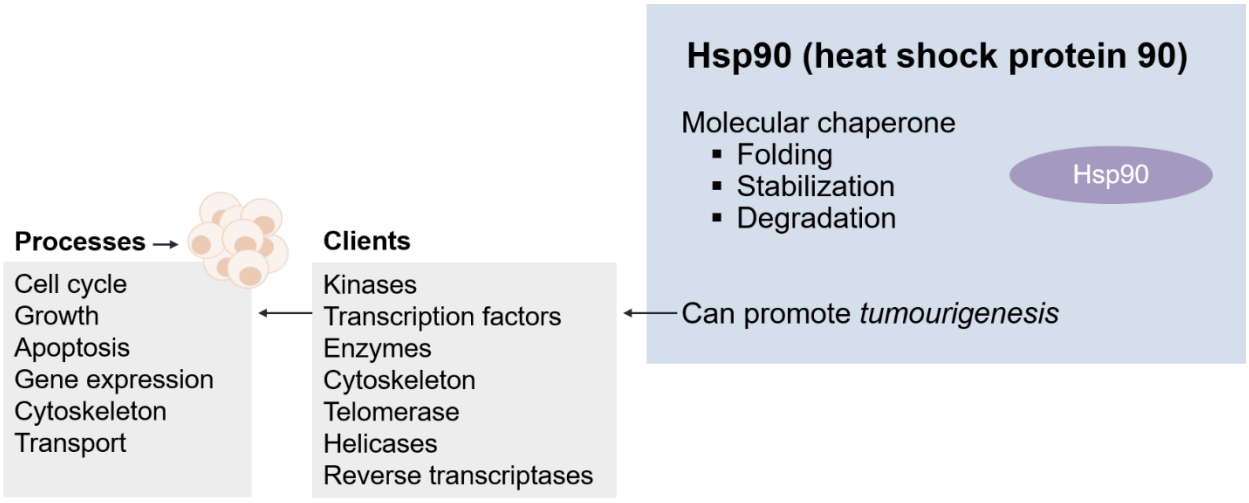

Figure 1.17: The role of Hsp90 in promoting tumourigenesis. In addition to its role as a molecular chaperone, many Hsp90 client proteins are involved in critical processes that may contribute to tumourigenesis when Hsp90 function is subverted in cancer.

1.5.4 Protein Degradation

Cytosolic misfolded proteins that cannot be refolded by molecular chaperones may be targeted for degradation by the ubiquitin-proteasome system (UPS), the major proteolytic pathway in eukaryotes (Ciechanover et al., 1980; Hershko et al., 1980). Proteasomal degradation occurs by two successive steps: (1) the enzymatic cascade of E1, E2, and E3 ubiquitin-associated enzymes which polyubiquitinate old, damaged, and misfolded proteins for degradation; and (2) degradation of the polyubiquitin-tagged protein by the downstream 26S proteasome. Evidence suggests that E3 ubiquitin ligases interact with molecular chaperones. For instance, the mammalian E3 ubiquitin

ligase carboxy-terminal Hsp70 interacting protein (CHIP) has been shown to tag Hsp90- and Hsp70-bound substrates for degradation (Zhang et al., 2015a; Quintana-Gallardo et al., 2019). Misfolded proteins that cannot be degraded by the proteasome may be cleared by autophagy.

For misfolded proteins that are localized to the endoplasmic reticulum (ER), degradation occurs through the ER-associated protein degradation (ERAD) pathway (McCracken & Brodsky, 1996; Werner et al., 1996). The accumulation of misfolded proteins in the ER leads to activation of the unfolded protein response, a cell stress response related to ER stress (Kohno et al., 1993; Cox & Walter, 1996; Liu & Chang, 2008). This halts protein synthesis, initiates degradation, and activates signalling pathways for the recruitment of molecular chaperones. Degradation by the ERAD pathway consists of three major steps: (1) recognition of misfolded proteins through the detection of protein substructures (e.g., exposed hydrophobic regions); (2) retrotranslocation to the cytosol; and (3) degradation by the unfolded protein response (Meusser et al., 2005).

1.5.5 Protein Sequestration

In scenarios where protein quality control mechanisms are overloaded due to cell stress or increased misfolded protein loads, or in cases of irreversible misfolding, aggregation/inclusion formation, proteins may be sequestered into spatially distinct compartments. In humans, misfolded proteins may be sequestered into a dynamic pericentriolar structure called the aggresome (Johnston et al., 1998; García-Mata et al., 1999). Aggresome formation begins with the packaging of misfolded proteins into larger aggresomal particles throughout the cytosol. Shortly after their formation, aggresomal particles are transported in a microtubule-dependent manner along with dynein/dynactin motor complexes to the microtubule-organizing centre (MTOC) where they are sequestered into an aggresome. Aggresome formation is usually accompanied by a reorganization of the intermediate filament cytoskeleton to form a “cage-like” structure around the aggresome (Johnston et al., 1998; García-Mata et al., 1999). From here, cytosolic degradative machinery such as chaperones, ubiquitination enzymes, and proteasome components are recruited to the aggresome to facilitate the clearance of the misfolded proteins (Johnston et al., 1998; Wigley et al., 1999). The aggresome is eventually disassembled with the help of proteasomes (Hao et al., 2013) or targeted for autophagic clearance from the cell (Bjørkøy et al., 2005). Aggresome formation has also been implicated in numerous disease states wherein mutant forms of inclusion/aggregation-

prone proteins that cannot be degraded within the aggresome form pathological inclusion bodies that can contribute to cellular dysfunction and/or cell death (e.g., α-synuclein in Parkinson's disease (Masliah et al., 2000), huntingtin in Huntingtin's disease (Davies et al., 1997), and superoxide dismutase in ALS (Durham et al., 1997; Bruijn et al., 1998)).

Alternatively, in yeast, two intracellular compartments, the juxtanuclear quality control (JUNQ) and the insoluble protein deposit (IPOD), sort and sequester misfolded cytosolic proteins in a tightly regulated manner (Kaganovich et al., 2008). Soluble ubiquitinated misfolded proteins are sequestered into JUNQ compartments where they may be refolded by cytoplasmic molecular chaperones such as Hsp104 or degraded by the 26S proteasomes that are colocalized to JUNQ. On the other hand, non-ubiquitinated, insoluble aggregated proteins, including disease-associated proteins, are sorted into IPOD components. Instead of colocalizing with proteasomes, IPOD colocalizes with autophagy-related protein 8 (Atg8) for aggregate clearance through autophagic pathways. Delivery to these compartments is dependent on molecular chaperones, co-chaperones, and the actin cytoskeleton (Narayanaswamy et al., 2009). In both yeast and humans, misfolded proteins that cannot be cleared within compartments are also cleared by autophagy.

1.5.6 Autophagy

Autophagy, or lysosomal degradation, is a highly regulated intracellular degradation system that delivers degraded constituents from the cytosol, such as degraded misfolded proteins, to the lysosome for clearance (Yorimitsu & Klionsky, 2005; Mizushima, 2007; Glick et al., 2010). There are three types of autophagy in mammals: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). The mechanisms for CMA involve the sequential and targeted delivery of misfolded proteins from the cytosol to the lysosomal lumen by molecular chaperones (Dice, 2007; Cuervo, 2010; Kaushik et al., 2011). Degradation by CMA requires the pentapeptide motif related to the sequence 5'-KFERQ-3' that is recognized by the chaperone for transport to the lysosome (Dice, 1990). Currently, Hsp70 is the only chaperone that can recognize this KFERQ-like motif but may function with assistance from molecular chaperones (Chiang et al., 1989). Unlike macro- and microautophagy which requires vesicle formation with the lysosome, CMA involves the translocation of a substrate across the membrane of the lysosomal lumen (Mizushima, 2007). Notably, this translocation process requires that the protein substrate is in an unfolded state

(Salvador et al., 2000), implying that some insoluble protein aggregates, such as those present in neurodegenerative diseases, cannot be degraded by CMA.

1.5.7 Protein Oxidation

Misfolded proteins may accumulate due to protein oxidation. When the balance between ROS production and elimination is tipped, proteins may become modified and damaged. Most protein amino acids can be oxidized by ROS, but those containing sulfur (S) (i.e., cysteine and methionine) and aromatic rings (i.e., tyrosine and tryptophan) are particularly susceptible (Stadtman, 1993). Oxidation products of cysteines include disulfide (S−S) bonds and mixed disulfide bonds that are reduced by the thioredoxin/thioredoxin reductase system. Thioredoxin reductase system catalyzes the reduction of oxidized thioredoxin which then goes on to reduce protein disulfides (Laurent et al., 1964; Moore et al., 1964; Nordberg & Arnér, 2001). Oxidized methionine is converted to methionine sulfoxide which can be reverted to methionine by methionine sulfoxide reductase (Msr) (Caldwell et al., 1978; Jiang & Moskovitz, 2018). Oxidation of most other amino acids usually involves the irreversible addition of a hydroxyl or carbonyl group; the latter is a biomarker for protein oxidation in cells (Dalle-Donne et al., 2003).

Protein oxidation leads to decreased thermodynamic stability and impaired activity due to partial protein unfolding and disruption of the native state (Kim et al., 2001; Petrov et al., 2016). Protein oxidation may also cause hydrophobic residues to become exposed at the protein surface (Friguet et al., 1994) thereby targeting them for degradation by the proteasome which binds preferentially to hydrophobic residues (Davies, 2001). However, upon conditions of severe oxidative stress, oxidized and misfolded proteins may become severely cross-linked, misfold, and aggregate into insoluble protein inclusions that cannot be degraded by the proteasome. These protein inclusions may in fact bind to the 20S proteasome and irreversibly inhibit their function (Davies, 2001). Cysteines are of particular interest due to their strong propensity for oxidation and cross-linking through disulfide bond formation.

It is important to note, however, that not all forms of protein oxidation are harmful to the cell. Some cellular processes require protein oxidation, such as the oxidation of specific cysteine residues in Keap1 that is required for Nrf2 stabilization (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003; Wakabayashi et al., 2004).

1.5.8 Cysteine Thiol Oxidation

The susceptibility of cysteines to oxidation is due to the presence of a sulfur atom. The sulfur atom is a strong nucleophile contained within the thiol functional group (also known as a sulfhydryl group) of cysteine residues where it exists at the lowest oxidation state (Poole, 2015). A thiol (RSH) that is deprotonated becomes a thiolate anion (RS^-) (**Figure 1.18**).

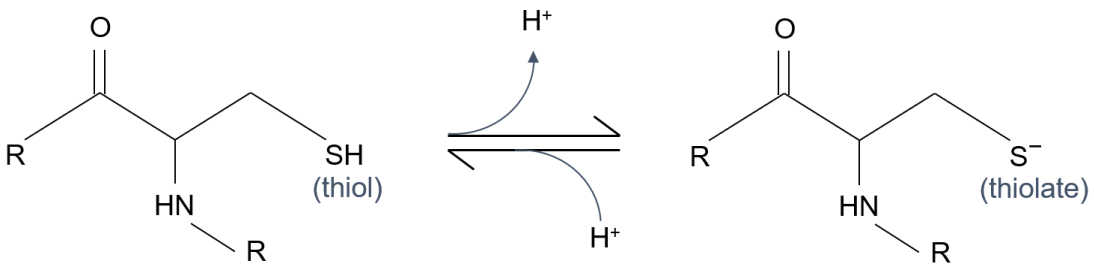

Figure 1.18: Forms of cysteine. The protonated form of cysteine contains a thiol group (left), while the deprotonated form contains a thiolate anion (right).

Oxidants can initiate a sulfur oxidation pathway of protein thiols through the step-wise oxidation to sulfenic acid (RSOH) and sulfinic acid (RSO_2H) and finally to sulfonic acid (RSO_3H) (van Bergen et al., 2014) (**Figure 1.19A**). This occurs in the following steps: (1) oxidation of a thiol group produces sulfenic acid; (2) disulfide bonds are formed by a nucleophilic attack of a thiol on a sulfenic acid; (3) further oxidation of sulfenic acid produces sulfinic acid; (4) even further oxidation of sulfinic acid produces sulfonic acid which is a very strong acid that is comparable to sulfuric acid (Bayse, 2011; van Bergen et al., 2014). The oxidation process towards sulfinic acid is considered mostly irreversible and can only be reverted by the Nrf2-regulated enzyme sulfiredoxin; however, no known enzymes can reverse the oxidation to sulfonic acid (van Bergen et al., 2014). A charged thiolate anion can also go through the sulfur oxidation pathway (**Figure 1.19B**). Within the sulfur oxidation pathway, disulfide bonds are formed through the nucleophilic attack of a thiol on sulfenic acid.

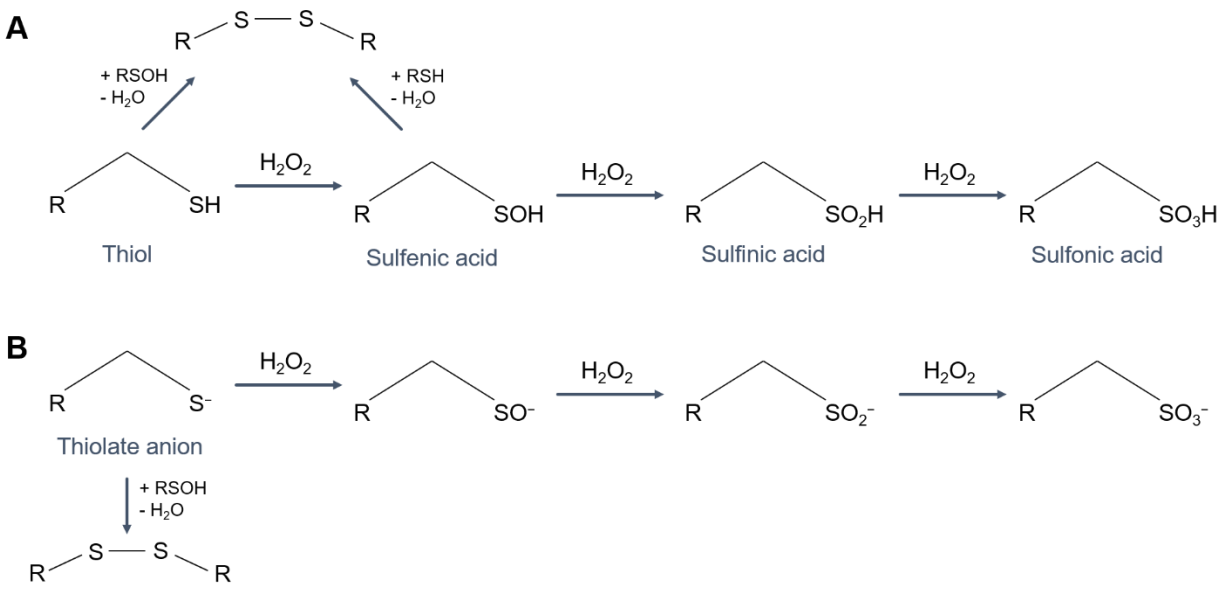

Figure 1.19: The sulfur oxidation pathway with oxidation by hydrogen peroxide (H_2O_2). Thiol oxidation produces sulfenic acid. A nucleophilic attack of a thiol on sulfenic acid and results in disulfide bond formation. Further oxidation of sulfenic acid produces sulfinic acid which can be further oxidized to sulfonic acid. **(B)** This same pathway is illustrated for the oxidation of a thiolate anion. Adapted from van Bergen et al. (van Bergen et al., 2014).

In redox-sensitive proteins such as Keap1, reactive cysteine residues tend to be located adjacent to basic amino acids which markedly lowers their pK_a value, resulting in increased reactivity (Wakabayashi et al., 2004). Additionally, reactive cysteine thiols tend to be situated in proximity to other cysteine thiols (Sanchez et al., 2008), thereby allowing thiols to more readily attack other thiols within the same protein or a different protein, leading to the formation of intramolecular or intermolecular disulfide bonds between cysteines, respectively:

$$2RSH \rightleftharpoons RS{-}SR + 2H^+ + 2e^-$$

This susceptibility of thiols to oxidation makes cysteine thiols very versatile with important roles in cell physiology (Poole, 2015); however, this high level of reactivity may also lead to negative events that impair normal function, such as protein inclusion formation by cysteine thiol oxidation and disulfide bond formation (**Figure 1.20**).

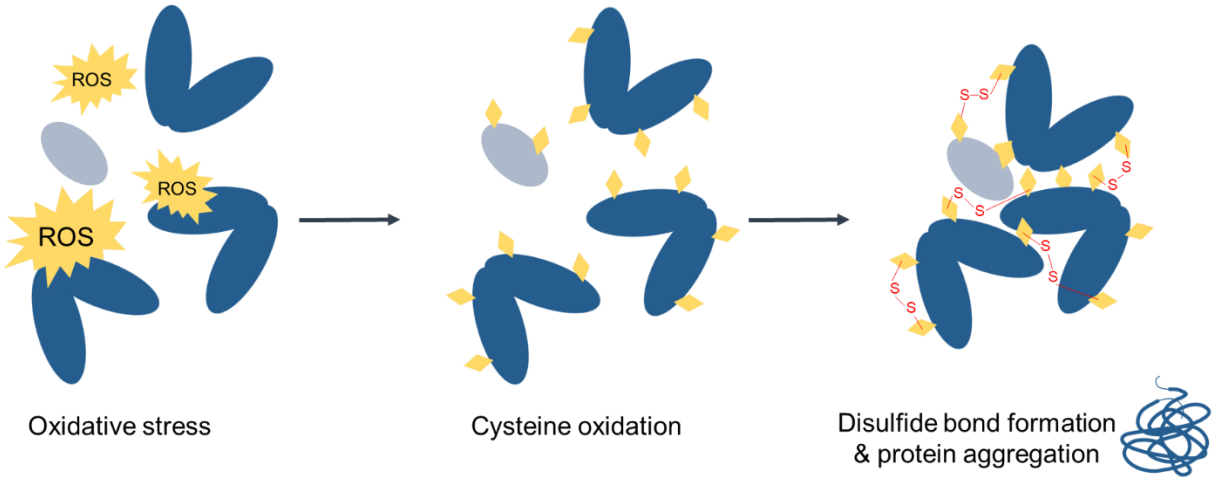

Figure 1.20: Oxidative stress-induced cysteine oxidation. Exposure to ROS results in the oxidation of cysteine thiol groups, leading to intra- and/or intermolecular disulfide bond formation which can contribute to protein misfolding and inclusion formation.

It is important to note that aberrant cysteine oxidation in the cytoplasm (which can be uncontrolled and harmful) differs from cysteine oxidation in the ER (which is controlled and intentional). Within the ER, temporary, non-native disulfide bonds can form within a protein that helps to stabilize proteins during the folding process, though the ultimate reduction of these non-native disulfide bonds is necessary for proper folding (Jansens et al., 2002). Non-native disulfide bond formation in the ER is therefore a normal part of the protein folding pathway, while non-native disulfide bonds are prevalent within aberrantly misfolded cytosolic proteins (Ellgaard et al., 2018). The delicate balance of cysteine oxidation is thus an important topic of study in protein redox biology.

1.6 Studying Protein Interactions

The study of proteins and their interactions is fundamental for understanding biochemical reactions and cellular pathways at the molecular level. For example, nearly all aspects of Nrf2 activity are regulated by its interactions with other proteins. Many techniques have been developed to decipher the genetic and physical interactions of proteins.

1.6.1 Genetic Interactions and Physical Protein-Protein Interactions

A genetic interaction can be defined as the phenomenon by which the phenotypic effects of the function of one gene modifies the phenotypic effects of another gene or group of genes (Mani et al., 2008). Genetic interaction studies have been used to study the functional relationship between genes and their protein products and have successfully been used to identify many protein partners, complexes, and pathways (Mani et al., 2008). It is important to note that although all proteins within a simple complex (e.g., the ribosomal complex) share a functional genetic interaction, not all proteins within a complex physically interact. This distinguishes genetic interactions from physical protein-protein interaction interactions.

A protein-protein interaction (PPI) is defined as the physical interaction between two proteins that occur through specific physical contact and molecular docking (De Las Rivas & Fontanillo, 2010). A protein interaction must meet two major criteria to be considered a PPI: (1) the contact must be specific and exclude chance interactions with other proteins in the cell; and (2) the contact is not generic and excludes any interactions experienced during protein synthesis, folding, and degradation (De Las Rivas & Fontanillo, 2010). Most PPIs are neither static nor permanent, as cells experience continuous turnover and PPIs will vary depending on the biological state of the cell (De Las Rivas & Fontanillo, 2010). All things considered; it is important to differentiate between *physical* PPIs with purely *functional* genetic interactions that do not involve direct physical contact. These interactions can be studied using various methods and models and can quite easily be studied using yeast models.

1.6.2 Yeast as a Model Organism to Study Protein Interactions

Saccharomyces cerevisiae, also known as budding yeast or baker's yeast, is a eukaryotic single-celled organism that has been frequently used as a model organism to study protein mechanisms, pathways, and interactions. Yeast is a powerful model organism because of its rapid growth, which allows for high throughput studies, its single-celled nature, which allows for simple *in vivo* analysis, and the fact that yeast and human cells share fundamental commonalities for many conserved cellular processes (Smith & Snyder, 2006; Mohammadi et al., 2015). About one-third of yeast genes have a human ortholog and over two-thirds share significant homology with human genes (Laurent et al., 2016). Notably, yeast was the first eukaryotic organism to have its complete genome sequenced (Goffeau et al., 1996) and subsequently, a wide range of yeast-associated genetic tools have been developed, including large strain libraries for deletion mutants (Giaever et al., 2002), overexpression mutants (Sopko et al., 2006), and strains with genes tagged by reporter genes (Huh et al., 2003). In this work, we utilize the yeast model to (1) study genetic interactions using yeast growth expression studies, and (2) study physical PPIs using the split-ubiquitin system (**Figure 1.21**).

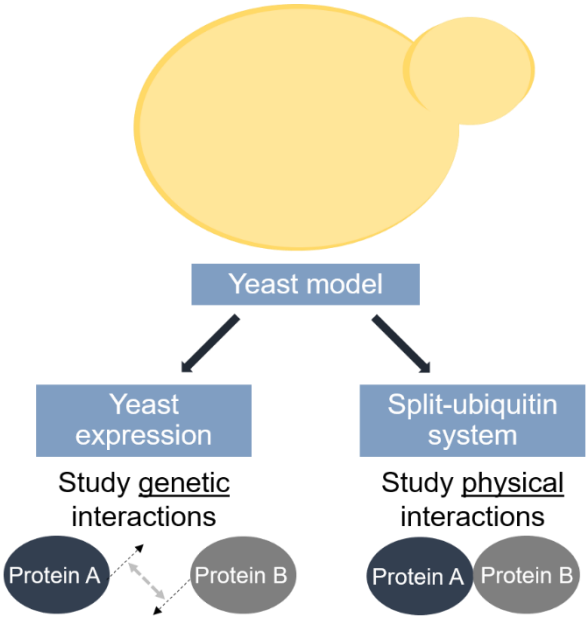

Figure 1.21: **Using a yeast model to study protein interactions.** Yeast growth expression studies and the split-ubiquitin system can be used to study genetic and physical interactions, respectively, for human proteins expressed in yeast.

To study genetic interactions using yeast, growth assays can be performed wherein two human proteins of interest are co-expressed together in yeast cells and their individual and combined effects can be observed from the resulting growth phenotype to determine possible genetic interactions. If their co-expression phenotype differs from the phenotype of either protein expressed alone, a genetic interaction may exist. For example, **Figure 1.22** demonstrates that the expression of 'protein A' is toxic in yeast (i.e., impaired growth compared to the control); however, the co-expression of protein A together with 'protein B' results in a partial rescue of protein A's toxicity. This indicates a genetic interaction between proteins A and B.

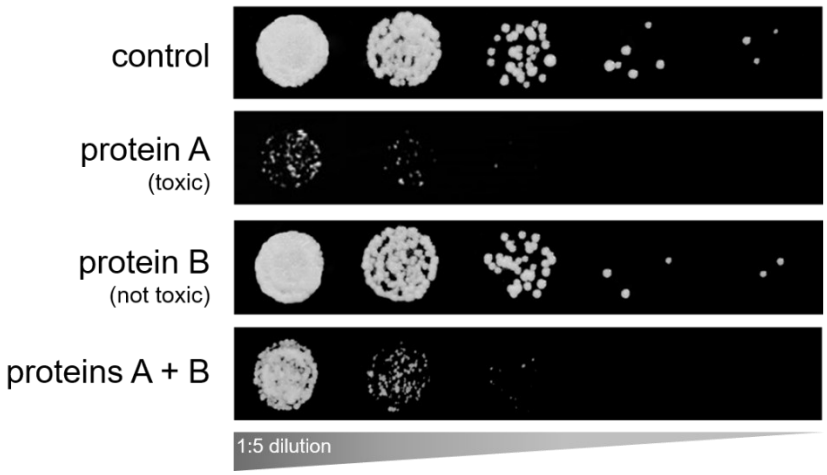

Figure 1.22: Yeast growth assay for detecting genetic interactions. The co-expression of proteins A and B together partially rescues the toxic phenotype observed for the expression of protein A alone, thereby indicating that a genetic interaction exists between them.

With regards to studying physical PPIs in yeast, several methods have been developed including the yeast two-hybrid (Y2H) system (Fields & Song, 1989; Brückner et al., 2009) and the split-ubiquitin system (Johnsson & Varshavsky, 1994; Müller & Johnsson, 2008). The Y2H system functions under the premise that the binding of a transcription factor to an upstream activating sequence (UAS) initiates the activation of a downstream reporter gene. The transcription factor is split into two fragments: the DNA-binding domain (DBD) which binds to the UAS, and the activating domain (AD) which activates transcription. Note that neither fragments alone can initiate transcription. For Y2H screening, protein fusions are prepared for two proteins of interest,

termed the 'bait' and the 'prey' (i.e., DBD-bait and AD-prey, or vice versa). If the bait protein interacts with the prey protein, then the DBD and AD reconstitute and transcription of the reporter gene occurs, resulting in a specific and detectable change in the yeast cell phenotype (**Figure 1.23**).

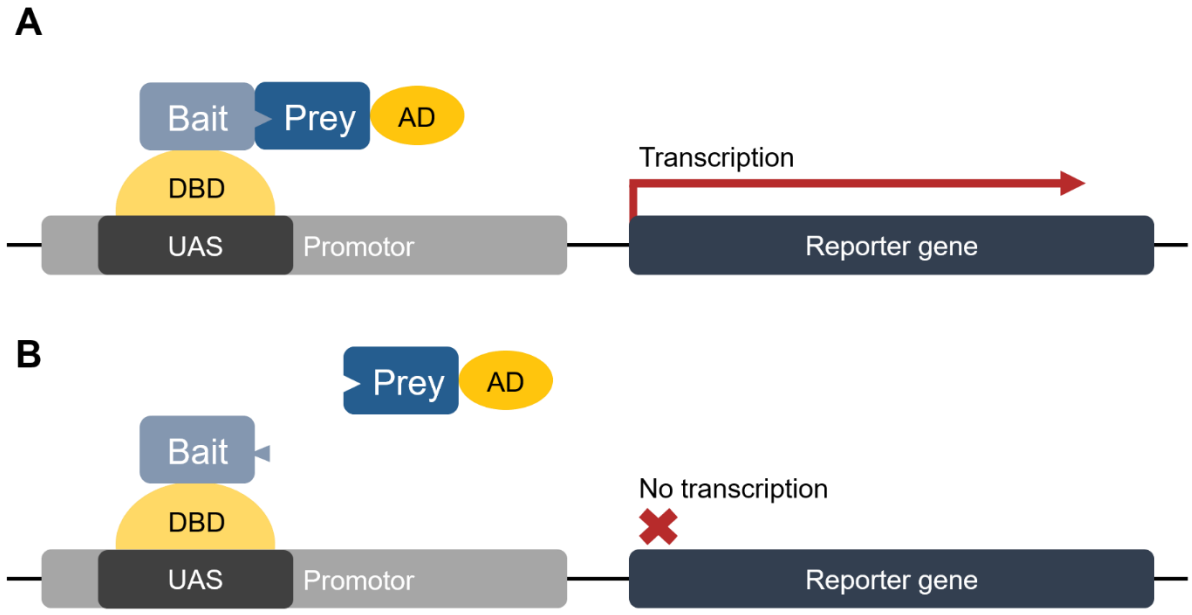

Figure 1.23: Yeast two-hybrid (Y2H) system for detecting PPIs. Two proteins of interest (the 'bait' and 'prey') are fused to the DNA-binding domain (DBD) and activating domain (AD), respectively, within the promoter region of a downstream reporter gene. The DBD is bound to the upstream activating sequence (UAS). **(A)** The bait and prey interact and the DBD and AD reconstitute. Transcription of the reporter gene occurs and a detectable change in cell phenotype is observed due to reporter gene activity. **(B)** The bait and prey do not interact. The reporter gene is not transcribed and no change in phenotype is observed.

The split-ubiquitin system was developed based on similar underlying principles as the Y2H system, but instead of utilizing two transcription factor fragments, the bait and prey are fused to two fragments of a ubiquitin molecule, wherein reconstitution of two ubiquitin fragments results in the cleavage of a reporter molecule, leading to a specific growth phenotype that can be detected on growth media (**Figure 1.24**). Since the split-ubiquitin system does not rely on the utilization of a transcription factor, it offers the great advantage of detecting interactions between non-nuclear,

insoluble, and hydrophobic proteins, and is sensitive enough to detect transient interactions, such as those with molecular chaperones (Stagljar et al., 1998).

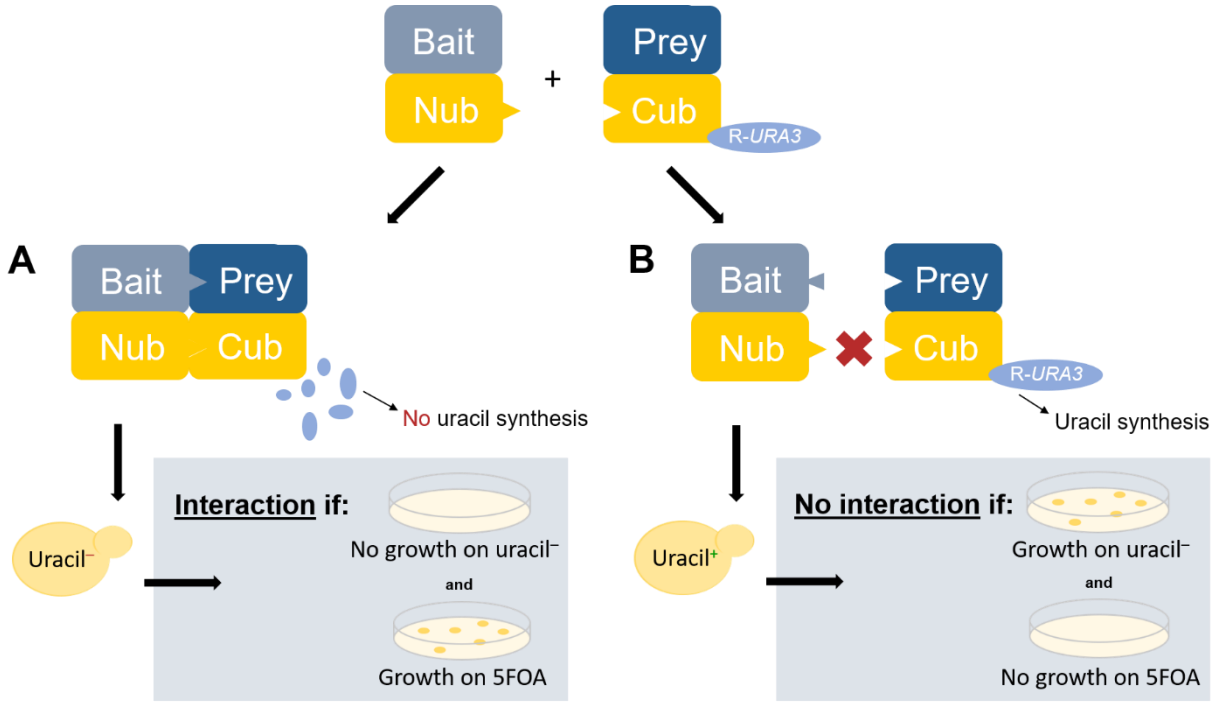

Figure 1.24: The split-ubiquitin system for detecting PPIs. Two proteins of interest (the 'bait' and 'prey') are fused to the N-terminal (Nub) and C-terminal (Cub) halves of a ubiquitin molecule. (**A**) The bait and prey interact and the two halves of ubiquitin re-associate to form a quasi-native ubiquitin. This leads to cleavage and degradation of an R-*URA3* reporter fused to the Cub by ubiquitin-specific proteases. In this scenario, yeast cells are *unable* to produce uracil and cannot grow on growth media that lacks uracil (uracil$^-$) but do grow on media selecting for the absence of *URA3*, i.e., media containing 5-fluoroorotic acid (5FOA). (**B**) The bait and prey do *not* interact. The R-*URA3* reporter remains intact and yeast cells retain their ability to synthesize uracil; however, in the presence of 5FOA, uracil is converted to 5-fluorouracil, which is toxic to yeast. This is detected by growth on uracil$^-$ media and no growth on media containing 5FOA.

Taken together, investigating protein interactions is very important for studying proteins such as Nrf2, whose regulation and activity are heavily dependent on its interactions with other proteins.

1.7 Hypothesis and Aims

Both the antioxidant response (mediated by Nrf2 and Keap1) and the heat shock response (facilitated by molecular chaperones such as Hsp90) are crucial for the maintenance of cellular homeostasis. We hypothesize that oxidative damage to Nrf2 and Keap1 and their interactions with Hsp90 alter their function within the cellular antioxidant stress response. This is explored throughout three chapters encompassing three aims that investigate important cellular and molecular aspects of the Keap1-Nrf2 antioxidant pathway.

Aim 1: *To establish and characterize a yeast model for studying human Nrf2*. Using the complementary experimental tools available for yeast, a previously unexplored interaction between Nrf2 and the heat shock response is detected. From there, further exploration into the nexus between the antioxidant and heat shock responses is studied with emphasis on protein oxidation and cellular responses to cancer therapy.

Aim 2: *To investigate protein oxidation and misfolding in Nrf2 regulation.* Using yeast, mammalian cells, and purified proteins, we describe and assess the oxidative stress-induced inclusion formation of Nrf2 and Keap1 to determine if protein oxidation and misfolding affect Nrf2 regulation.

Aim 3: *To explore potential crosstalk between cell stress responses in cancer.* By using a clinically relevant model of HER2+ breast cancer, we investigate the interplay between the antioxidant and heat shock responses and their dependence on Hsp90 chaperone activity in cancer therapy resistance.

The results from these studies suggest previously unexplored mechanisms by which the cysteine oxidation of Keap1 and Nrf2 alter their cellular function during oxidative stress, and that both Keap1 and Nrf2 folding and activity are regulated by Hsp90, implicating crosstalk between these two cell stress response pathways (**Figure 1.25**).

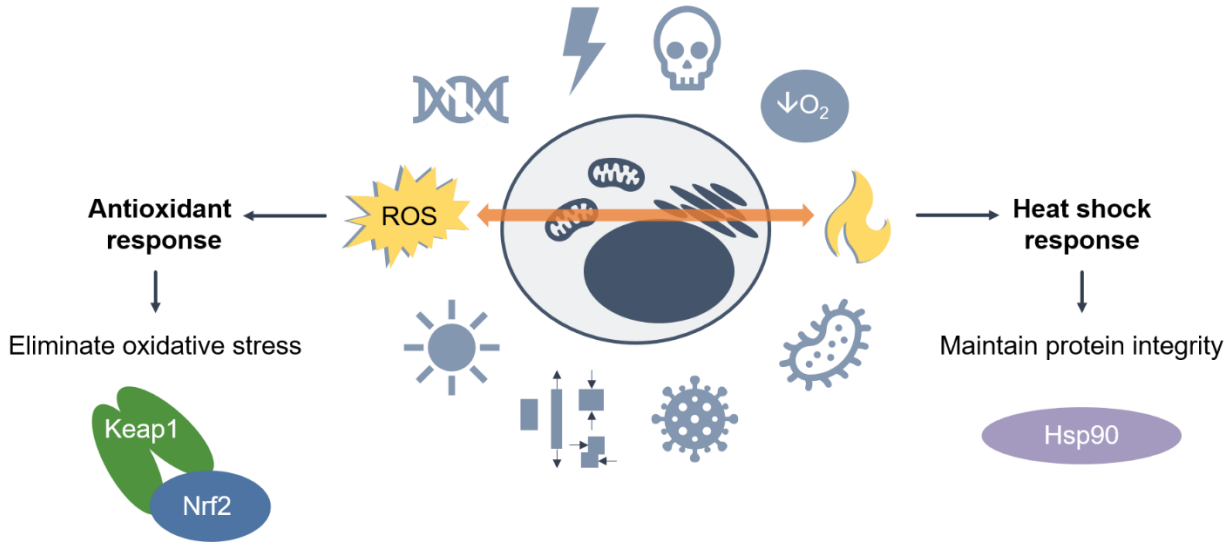

Figure 1.25: Investigating crosstalk between the antioxidant and heat shock responses. This work investigates key cellular and molecular aspects of the Keap1-Nrf2 antioxidant pathway, including its crosstalk with the heat shock response and its implications in protein folding and quality control and cancer.

1.8 References

Abreu, I. A., & Cabelli, D. E. (2010). Superoxide dismutases—a review of the metal-associated mechanistic variations. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1804*(2), 263-274.

Adam-Vizi, V., & Chinopoulos, C. (2006). Bioenergetics and the formation of mitochondrial reactive oxygen species. *Trends Pharmacol Sci, 27*(12), 639-645.

Ahmed, S. M. U., Luo, L., Namani, A., Wang, X. J., & Tang, X. (2017). Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1863*(2), 585-597.

Al-Mehdi, A. B., Pastukh, V. M., Swiger, B. M., Reed, D. J., Patel, M. R., Bardwell, G. C., Pastukh, V. V., Alexeyev, M. F., & Gillespie, M. N. (2012). Perinuclear mitochondrial clustering creates an oxidant-rich nuclear domain required for hypoxia-induced transcription. *Sci Signal, 5*(231), ra47.

Albanèse, V., Reissmann, S., & Frydman, J. (2010). A ribosome-anchored chaperone network that facilitates eukaryotic ribosome biogenesis. *Journal of Cell Biology, 189*(1), 69-81.

Alfadda, A. A., & Sallam, R. M. (2012). Reactive Oxygen Species in Health and Disease. *Journal of Biomedicine and Biotechnology, 2012*, 936486.

Ali, A., Bharadwaj, S., O'Carroll, R., & Ovsenek, N. (1998). HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. *Mol Cell Biol, 18*(9), 4949-4960.

Amm, I., Sommer, T., & Wolf, D. H. (2014). Protein quality control and elimination of protein waste: The role of the ubiquitin–proteasome system. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1843*(1), 182-196.

Anckar, J., & Sistonen, L. (2011). Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annu Rev Biochem, 80*, 1089-1115.

Andreyev, A. Y., Kushnareva, Y. E., & Starkov, A. A. (2005). Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc), 70*(2), 200-214.

Aoki, Y., Sato, H., Nishimura, N., Takahashi, S., Itoh, K., & Yamamoto, M. (2001). Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. *Toxicol Appl Pharmacol, 173*(3), 154-160.

Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol, 55*, 373-399.

Asmat, U., Abad, K., & Ismail, K. (2016). Diabetes mellitus and oxidative stress—A concise review. *Saudi Pharmaceutical Journal, 24*(5), 547-553.

Bae, Y. S., Oh, H., Rhee, S. G., & Yoo, Y. D. (2011). Regulation of reactive oxygen species generation in cell signaling. *Mol Cells, 32*(6), 491-509.

Barber, S. C., Mead, R. J., & Shaw, P. J. (2006). Oxidative stress in ALS: a mechanism of neurodegeneration and a therapeutic target. *Biochim Biophys Acta, 1762*(11-12), 1051-1067.

Barnham, K. J., Masters, C. L., & Bush, A. I. (2004). Neurodegenerative diseases and oxidative stress. *Nature Reviews Drug Discovery, 3*(3), 205-214.

Bayse, C. A. (2011). Transition states for cysteine redox processes modeled by DFT and solvent-assisted proton exchange. *Org Biomol Chem, 9*(13), 4748-4751.

Beal, M. F. (2002). Oxidatively modified proteins in aging and disease1, 2 1Guest Editor: Earl Stadtman 2This article is part of a series of reviews on "Oxidatively Modified Proteins in Aging and Disease." The full list of papers may be found on the homepage of the journal. *Free Radical Biology and Medicine, 32*(9), 797-803.

Bennett, J. M., Reeves, G., Billman, G. E., & Sturmberg, J. P. (2018). Inflammation–Nature's Way to Efficiently Respond to All Types of Challenges: Implications for Understanding and Managing "the Epidemic" of Chronic Diseases. *Frontiers in Medicine, 5*(316).

Bitto, A., Lerner, C. A., Nacarelli, T., Crowe, E., Torres, C., & Sell, C. (2014). p62/SQSTM1 at the interface of aging, autophagy, and disease. *AGE, 36*(3), 9626.

Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Øvervatn, A., Stenmark, H., & Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *Journal of Cell Biology, 171*(4), 603-614.

Blesa, J., Trigo-Damas, I., Quiroga-Varela, A., & Jackson-Lewis, V. R. (2015). Oxidative stress and Parkinson's disease. *Frontiers in Neuroanatomy, 9*, 91.

Bonifati, V., Rizzu, P., van Baren, M. J., Schaap, O., Breedveld, G. J., Krieger, E., Dekker, M. C., Squitieri, F., Ibanez, P., Joosse, M., van Dongen, J. W., Vanacore, N., van Swieten, J. C., Brice, A., Meco, G., van Duijn, C. M., Oostra, B. A., & Heutink, P. (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science, 299*(5604), 256-259.

Bota, D. A., & Davies, K. J. A. (2002). Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nature Cell Biology, 4*(9), 674-680.

Bota, D. A., Van Remmen, H., & Davies, K. J. A. (2002). Modulation of Lon protease activity and aconitase turnover during aging and oxidative stress. *FEBS Lett, 532*(1), 103-106.

Braun, S., Hanselmann, C., Gassmann, M. G., auf dem Keller, U., Born-Berclaz, C., Chan, K., Kan, Y. W., & Werner, S. (2002). Nrf2 transcription factor, a novel target of keratinocyte

growth factor action which regulates gene expression and inflammation in the healing skin wound. *Mol Cell Biol, 22*(15), 5492-5505.

Brigelius-Flohé, R., & Maiorino, M. (2013). Glutathione peroxidases. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1830*(5), 3289-3303.

Browne, S. E., Ferrante, R. J., & Beal, M. F. (1999). Oxidative stress in Huntington's disease. *Brain Pathol, 9*(1), 147-163.

Brückner, A., Polge, C., Lentze, N., Auerbach, D., & Schlattner, U. (2009). Yeast Two-Hybrid, a Powerful Tool for Systems Biology. *International Journal of Molecular Sciences, 10*(6), 2763-2788.

Brugarolas, J., Moberg, K., Boyd, S. D., Taya, Y., Jacks, T., & Lees, J. A. (1999). Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after gamma-irradiation. *Proc Natl Acad Sci U S A, 96*(3), 1002-1007.

Bruijn, L. I., Houseweart, M. K., Kato, S., Anderson, K. L., Anderson, S. D., Ohama, E., Reaume, A. G., Scott, R. W., & Cleveland, D. W. (1998). Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. *Science, 281*(5384), 1851-1854.

Bukau, B., Weissman, J., & Horwich, A. (2006). Molecular Chaperones and Protein Quality Control. *Cell, 125*(3), 443-451.

Butterfield, A. D., Castegna, A., Lauderback, C. M., & Drake, J. (2002). Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol Aging, 23*(5), 655-664.

Caldwell, P., Luk, D. C., Weissbach, H., & Brot, N. (1978). Oxidation of the methionine residues of Escherichia coli ribosomal protein L12 decreases the protein's biological activity. *Proc Natl Acad Sci U S A, 75*(11), 5349-5352.

Calkins, M. J., Jakel, R. J., Johnson, D. A., Chan, K., Kan, Y. W., & Johnson, J. A. (2005). Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. *Proc Natl Acad Sci U S A, 102*(1), 244-249.

Camp, N. D., James, R. G., Dawson, D. W., Yan, F., Davison, J. M., Houck, S. A., Tang, X., Zheng, N., Major, M. B., & Moon, R. T. (2012). Wilms tumor gene on X chromosome (WTX) inhibits degradation of NRF2 protein through competitive binding to KEAP1 protein. *J Biol Chem, 287*(9), 6539-6550.

Canning, P., Cooper, C. D., Krojer, T., Murray, J. W., Pike, A. C., Chaikuad, A., Keates, T., Thangaratnarajah, C., Hojzan, V., Ayinampudi, V., Marsden, B. D., Gileadi, O., Knapp, S., von Delft, F., & Bullock, A. N. (2013). Structural basis for Cul3 protein assembly with the BTB-Kelch family of E3 ubiquitin ligases. *J Biol Chem, 288*(11), 7803-7814.

Cao, Z., Zhu, H., Zhang, L., Zhao, X., Zweier, J. L., & Li, Y. (2006). Antioxidants and phase 2 enzymes in cardiomyocytes: Chemical inducibility and chemoprotection against oxidant and simulated ischemia-reperfusion injury. *Exp Biol Med (Maywood), 231*(8), 1353-1364.

Ceriello, A. (2008). Possible role of oxidative stress in the pathogenesis of hypertension. *Diabetes Care, 31 Suppl 2*, S181-184.

Chan, K., Han, X.-D., & Kan, Y. W. (2001). An important function of Nrf2 in combating oxidative stress: Detoxification of acetaminophen. *Proceedings of the National Academy of Sciences, 98*(8), 4611.

Chelikani, P., Fita, I., & Loewen, P. C. (2004). Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences CMLS, 61*(2), 192-208.

Chen, B., Zhong, D., & Monteiro, A. (2006a). Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms. *BMC genomics, 7*, 156-156.

Chen, P. C., Vargas, M. R., Pani, A. K., Smeyne, R. J., Johnson, D. A., Kan, Y. W., & Johnson, J. A. (2009a). Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. *Proc Natl Acad Sci U S A, 106*(8), 2933-2938.

Chen, W., Sun, Z., Wang, X. J., Jiang, T., Huang, Z., Fang, D., & Zhang, D. D. (2009b). Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol Cell, 34*(6), 663-673.

Chen, X.-L., Dodd, G., Thomas, S., Zhang, X., Wasserman, M. A., Rovin, B. H., & Kunsch, C. (2006b). Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. *American Journal of Physiology-Heart and Circulatory Physiology, 290*(5), H1862-H1870.

Chen, Y., McMillan-Ward, E., Kong, J., Israels, S. J., & Gibson, S. B. (2008). Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death & Differentiation, 15*(1), 171-182.

Chiang, H. L., Terlecky, S. R., Plant, C. P., & Dice, J. F. (1989). A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science, 246*(4928), 382-385.

Chowdhry, S., Zhang, Y., McMahon, M., Sutherland, C., Cuadrado, A., & Hayes, J. D. (2013). Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene, 32*(32), 3765-3781.

Ciechanover, A., Heller, H., Elias, S., Haas, A. L., & Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proceedings of the National Academy of Sciences, 77*(3), 1365.

Ciechanover, A., & Kwon, Y. T. (2015). Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. *Experimental & molecular medicine, 47*(3), e147-e147.

Cioffi, F., Adam, R. H. I., & Broersen, K. (2019). Molecular Mechanisms and Genetics of Oxidative Stress in Alzheimer's Disease. *Journal of Alzheimer's disease : JAD, 72*(4), 981-1017.

Cleasby, A., Yon, J., Day, P. J., Richardson, C., Tickle, I. J., Williams, P. A., Callahan, J. F., Carr, R., Concha, N., Kerns, J. K., Qi, H., Sweitzer, T., Ward, P., & Davies, T. G. (2014). Structure of the BTB domain of Keap1 and its interaction with the triterpenoid antagonist CDDO. *PLoS ONE, 9*(6), e98896.

Clements, C. M., McNally, R. S., Conti, B. J., Mak, T. W., & Ting, J. P. (2006). DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proc Natl Acad Sci U S A, 103*(41), 15091-15096.

ClinicalTrials.gov. ClinicalTrials.gov Available from U.S. National Library of Medicine ClinicalTrials.gov Retrieved December 16, 2020, from U.S. National Library of Medicine https://www.clinicaltrials.gov/

Copple, I. M., Lister, A., Obeng, A. D., Kitteringham, N. R., Jenkins, R. E., Layfield, R., Foster, B. J., Goldring, C. E., & Park, B. K. (2010). Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway. *J Biol Chem, 285*(22), 16782-16788.

Cox, J. S., & Walter, P. (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell, 87*(3), 391-404.

Cuervo, A. M. (2010). Chaperone-mediated autophagy: selectivity pays off. *Trends in Endocrinology & Metabolism, 21*(3), 142-150.

Cullinan, S. B., & Diehl, J. A. (2004). PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem, 279*(19), 20108-20117.

Cullinan, S. B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R. J., & Diehl, J. A. (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol, 23*(20), 7198-7209.

Dai, C., Santagata, S., Tang, Z., Shi, J., Cao, J., Kwon, H., Bronson, R. T., Whitesell, L., & Lindquist, S. (2012). Loss of tumor suppressor NF1 activates HSF1 to promote carcinogenesis. *The Journal of Clinical Investigation, 122*(10), 3742-3754.

Dai, C., Whitesell, L., Rogers, A. B., & Lindquist, S. (2007). Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell, 130*(6), 1005-1018.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta, 329*(1), 23-38.

Davies, K. J. A. (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie, 83*(3), 301-310.

Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L., & Bates, G. P. (1997). Formation of Neuronal Intranuclear Inclusions Underlies the Neurological Dysfunction in Mice Transgenic for the HD Mutation. *Cell, 90*(3), 537-548.

De Las Rivas, J., & Fontanillo, C. (2010). Protein-protein interactions essentials: key concepts to building and analyzing interactome networks. *PLoS computational biology, 6*(6), e1000807-e1000807.

Deng, C.-X., & Wang, R.-H. (2003). Roles of BRCA1 in DNA damage repair: a link between development and cancer. *Human Molecular Genetics, 12*(suppl_1), R113-R123.

Dhakshinamoorthy, S., Jain, A. K., Bloom, D. A., & Jaiswal, A. K. (2005). Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. *J Biol Chem, 280*(17), 16891-16900.

Dias, V., Junn, E., & Mouradian, M. M. (2013). The Role of Oxidative Stress in Parkinson's Disease. *Journal of Parkinson's disease, 3*(4), 461-491.

Dice, J. F. (1990). Peptide sequences that target cytosolic proteins for lysosomal proteolysis. *Trends Biochem Sci, 15*(8), 305-309.

Dice, J. F. (2007). Chaperone-Mediated Autophagy. *Autophagy, 3*(4), 295-299.

Dill, K. A., & MacCallum, J. L. (2012). The Protein-Folding Problem, 50 Years On. *Science, 338*(6110), 1042.

Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., & Talalay, P. (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences, 99*(18), 11908.

Dinkova-Kostova, A. T., Kostov, R. V., & Kazantsev, A. G. (2018). The role of Nrf2 signaling in counteracting neurodegenerative diseases. *The FEBS Journal, 285*(19), 3576-3590.

Dinkova-Kostova, A. T., & Talalay, P. (2008). Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Mol Nutr Food Res, 52 Suppl 1*, S128-138.

Dobson, C. M. (2003). Protein folding and misfolding. *Nature, 426*(6968), 884-890.

Douglas, P. M., Summers, D. W., & Cyr, D. M. (2009). Molecular chaperones antagonize proteotoxicity by differentially modulating protein aggregation pathways. *Prion, 3*(2), 51-58.

Drubin, D. G., Jones, H. D., & Wertman, K. F. (1993). Actin structure and function: roles in mitochondrial organization and morphogenesis in budding yeast and identification of the phalloidin-binding site. *Mol Biol Cell, 4*(12), 1277-1294.

Durham, H. D., Roy, J., Dong, L., & Figlewicz, D. A. (1997). Aggregation of mutant Cu/Zn superoxide dismutase proteins in a culture model of ALS. *J Neuropathol Exp Neurol, 56*(5), 523-530.

Ellgaard, L., Sevier, C. S., & Bulleid, N. J. (2018). How Are Proteins Reduced in the Endoplasmic Reticulum? *Trends Biochem Sci, 43*(1), 32-43.

Enomoto, A., Itoh, K., Nagayoshi, E., Haruta, J., Kimura, T., O'Connor, T., Harada, T., & Yamamoto, M. (2001). High Sensitivity of Nrf2 Knockout Mice to Acetaminophen Hepatotoxicity Associated with Decreased Expression of ARE-Regulated Drug Metabolizing Enzymes and Antioxidant Genes. *Toxicological Sciences, 59*(1), 169-177.

Eskelinen, E.-L., & Saftig, P. (2009). Autophagy: A lysosomal degradation pathway with a central role in health and disease. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1793*(4), 664-673.

Fan, W., Tang, Z., Chen, D., Moughon, D., Ding, X., Chen, S., Zhu, M., & Zhong, Q. (2010). Keap1 facilitates p62-mediated ubiquitin aggregate clearance via autophagy. *Autophagy, 6*(5), 614-621.

Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol, 61*, 243-282.

Fields, S., & Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature, 340*(6230), 245-246.

Finkel, T. (2011). Signal transduction by reactive oxygen species. *The Journal of Cell Biology, 194*(1), 7-15.

Forman, H. J., Zhang, H., & Rinna, A. (2009). Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med, 30*(1-2), 1-12.

Fransen, M., Nordgren, M., Wang, B., & Apanasets, O. (2012). Role of peroxisomes in ROS/RNS-metabolism: Implications for human disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1822*(9), 1363-1373.

Freigang, S., Ampenberger, F., Spohn, G., Heer, S., Shamshiev, A. T., Kisielow, J., Hersberger, M., Yamamoto, M., Bachmann, M. F., & Kopf, M. (2011). Nrf2 is essential for cholesterol crystal-induced inflammasome activation and exacerbation of atherosclerosis. *European Journal of Immunology, 41*(7), 2040-2051.

Friguet, B., Szweda, L. I., & Stadtman, E. R. (1994). Susceptibility of Glucose-6-Phosphate Dehydrogenase Modified by 4-Hydroxy-2-nonenal and Metal-Catalyzed Oxidation to Proteolysis by the Multicatalytic Protease. *Archives of Biochemistry and Biophysics, 311*(1), 168-173.

Frydman, J. (2001). Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annu Rev Biochem, 70*, 603-647.

Fukutomi, T., Takagi, K., Mizushima, T., Ohuchi, N., & Yamamoto, M. (2014). Kinetic, thermodynamic, and structural characterizations of the association between Nrf2-DLGex degron and Keap1. *Mol Cell Biol, 34*(5), 832-846.

García-Mata, R., Bebök, Z., Sorscher, E. J., & Sztul, E. S. (1999). Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J Cell Biol, 146*(6), 1239-1254.

Gartel, A. L., & Radhakrishnan, S. K. (2005). Lost in Transcription: p21 Repression, Mechanisms, and Consequences. *Cancer Res, 65*(10), 3980.

George, E. M., & Brown, D. T. (2010). Prothymosin alpha is a component of a linker histone chaperone. *FEBS Lett, 584*(13), 2833-2836.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Véronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., André, B., Arkin, A. P., Astromoff, A., El Bakkoury, M., Bangham, R., Benito, R., Brachat, S., Campanaro, S., Curtiss, M., Davis, K., Deutschbauer, A., Entian, K.-D., Flaherty, P., Foury, F., Garfinkel, D. J., Gerstein, M., Gotte, D., Güldener, U., Hegemann, J. H., Hempel, S., Herman, Z., Jaramillo, D. F., Kelly, D. E., Kelly, S. L., Kötter, P., LaBonte, D., Lamb, D. C., Lan, N., Liang, H., Liao, H., Liu, L., Luo, C., Lussier, M., Mao, R., Menard, P., Ooi, S. L., Revuelta, J. L., Roberts, C. J., Rose, M., Ross-Macdonald, P., Scherens, B., Schimmack, G., Shafer, B., Shoemaker, D. D., Sookhai-Mahadeo, S., Storms, R. K., Strathern, J. N., Valle, G., Voet, M., Volckaert, G., Wang, C.-y., Ward, T. R., Wilhelmy, J., Winzeler, E. A., Yang, Y., Yen, G., Youngman, E., Yu, K., Bussey, H., Boeke, J. D., Snyder, M., Philippsen, P., Davis, R. W., & Johnston, M. (2002). Functional profiling of the Saccharomyces cerevisiae genome. *Nature, 418*(6896), 387-391.

Gilgun-Sherki, Y., Melamed, E., & Offen, D. (2004). The role of oxidative stress in the pathogenesis of multiple sclerosis: the need for effective antioxidant therapy. *J Neurol, 251*(3), 261-268.

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol, 221*(1), 3-12.

Glickman, M. H., & Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev, 82*(2), 373-428.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y.,

Philippsen, P., Tettelin, H., & Oliver, S. G. (1996). Life with 6000 genes. *Science, 274*(5287), 546, 563-547.

Gorrini, C., Baniasadi, P. S., Harris, I. S., Silvester, J., Inoue, S., Snow, B., Joshi, P. A., Wakeham, A., Molyneux, S. D., Martin, B., Bouwman, P., Cescon, D. W., Elia, A. J., Winterton-Perks, Z., Cruickshank, J., Brenner, D., Tseng, A., Musgrave, M., Berman, H. K., Khokha, R., Jonkers, J., Mak, T. W., & Gauthier, M. L. (2013). BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival. *Journal of Experimental Medicine, 210*(8), 1529-1544.

Hainzl, O., Lapina, M. C., Buchner, J., & Richter, K. (2009). The charged linker region is an important regulator of Hsp90 function. *J Biol Chem, 284*(34), 22559-22567.

Halliwell, B. (2001). Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging, 18*(9), 685-716.

Halliwell, B., & Gutteridge, J. M. (1995). The definition and measurement of antioxidants in biological systems. *Free Radic Biol Med, 18*(1), 125-126.

Halliwell, B., & Gutteridge, J. M. C. (2015). *Free Radicals in Biology and Medicine* (5 ed.). Oxford: Oxford University Press.

Hallmann, A., Milczarek, R., Lipiński, M., Kossowska, E., Spodnik, J. H., Woźniak, M., Wakabayashi, T., & Klimek, J. (2004). Fast perinuclear clustering of mitochondria in oxidatively stressed human choriocarcinoma cells. *Folia Morphol (Warsz), 63*(4), 407-412.

Hammond, P. W., Alpin, J., Rise, C. E., Wright, M., & Kreider, B. L. (2001). In vitro selection and characterization of Bcl-X(L)-binding proteins from a mix of tissue-specific mRNA display libraries. *J Biol Chem, 276*(24), 20898-20906.

Hao, R., Nanduri, P., Rao, Y., Panichelli, R. S., Ito, A., Yoshida, M., & Yao, T. P. (2013). Proteasomes activate aggresome disassembly and clearance by producing unanchored ubiquitin chains. *Mol Cell, 51*(6), 819-828.

Harding, H. P., Zhang, Y., & Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature, 397*(6716), 271-274.

Harris, S. F., Shiau, A. K., & Agard, D. A. (2004). The Crystal Structure of the Carboxy-Terminal Dimerization Domain of htpG, the Escherichia coli Hsp90, Reveals a Potential Substrate Binding Site. *Structure, 12*(6), 1087-1097.

Hartikainen, J. M., Tengström, M., Kosma, V.-M., Kinnula, V. L., Mannermaa, A., & Soini, Y. (2012). Genetic Polymorphisms and Protein Expression of NRF2 and Sulfiredoxin Predict Survival Outcomes in Breast Cancer. *Cancer Res, 72*(21), 5537.

Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature, 475*(7356), 324-332.

Hartl, F. U., & Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science, 295*(5561), 1852-1858.

Hast, B. E., Goldfarb, D., Mulvaney, K. M., Hast, M. A., Siesser, P. F., Yan, F., Hayes, D. N., & Major, M. B. (2013). Proteomic Analysis of Ubiquitin Ligase KEAP1 Reveals Associated Proteins That Inhibit NRF2 Ubiquitination. *Cancer Res, 73*(7), 2199.

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., & Rose, I. A. (1980). Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proceedings of the National Academy of Sciences, 77*(4), 1783.

Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nature Reviews Molecular Cell Biology, 13*(2), 89-102.

Hetz, C., Zhang, K., & Kaufman, R. J. (2020). Mechanisms, regulation and functions of the unfolded protein response. *Nature Reviews Molecular Cell Biology, 21*(8), 421-438.

Huang, H. C., Nguyen, T., & Pickett, C. B. (2002). Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J Biol Chem, 277*(45), 42769-42774.

Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., & O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature, 425*(6959), 686-691.

Hyttinen, J. M., Amadio, M., Viiri, J., Pascale, A., Salminen, A., & Kaarniranta, K. (2014). Clearance of misfolded and aggregated proteins by aggrephagy and implications for aggregation diseases. *Ageing Res Rev, 18*, 16-28.

Ichikawa, T., Li, J., Meyer, C. J., Janicki, J. S., Hannink, M., & Cui, T. (2009). Dihydro-CDDO-trifluoroethyl amide (dh404), a novel Nrf2 activator, suppresses oxidative stress in cardiomyocytes. *PLoS ONE, 4*(12), e8391.

Ichimura, Y., Kumanomidou, T., Sou, Y.-s., Mizushima, T., Ezaki, J., Ueno, T., Kominami, E., Yamane, T., Tanaka, K., & Komatsu, M. (2008). Structural Basis for Sorting Mechanism of p62 in Selective Autophagy. *Journal of Biological Chemistry, 283*(33), 22847-22857.

Ichimura, Y., Waguri, S., Sou, Y.-s., Kageyama, S., Hasegawa, J., Ishimura, R., Saito, T., Yang, Y., Kouno, T., Fukutomi, T., Hoshii, T., Hirao, A., Takagi, K., Mizushima, T., Motohashi, H., Lee, M.-S., Yoshimori, T., Tanaka, K., Yamamoto, M., & Komatsu, M. (2013). Phosphorylation of p62 Activates the Keap1-Nrf2 Pathway during Selective Autophagy. *Mol Cell, 51*(5), 618-631.

Ikeda, H., Nishi, S., & Sakai, M. (2004). Transcription factor Nrf2/MafK regulates rat placental glutathione S-transferase gene during hepatocarcinogenesis. *Biochemical Journal, 380*(2), 515.

Imlay, J. A., Chin, S. M., & Linn, S. (1988). Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science, 240*(4852), 640.

Irby, R. B., & Yeatman, T. J. (2000). Role of Src expression and activation in human cancer. *Oncogene, 19*(49), 5636-5642.

Itabe, H. (2012). Oxidized low-density lipoprotein as a biomarker of in vivo oxidative stress: from atherosclerosis to periodontitis. *Journal of clinical biochemistry and nutrition, 51*(1), 1-8.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., & Nabeshima, Y.-i. (1997). An Nrf2/Small Maf Heterodimer Mediates the Induction of Phase II Detoxifying Enzyme Genes through Antioxidant Response Elements. *Biochem Biophys Res Commun, 236*(2), 313-322.

Itoh, K., Mochizuki, M., Ishii, Y., Ishii, T., Shibata, T., Kawamoto, Y., Kelly, V., Sekizawa, K., Uchida, K., & Yamamoto, M. (2004). Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta(12,14)-prostaglandin j(2). *Mol Cell Biol, 24*(1), 36-45.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., & Yamamoto, M. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev, 13*(1), 76-86.

Jacinto, T. A., Meireles, G. S., Dias, A. T., Aires, R., Porto, M. L., Gava, A. L., Vasquez, E. C., Pereira, T. M. C., Campagnaro, B. P., & Meyrelles, S. S. (2018). Increased ROS production and DNA damage in monocytes are biomarkers of aging and atherosclerosis. *Biological Research, 51*(1), 33.

Jain, A., Lamark, T., Sjøttem, E., Larsen, K. B., Awuh, J. A., Øvervatn, A., McMahon, M., Hayes, J. D., & Johansen, T. (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J Biol Chem, 285*(29), 22576-22591.

Jansens, A., van Duijn, E., & Braakman, I. (2002). Coordinated nonvectorial folding in a newly synthesized multidomain protein. *Science, 298*(5602), 2401-2403.

Jiang, B., & Moskovitz, J. (2018). The Functions of the Mammalian Methionine Sulfoxide Reductase System and Related Diseases. *Antioxidants (Basel, Switzerland), 7*(9), 122.

Jiang, T., Chen, N., Zhao, F., Wang, X.-J., Kong, B., Zheng, W., & Zhang, D. D. (2010). High Levels of Nrf2 Determine Chemoresistance in Type II Endometrial Cancer. *Cancer Res, 70*(13), 5486-5496.

Johnson, B. D., Schumacher, R. J., Ross, E. D., & Toft, D. O. (1998). Hop Modulates hsp70/hsp90 Interactions in Protein Folding. *Journal of Biological Chemistry, 273*(6), 3679-3686.

Johnsson, N., & Varshavsky, A. (1994). Split ubiquitin as a sensor of protein interactions in vivo. *Proc Natl Acad Sci U S A, 91*(22), 10340-10344.

Johnston, J. A., Ward, C. L., & Kopito, R. R. (1998). Aggresomes: A Cellular Response to Misfolded Proteins. *The Journal of Cell Biology, 143*(7), 1883-1898.

Jun, J. C., Rathore, A., Younas, H., Gilkes, D., & Polotsky, V. Y. (2017). Hypoxia-Inducible Factors and Cancer. *Current sleep medicine reports, 3*(1), 1-10.

Kaganovich, D., Kopito, R., & Frydman, J. (2008). Misfolded proteins partition between two distinct quality control compartments. *Nature, 454*(7208), 1088-1095.

Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., Cheetham, M. E., Chen, B., & Hightower, L. E. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones, 14*(1), 105-111.

Karapetian, R. N., Evstafieva, A. G., Abaeva, I. S., Chichkova, N. V., Filonov, G. S., Rubtsov, Y. P., Sukhacheva, E. A., Melnikov, S. V., Schneider, U., Wanker, E. E., & Vartapetian, A. B. (2005). Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Mol Cell Biol, 25*(3), 1089-1099.

Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A., & Yamamoto, M. (2001). Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells, 6*(10), 857-868.

Kaushik, S., Bandyopadhyay, U., Sridhar, S., Kiffin, R., Martinez-Vicente, M., Kon, M., Orenstein, S. J., Wong, E., & Cuervo, A. M. (2011). Chaperone-mediated autophagy at a glance. *Journal of Cell Science, 124*(Pt 4), 495-499.

Kerins, M. J., & Ooi, A. (2018). A catalogue of somatic NRF2 gain-of-function mutations in cancer. *Scientific Reports, 8*(1), 12846-12846.

Khan, H., Cino, E. A., Brickenden, A., Fan, J., Yang, D., & Choy, W.-Y. (2013). Fuzzy Complex Formation between the Intrinsically Disordered Prothymosin α and the Kelch Domain of Keap1 Involved in the Oxidative Stress Response. *Journal of Molecular Biology, 425*(6), 1011-1027.

Kim, J. H., Yu, S., Chen, J. D., & Kong, A. N. (2013a). The nuclear cofactor RAC3/AIB1/SRC-3 enhances Nrf2 signaling by interacting with transactivation domains. *Oncogene, 32*(4), 514-527.

Kim, Y. E., Hipp, M. S., Bracher, A., Hayer-Hartl, M., & Hartl, F. U. (2013b). Molecular chaperone functions in protein folding and proteostasis. *Annu Rev Biochem, 82*, 323-355.

Kim, Y. H., Berry, A. H., Spencer, D. S., & Stites, W. E. (2001). Comparing the effect on protein stability of methionine oxidation versus mutagenesis: steps toward engineering oxidative resistance in proteins. *Protein Engineering, Design and Selection, 14*(5), 343-347.

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., & Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-

based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol, 24*(16), 7130-7139.

Kobayashi, A., Kang, M. I., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., & Yamamoto, M. (2006). Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol Cell Biol, 26*(1), 221-229.

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., Tanaka, N., Moriguchi, T., Motohashi, H., Nakayama, K., & Yamamoto, M. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nature Communications, 7*(1), 11624.

Kohno, K., Normington, K., Sambrook, J., Gething, M. J., & Mori, K. (1993). The promoter region of the yeast KAR2 (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. *Mol Cell Biol, 13*(2), 877.

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y.-S., Ueno, I., Sakamoto, A., Tong, K. I., Kim, M., Nishito, Y., Iemura, S.-i., Natsume, T., Ueno, T., Kominami, E., Motohashi, H., Tanaka, K., & Yamamoto, M. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nature Cell Biology, 12*, 213.

Komatsu, M., Waguri, S., Koike, M., Sou, Y.-s., Ueno, T., Hara, T., Mizushima, N., Iwata, J.-i., Ezaki, J., Murata, S., Hamazaki, J., Nishito, Y., Iemura, S.-i., Natsume, T., Yanagawa, T., Uwayama, J., Warabi, E., Yoshida, H., Ishii, T., Kobayashi, A., Yamamoto, M., Yue, Z., Uchiyama, Y., Kominami, E., & Tanaka, K. (2007). Homeostatic Levels of p62 Control Cytoplasmic Inclusion Body Formation in Autophagy-Deficient Mice. *Cell, 131*(6), 1149-1163.

Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis, 4*(2), 68-75.

Kovac, S., Angelova, P. R., Holmström, K. M., Zhang, Y., Dinkova-Kostova, A. T., & Abramov, A. Y. (2015). Nrf2 regulates ROS production by mitochondria and NADPH oxidase. *Biochim Biophys Acta, 1850*(4), 794-801.

Kröncke, K. D. (2003). Nitrosative stress and transcription. *Biol Chem, 384*(10-11), 1365-1377.

Kumar, A., & Ratan, R. R. (2016). Oxidative Stress and Huntington's Disease: The Good, The Bad, and The Ugly. *J Huntingtons Dis, 5*(3), 217-237.

Kundrat, L., & Regan, L. (2010). Balance between folding and degradation for Hsp90-dependent client proteins: a key role for CHIP. *Biochemistry, 49*(35), 7428-7438.

Lara-Guzmán, O. J., Gil-Izquierdo, Á., Medina, S., Osorio, E., Álvarez-Quintero, R., Zuluaga, N., Oger, C., Galano, J. M., Durand, T., & Muñoz-Durango, K. (2018). Oxidized LDL triggers changes in oxidative stress and inflammatory biomarkers in human macrophages. *Redox Biol, 15*, 1-11.

Lau, A., Wang, X. J., Zhao, F., Villeneuve, N. F., Wu, T., Jiang, T., Sun, Z., White, E., & Zhang, D. D. (2010). A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol Cell Biol, 30*(13), 3275-3285.

Laurent, J. M., Young, J. H., Kachroo, A. H., & Marcotte, E. M. (2016). Efforts to make and apply humanized yeast. *Brief Funct Genomics, 15*(2), 155-163.

Laurent, T. C., Moore, E. C., & Reichard, P. (1964). Enzymatic synthesis of deoxyribonucleotides. II. Isolation and characterization of thioredoxin, the hydrogen donor from Escherichia coli B. *J Biol Chem, 239*, 3436-3444.

Lazzarino, D. A., Boldogh, I., Smith, M. G., Rosand, J., & Pon, L. A. (1994). Yeast mitochondria contain ATP-sensitive, reversible actin-binding activity. *Mol Biol Cell, 5*(7), 807-818.

Lecker, S. H., Goldberg, A. L., & Mitch, W. E. (2006). Protein Degradation by the Ubiquitin–Proteasome Pathway in Normal and Disease States. *Journal of the American Society of Nephrology, 17*(7), 1807.

Lee, H. C., Kim, D. W., Jung, K. Y., Park, I. C., Park, M. J., Kim, M. S., Woo, S. H., Rhee, C. H., Yoo, H., Lee, S. H., & Hong, S. I. (2004). Increased expression of antioxidant enzymes in radioresistant variant from U251 human glioblastoma cell line. *Int J Mol Med, 13*(6), 883-887.

Leopold, P. L., McDowall, A. W., Pfister, K. K., Bloom, G. S., & Brady, S. T. (1992). Association of kinesin with characterized membrane-bounded organelles. *Cell Motility, 23*(1), 19-33.

Li, J., Soroka, J., & Buchner, J. (2012). The Hsp90 chaperone machinery: Conformational dynamics and regulation by co-chaperones. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1823*(3), 624-635.

Li, Q. K., Singh, A., Biswal, S., Askin, F., & Gabrielson, E. (2011). KEAP1 gene mutations and NRF2 activation are common in pulmonary papillary adenocarcinoma. *J Hum Genet, 56*(3), 230-234.

Li, W., Khor, T. O., Xu, C., Shen, G., Jeong, W.-S., Yu, S., & Kong, A.-N. (2008). Activation of Nrf2-antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. *Biochemical Pharmacology, 76*(11), 1485-1489.

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Gargiulo, G., Testa, G., Cacciatore, F., Bonaduce, D., & Abete, P. (2018). Oxidative stress, aging, and diseases. *Clinical interventions in aging, 13*, 757-772.

Lin, M. T., & Flint Beal, M. (2003). The oxidative damage theory of aging. *Clinical Neuroscience Research, 2*(5), 305-315.

Lin, X., Li, S., Zhao, Y., Ma, X., Zhang, K., He, X., & Wang, Z. (2013). Interaction domains of p62: a bridge between p62 and selective autophagy. *DNA Cell Biol, 32*(5), 220-227.

Linker, R. A., Lee, D. H., Ryan, S., van Dam, A. M., Conrad, R., Bista, P., Zeng, W., Hronowsky, X., Buko, A., Chollate, S., Ellrichmann, G., Brück, W., Dawson, K., Goelz, S., Wiese, S., Scannevin, R. H., Lukashev, M., & Gold, R. (2011). Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain, 134*(Pt 3), 678-692.

Liou, G. Y., & Storz, P. (2010). Reactive oxygen species in cancer. *Free Radic Res, 44*(5), 479-496.

Liu, M., Yao, X. D., Li, W., Geng, J., Yan, Y., Che, J. P., Xu, Y. F., & Zheng, J. H. (2015). Nrf2 sensitizes prostate cancer cells to radiation via decreasing basal ROS levels. *Biofactors, 41*(1), 52-57.

Liu, Y., & Chang, A. (2008). Heat shock response relieves ER stress. *Embo j, 27*(7), 1049-1059.

Lo, S.-C., & Hannink, M. (2008). PGAM5 tethers a ternary complex containing Keap1 and Nrf2 to mitochondria. *Experimental Cell Research, 314*(8), 1789-1803.

Lo, S. C., & Hannink, M. (2006). PGAM5, a Bcl-XL-interacting protein, is a novel substrate for the redox-regulated Keap1-dependent ubiquitin ligase complex. *J Biol Chem, 281*(49), 37893-37903.

Lo, S. C., Li, X., Henzl, M. T., Beamer, L. J., & Hannink, M. (2006). Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *Embo j, 25*(15), 3605-3617.

Lu, K., Alcivar, A. L., Ma, J., Foo, T. K., Zywea, S., Mahdi, A., Huo, Y., Kensler, T. W., Gatza, M. L., & Xia, B. (2017). NRF2 Induction Supporting Breast Cancer Cell Survival Is Enabled by Oxidative Stress-Induced DPP3-KEAP1 Interaction. *Cancer Res, 77*(11), 2881-2892.

Luceri, C., Bigagli, E., Femia, A. P., Caderni, G., Giovannelli, L., & Lodovici, M. (2018). Aging related changes in circulating reactive oxygen species (ROS) and protein carbonyls are indicative of liver oxidative injury. *Toxicology Reports, 5*, 141-145.

Ma, J., Cai, H., Wu, T., Sobhian, B., Huo, Y., Alcivar, A., Mehta, M., Cheung, K. L., Ganesan, S., Kong, A. N., Zhang, D. D., & Xia, B. (2012). PALB2 interacts with KEAP1 to promote NRF2 nuclear accumulation and function. *Mol Cell Biol, 32*(8), 1506-1517.

Ma, Q., Kinneer, K., Ye, J., & Chen, B. J. (2003). Inhibition of nuclear factor kappaB by phenolic antioxidants: interplay between antioxidant signaling and inflammatory cytokine expression. *Mol Pharmacol, 64*(2), 211-219.

Major, M. B., Camp, N. D., Berndt, J. D., Yi, X., Goldenberg, S. J., Hubbert, C., Biechele, T. L., Gingras, A. C., Zheng, N., Maccoss, M. J., Angers, S., & Moon, R. T. (2007). Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin signaling. *Science, 316*(5827), 1043-1046.

Malicet, C., Giroux, V., Vasseur, S., Dagorn, J. C., Neira, J. L., & Iovanna, J. L. (2006). Regulation of apoptosis by the p8/prothymosin α complex. *Proc Natl Acad Sci U S A, 103*(8), 2671-2676.

Manandhar, S., Choi, B. H., Jung, K. A., Ryoo, I. G., Song, M., Kang, S. J., Choi, H. G., Kim, J. A., Park, P. H., & Kwak, M. K. (2012). NRF2 inhibition represses ErbB2 signaling in ovarian carcinoma cells: implications for tumor growth retardation and docetaxel sensitivity. *Free Radic Biol Med, 52*(9), 1773-1785.

Mani, R., St.Onge, R. P., Hartman, J. L., Giaever, G., & Roth, F. P. (2008). Defining genetic interaction. *Proceedings of the National Academy of Sciences, 105*(9), 3461.

Martínez, M. C., & Andriantsitohaina, R. (2009). Reactive nitrogen species: molecular mechanisms and potential significance in health and disease. *Antioxid Redox Signal, 11*(3), 669-702.

Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., & Mucke, L. (2000). Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science, 287*(5456), 1265-1269.

Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci, 62*(6), 670-684.

McCracken, A. A., & Brodsky, J. L. (1996). Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. *J Cell Biol, 132*(3), 291-298.

McMahon, M., Itoh, K., Yamamoto, M., & Hayes, J. D. (2003). Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem, 278*(24), 21592-21600.

McMahon, M., Lamont, D. J., Beattie, K. A., & Hayes, J. D. (2010). Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. *Proceedings of the National Academy of Sciences, 107*(44), 18838-18843.

Mealey, G. B., Plafker, K. S., Berry, W. L., Janknecht, R., Chan, J. Y., & Plafker, S. M. (2017). A PGAM5–KEAP1–Nrf2 complex is required for stress-induced mitochondrial retrograde trafficking. *Journal of Cell Science, 130*(20), 3467.

Meusser, B., Hirsch, C., Jarosch, E., & Sommer, T. (2005). ERAD: the long road to destruction. *Nature Cell Biology, 7*(8), 766-772.

Meyer, P., Prodromou, C., Hu, B., Vaughan, C., Roe, S. M., Panaretou, B., Piper, P. W., & Pearl, L. H. (2003). Structural and Functional Analysis of the Middle Segment of Hsp90: Implications for ATP Hydrolysis and Client Protein and Cochaperone Interactions. *Mol Cell, 11*(3), 647-658.

Meyer, P., Prodromou, C., Liao, C., Hu, B., Mark Roe, S., Vaughan, C. K., Vlasic, I., Panaretou, B., Piper, P. W., & Pearl, L. H. (2004). Structural basis for recruitment of the ATPase activator Aha1 to the Hsp90 chaperone machinery. *Embo j, 23*(3), 511-519.

Miseta, A., & Csutora, P. (2000). Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Mol Biol Evol, 17*(8), 1232-1239.

Mizushima, N. (2007). Autophagy: process and function. *Genes Dev, 21*(22), 2861-2873.

Mohammadi, S., Saberidokht, B., Subramaniam, S., & Grama, A. (2015). Scope and limitations of yeast as a model organism for studying human tissue-specific pathways. *BMC Systems Biology, 9*(1), 96.

Moi, P., Chan, K., Asunis, I., Cao, A., & Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Sciences, 91*(21), 9926-9930.

Moore, E. C., Reichard, P., & Thelander, L. (1964). Enzymatic synthesis of deoxyribonucleotides. V. Purification and properties of thioredoxin reductase from Escherichia coli B. *J Biol Chem, 239*, 3445-3452.

Morimoto, R. I. (2008). Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes Dev, 22*(11), 1427-1438.

Morimoto, R. I., & Cuervo, A. M. (2014). Proteostasis and the aging proteome in health and disease. *J Gerontol A Biol Sci Med Sci, 69 Suppl 1*(Suppl 1), S33-38.

Morley, J. F., Brignull, H. R., Weyers, J. J., & Morimoto, R. I. (2002). The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences, 99*(16), 10417.

Morley, J. F., & Morimoto, R. I. (2003). Regulation of Longevity in Caenorhabditis elegans by Heat Shock Factor and Molecular Chaperones. *Mol Biol Cell, 15*(2), 657-664.

Muller, F. (2000). The nature and mechanism of superoxide production by the electron transport chain: Its relevance to aging. *J Am Aging Assoc, 23*(4), 227-253.

Müller, J., & Johnsson, N. (2008). Split-Ubiquitin and the Split-Protein Sensors: Chessman for the Endgame. *ChemBioChem, 9*(13), 2029-2038.

Nabel, E. G. (2003). Cardiovascular Disease. *New England Journal of Medicine, 349*(1), 60-72.

Nakata, K., Tanaka, Y., Nakano, T., Adachi, T., Tanaka, H., Kaminuma, T., & Ishikawa, T. (2006). Nuclear receptor-mediated transcriptional regulation in Phase I, II, and III xenobiotic metabolizing systems. *Drug Metab Pharmacokinet, 21*(6), 437-457.

Narayanaswamy, R., Levy, M., Tsechansky, M., Stovall, G. M., Connell, J. D., Mirrielees, J., Ellington, A. D., & Marcotte, E. M. (2009). Widespread reorganization of metabolic enzymes into reversible assemblies upon nutrient starvation. *Proceedings of the National Academy of Sciences, 106*(25), 10147.

Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S., & Pickett, C. B. (2003). Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem, 278*(7), 4536-4541.

Niedzielska, E., Smaga, I., Gawlik, M., Moniczewski, A., Stankowicz, P., Pera, J., & Filip, M. (2016). Oxidative Stress in Neurodegenerative Diseases. *Molecular Neurobiology, 53*(6), 4094-4125.

Nioi, P., McMahon, M., Itoh, K., Yamamoto, M., & Hayes, J. D. (2003). Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. *Biochem J, 374*(Pt 2), 337-348.

Nioi, P., & Nguyen, T. (2007). A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity. *Biochem Biophys Res Commun, 362*(4), 816-821.

Nioi, P., Nguyen, T., Sherratt, P. J., & Pickett, C. B. (2005). The Carboxy-Terminal Neh3 Domain of Nrf2 Is Required for Transcriptional Activation. *Mol Cell Biol, 25*(24), 10895.

Nordberg, J., & Arnér, E. S. J. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system1 1This review is based on the licentiate thesis "Thioredoxin reductase—interactions with the redox active compounds 1-chloro-2,4-dinitrobenzene and lipoic acid" by Jonas Nordberg, 2001, Karolinska Institute, Stockholm, ISBN 91-631-1064-4. *Free Radical Biology and Medicine, 31*(11), 1287-1312.

O'Dell, T. J., Hawkins, R. D., Kandel, E. R., & Arancio, O. (1991). Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc Natl Acad Sci U S A, 88*(24), 11285-11289.

Olayanju, A., Copple, I. M., Bryan, H. K., Edge, G. T., Sison, R. L., Wong, M. W., Lai, Z.-Q., Lin, Z.-X., Dunn, K., Sanderson, C. M., Alghanem, A. F., Cross, M. J., Ellis, E. C., Ingelman-Sundberg, M., Malik, H. Z., Kitteringham, N. R., Goldring, C. E., & Park, B. K. (2015). Brusatol provokes a rapid and transient inhibition of Nrf2 signaling and sensitizes mammalian cells to chemical toxicity—implications for therapeutic targeting of Nrf2. *Free Radical Biology and Medicine, 78*, 202-212.

Onuchic, J. N., Luthey-Schulten, Z., & Wolynes, P. G. (1997). Theory of protein folding: the energy landscape perspective. *Annu Rev Phys Chem, 48*, 545-600.

Ooi, A., Dykema, K., Ansari, A., Petillo, D., Snider, J., Kahnoski, R., Anema, J., Craig, D., Carpten, J., Teh, B. T., & Furge, K. A. (2013). CUL3 and NRF2 mutations confer an NRF2

activation phenotype in a sporadic form of papillary renal cell carcinoma. *Cancer Res, 73*(7), 2044-2051.

Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsuji, M., Kang, M. I., Kobayashi, A., Yokoyama, S., & Yamamoto, M. (2006). Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol Cell, 21*(5), 689-700.

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J.-A., Outzen, H., Øvervatn, A., Bjørkøy, G., & Johansen, T. (2007). p62/SQSTM1 Binds Directly to Atg8/LC3 to Facilitate Degradation of Ubiquitinated Protein Aggregates by Autophagy. *Journal of Biological Chemistry, 282*(33), 24131-24145.

Petrov, D., Daura, X., & Zagrovic, B. (2016). Effect of Oxidative Damage on the Stability and Dimerization of Superoxide Dismutase 1. *Biophysical journal, 110*(7), 1499-1509.

Piantadosi Claude, A., Carraway Martha, S., Babiker, A., & Suliman Hagir, B. (2008). Heme Oxygenase-1 Regulates Cardiac Mitochondrial Biogenesis via Nrf2-Mediated Transcriptional Control of Nuclear Respiratory Factor-1. *Circulation Research, 103*(11), 1232-1240.

Pick, E., Kluger, Y., Giltnane, J. M., Moeder, C., Camp, R. L., Rimm, D. L., & Kluger, H. M. (2007). High HSP90 expression is associated with decreased survival in breast cancer. *Cancer Res, 67*(7), 2932-2937.

Pompella, A., Visvikis, A., Paolicchi, A., Tata, V. D., & Casini, A. F. (2003). The changing faces of glutathione, a cellular protagonist. *Biochemical Pharmacology, 66*(8), 1499-1503.

Poole, L. B. (2015). The basics of thiols and cysteines in redox biology and chemistry. *Free Radic Biol Med, 80*, 148-157.

Prajapati, S. C., & Chauhan, S. S. (2011). Dipeptidyl peptidase III: a multifaceted oligopeptide N-end cutter. *The FEBS Journal, 278*(18), 3256-3276.

Praslicka, B. J., Kerins, M. J., & Ooi, A. (2016). The complex role of NRF2 in cancer: A genomic view. *Current opinion in toxicology, 1*, 37-45.

Prodromou, C., Panaretou, B., Chohan, S., Siligardi, G., O'Brien, R., Ladbury, J. E., Roe, S. M., Piper, P. W., & Pearl, L. H. (2000). The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. *Embo j, 19*(16), 4383-4392.

Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., & Pearl, L. H. (1997). Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell, 90*(1), 65-75.

Ptitsyn, O. B. (1994). Kinetic and equilibrium intermediates in protein folding. *Protein Engineering, Design and Selection, 7*(5), 593-596.

Quintana-Gallardo, L., Martín-Benito, J., Marcilla, M., Espadas, G., Sabidó, E., & Valpuesta, J. M. (2019). The cochaperone CHIP marks Hsp70- and Hsp90-bound substrates for degradation through a very flexible mechanism. *Scientific Reports, 9*(1), 5102.

Rada, P., Rojo, A. I., Chowdhry, S., McMahon, M., Hayes, J. D., & Cuadrado, A. (2011). SCF/β-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Mol Cell Biol, 31*(6), 1121-1133.

Radi, R. (2018). Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. *Proceedings of the National Academy of Sciences, 115*(23), 5839.

Rahman, M. M., Sykiotis, G. P., Nishimura, M., Bodmer, R., & Bohmann, D. (2013). Declining signal dependence of Nrf2-MafS-regulated gene expression correlates with aging phenotypes. *Aging Cell, 12*(4), 554-562.

Ramos-Gomez, M., Kwak, M. K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., & Kensler, T. W. (2001). Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc Natl Acad Sci U S A, 98*(6), 3410-3415.

Ramsey, C. P., Glass, C. A., Montgomery, M. B., Lindl, K. A., Ritson, G. P., Chia, L. A., Hamilton, R. L., Chu, C. T., & Jordan-Sciutto, K. L. (2007). Expression of Nrf2 in neurodegenerative diseases. *J Neuropathol Exp Neurol, 66*(1), 75-85.

Redza-Dutordoir, M., & Averill-Bates, D. A. (2016). Activation of apoptosis signalling pathways by reactive oxygen species. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1863*(12), 2977-2992.

Ren, D., Villeneuve, N. F., Jiang, T., Wu, T., Lau, A., Toppin, H. A., & Zhang, D. D. (2011). Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proceedings of the National Academy of Sciences, 108*(4), 1433-1438.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med, 49*(11), 1603-1616.

Robledinos-Antón, N., Fernández-Ginés, R., Manda, G., & Cuadrado, A. (2019). Activators and Inhibitors of NRF2: A Review of Their Potential for Clinical Development. *Oxid Med Cell Longev, 2019*, 9372182-9372182.

Rodrigo, R., González, J., & Paoletto, F. (2011). The role of oxidative stress in the pathophysiology of hypertension. *Hypertension Research, 34*(4), 431-440.

Roe, S. M., Prodromou, C., O'Brien, R., Ladbury, J. E., Piper, P. W., & Pearl, L. H. (1999). Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem, 42*(2), 260-266.

Ron, D., & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews Molecular Cell Biology, 8*(7), 519-529.

Roos, D., van Bruggen, R., & Meischl, C. (2003). Oxidative killing of microbes by neutrophils. *Microbes and Infection, 5*(14), 1307-1315.

Ruotsalainen, A. K., Inkala, M., Partanen, M. E., Lappalainen, J. P., Kansanen, E., Mäkinen, P. I., Heinonen, S. E., Laitinen, H. M., Heikkilä, J., Vatanen, T., Hörkkö, S., Yamamoto, M., Ylä-Herttuala, S., Jauhiainen, M., & Levonen, A. L. (2013). The absence of macrophage Nrf2 promotes early atherogenesis. *Cardiovasc Res, 98*(1), 107-115.

Rushmore, T. H., Morton, M. R., & Pickett, C. B. (1991). The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem, 266*(18), 11632-11639.

Rushworth, S. A., Bowles, K. M., & MacEwan, D. J. (2011). High basal nuclear levels of Nrf2 in acute myeloid leukemia reduces sensitivity to proteasome inhibitors. *Cancer Res, 71*(5), 1999-2009.

Rushworth, S. A., MacEwan, D. J., & O'Connell, M. A. (2008). Lipopolysaccharide-induced expression of NAD(P)H:quinone oxidoreductase 1 and heme oxygenase-1 protects against excessive inflammatory responses in human monocytes. *Journal of immunology (Baltimore, Md. : 1950), 181*(10), 6730-6737.

Sachdeva, M. M., Cano, M., & Handa, J. T. (2014). Nrf2 signaling is impaired in the aging RPE given an oxidative insult. *Exp Eye Res, 119*, 111-114.

Saibil, H. (2013). Chaperone machines for protein folding, unfolding and disaggregation. *Nature Reviews Molecular Cell Biology, 14*(10), 630-642.

Saito, R., Suzuki, T., Hiramoto, K., Asami, S., Naganuma, E., Suda, H., Iso, T., Yamamoto, H., Morita, M., Baird, L., Furusawa, Y., Negishi, T., Ichinose, M., & Yamamoto, M. (2016). Characterizations of Three Major Cysteine Sensors of Keap1 in Stress Response. *Mol Cell Biol, 36*(2), 271.

Salvador, N., Aguado, C., Horst, M., & Knecht, E. (2000). Import of a cytosolic protein into lysosomes by chaperone-mediated autophagy depends on its folding state. *J Biol Chem, 275*(35), 27447-27456.

Sanchez, R., Riddle, M., Woo, J., & Momand, J. (2008). Prediction of reversibly oxidized protein cysteine thiols using protein structure properties. *Protein science : a publication of the Protein Society, 17*(3), 473-481.

Santhanam, A. V. R., d'Uscio, L. V., Smith, L. A., & Katusic, Z. S. (2012). Uncoupling of eNOS causes superoxide anion production and impairs NO signaling in the cerebral microvessels of hph-1 mice. *J Neurochem, 122*(6), 1211-1218.

Santra, M., Dill, K. A., & de Graff, A. M. R. (2019). Proteostasis collapse is a driver of cell aging and death. *Proceedings of the National Academy of Sciences, 116*(44), 22173.

Schopf, F. H., Biebl, M. M., & Buchner, J. (2017). The HSP90 chaperone machinery. *Nature Reviews Molecular Cell Biology, 18*(6), 345-360.

Schuman, E. M., & Madison, D. V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science, 254*(5037), 1503-1506.

Schwarz, T. L. (2013). Mitochondrial trafficking in neurons. *Cold Spring Harbor perspectives in biology, 5*(6).

Shibata, T., Kokubu, A., Saito, S., Narisawa-Saito, M., Sasaki, H., Aoyagi, K., Yoshimatsu, Y., Tachimori, Y., Kushima, R., Kiyono, T., & Yamamoto, M. (2011). NRF2 mutation confers malignant potential and resistance to chemoradiation therapy in advanced esophageal squamous cancer. *Neoplasia, 13*(9), 864-873.

Shibata, T., Ohta, T., Tong, K. I., Kokubu, A., Odogawa, R., Tsuta, K., Asamura, H., Yamamoto, M., & Hirohashi, S. (2008). Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci U S A, 105*(36), 13568-13573.

Shimamori, Y., Watanabe, Y., & Fujimoto, Y. (1988). Human placental dipeptidyl aminopeptidase III: hydrolysis of enkephalins and its stimulation by cobaltous ion. *Biochem Med Metab Biol, 40*(3), 305-310.

Siligardi, G., Panaretou, B., Meyer, P., Singh, S., Woolfson, D. N., Piper, P. W., Pearl, L. H., & Prodromou, C. (2002). Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37. *J Biol Chem, 277*(23), 20151-20159.

Singh, A., Misra, V., Thimmulappa, R. K., Lee, H., Ames, S., Hoque, M. O., Herman, J. G., Baylin, S. B., Sidransky, D., Gabrielson, E., Brock, M. V., & Biswal, S. (2006). Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. *PLoS Med, 3*(10), e420.

Smith, M. A., Rottkamp, C. A., Nunomura, A., Raina, A. K., & Perry, G. (2000). Oxidative stress in Alzheimer's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1502*(1), 139-144.

Smith, M. G., & Snyder, M. (2006). Yeast as a model for human disease. *Curr Protoc Hum Genet, Chapter 15*, Unit 15.16.

Soga, S., Shiotsu, Y., Akinaga, S., & Sharma, S. V. (2003). Development of radicicol analogues. *Curr Cancer Drug Targets, 3*(5), 359-369.

Sohal, R. S., & Orr, W. C. (2012). The redox stress hypothesis of aging. *Free Radical Biology and Medicine, 52*(3), 539-555.

Sopko, R., Huang, D., Preston, N., Chua, G., Papp, B., Kafadar, K., Snyder, M., Oliver, S. G., Cyert, M., Hughes, T. R., Boone, C., & Andrews, B. (2006). Mapping pathways and phenotypes by systematic gene overexpression. *Mol Cell, 21*(3), 319-330.

Soto, C. (2003). Unfolding the role of protein misfolding in neurodegenerative diseases. *Nature Reviews Neuroscience, 4*(1), 49-60.

Sreedhar, A. S., Kalmar, E., Csermely, P., & Shen, Y. F. (2004). Hsp90 isoforms: functions, expression and clinical importance. *FEBS Lett, 562*(1-3), 11-15.

Stadtman, E. R. (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem, 62*, 797-821.

Stagljar, I., Korostensky, C., Johnsson, N., & te Heesen, S. (1998). A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. *Proc Natl Acad Sci U S A, 95*(9), 5187-5192.

Stewart, D., Killeen, E., Naquin, R., Alam, S., & Alam, J. (2003). Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. *J Biol Chem, 278*(4), 2396-2402.

Strom, J., Xu, B., Tian, X., & Chen, Q. M. (2016). Nrf2 protects mitochondrial decay by oxidative stress. *Faseb j, 30*(1), 66-80.

Suh, J. H., Shenvi, S. V., Dixon, B. M., Liu, H., Jaiswal, A. K., Liu, R. M., & Hagen, T. M. (2004). Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. *Proc Natl Acad Sci U S A, 101*(10), 3381-3386.

Sun, Z., Wu, T., Zhao, F., Lau, A., Birch, C. M., & Zhang, D. D. (2011). KPNA6 (Importin α7)-Mediated Nuclear Import of Keap1 Represses the Nrf2-Dependent Antioxidant Response. *Mol Cell Biol, 31*(9), 1800-1811.

Suzuki, T., Motohashi, H., & Yamamoto, M. (2013). Toward clinical application of the Keap1-Nrf2 pathway. *Trends Pharmacol Sci, 34*(6), 340-346.

Suzuki, T., Muramatsu, A., Saito, R., Iso, T., Shibata, T., Kuwata, K., Kawaguchi, S.-i., Iwawaki, T., Adachi, S., Suda, H., Morita, M., Uchida, K., Baird, L., & Yamamoto, M. (2019). Molecular Mechanism of Cellular Oxidative Stress Sensing by Keap1. *Cell Reports, 28*(3), 746-758.e744.

Sykiotis, G. P., & Bohmann, D. (2010). Stress-activated cap'n'collar transcription factors in aging and human disease. *Sci Signal, 3*(112), re3.

Szanto, A., Narkar, V., Shen, Q., Uray, I. P., Davies, P. J. A., & Nagy, L. (2004). Retinoid X receptors: X-ploring their (patho)physiological functions. *Cell Death & Differentiation, 11*(2), S126-S143.

Taipale, M., Jarosz, D. F., & Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat Rev Mol Cell Biol, 11*(7), 515-528.

Takahashi, T., Sonobe, M., Menju, T., Nakayama, E., Mino, N., Iwakiri, S., Nagai, S., Sato, K., Miyahara, R., Okubo, K., Hirata, T., Date, H., & Wada, H. (2010). Mutations in Keap1 are

a potential prognostic factor in resected non-small cell lung cancer. *J Surg Oncol, 101*(6), 500-506.

Tapley, T. L., Franzmann, T. M., Chakraborty, S., Jakob, U., & Bardwell, J. C. (2010). Protein refolding by pH-triggered chaperone binding and release. *Proc Natl Acad Sci U S A, 107*(3), 1071-1076.

The Cancer Genome Atlas Research, N. (2012). Comprehensive genomic characterization of squamous cell lung cancers. *Nature, 489*, 519.

Tong, K. I., Katoh, Y., Kusunoki, H., Itoh, K., Tanaka, T., & Yamamoto, M. (2006a). Keap1 Recruits Neh2 through Binding to ETGE and DLG Motifs: Characterization of the Two-Site Molecular Recognition Model. *Mol Cell Biol, 26*(8), 2887-2900.

Tong, K. I., Kobayashi, A., Katsuoka, F., & Yamamoto, M. (2006b). Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol Chem, 387*(10-11), 1311-1320.

Tong, K. I., Padmanabhan, B., Kobayashi, A., Shang, C., Hirotsu, Y., Yokoyama, S., & Yamamoto, M. (2007). Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol Cell Biol, 27*(21), 7511-7521.

Tsutsumi, S., Mollapour, M., Prodromou, C., Lee, C. T., Panaretou, B., Yoshida, S., Mayer, M. P., & Neckers, L. M. (2012). Charged linker sequence modulates eukaryotic heat shock protein 90 (Hsp90) chaperone activity. *Proc Natl Acad Sci U S A, 109*(8), 2937-2942.

Turner, M. D., Nedjai, B., Hurst, T., & Pennington, D. J. (2014). Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1843*(11), 2563-2582.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J Physiol, 552*(Pt 2), 335-344.

Ungvari, Z., Bailey-Downs, L., Gautam, T., Sosnowska, D., Wang, M., Monticone, R. E., Telljohann, R., Pinto, J. T., de Cabo, R., Sonntag, W. E., Lakatta, E. G., & Csiszar, A. (2011a). Age-associated vascular oxidative stress, Nrf2 dysfunction, and NF-{kappa}B activation in the nonhuman primate Macaca mulatta. *J Gerontol A Biol Sci Med Sci, 66*(8), 866-875.

Ungvari, Z., Bailey-Downs, L., Sosnowska, D., Gautam, T., Koncz, P., Losonczy, G., Ballabh, P., de Cabo, R., Sonntag, W. E., & Csiszar, A. (2011b). Vascular oxidative stress in aging: a homeostatic failure due to dysregulation of NRF2-mediated antioxidant response. *Am J Physiol Heart Circ Physiol, 301*(2), H363-372.

Uttara, B., Singh, A. V., Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current neuropharmacology, 7*(1), 65-74.

Valcarcel-Ares, M. N., Gautam, T., Warrington, J. P., Bailey-Downs, L., Sosnowska, D., de Cabo, R., Losonczy, G., Sonntag, W. E., Ungvari, Z., & Csiszar, A. (2012). Disruption of Nrf2 signaling impairs angiogenic capacity of endothelial cells: implications for microvascular aging. *J Gerontol A Biol Sci Med Sci, 67*(8), 821-829.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*(1), 1-40.

Vallance, P., Collier, J., & Moncada, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet, 2*(8670), 997-1000.

van Bergen, L. A. H., Roos, G., & De Proft, F. (2014). From Thiol to Sulfonic Acid: Modeling the Oxidation Pathway of Protein Thiols by Hydrogen Peroxide. *The Journal of Physical Chemistry A, 118*(31), 6078-6084.

Vargas, M. R., Johnson, D. A., Sirkis, D. W., Messing, A., & Johnson, J. A. (2008). Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. *J Neurosci, 28*(50), 13574-13581.

Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., & Talalay, P. (2004). Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci U S A, 101*(7), 2040-2045.

Wang, H., Liu, K., Geng, M., Gao, P., Wu, X., Hai, Y., Li, Y., Li, Y., Luo, L., Hayes, J. D., Wang, X. J., & Tang, X. (2013a). RXRalpha inhibits the NRF2-ARE signaling pathway through a direct interaction with the Neh7 domain of NRF2. *Cancer Res, 73*(10), 3097-3108.

Wang, Q., Li, J., Yang, X., Sun, H., Gao, S., Zhu, H., Wu, J., & Jin, W. (2013b). Nrf2 is associated with the regulation of basal transcription activity of the BRCA1 gene. *Acta Biochimica et Biophysica Sinica, 45*(3), 179-187.

Wang, X. J., Sun, Z., Villeneuve, N. F., Zhang, S., Zhao, F., Li, Y., Chen, W., Yi, X., Zheng, W., Wondrak, G. T., Wong, P. K., & Zhang, D. D. (2008). Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis, 29*(6), 1235-1243.

Warrick, J. M., Chan, H. Y. E., Gray-Board, G. L., Chai, Y., Paulson, H. L., & Bonini, N. M. (1999). Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. *Nat Genet, 23*(4), 425-428.

Wayne, N., & Bolon, D. N. (2007). Dimerization of Hsp90 is required for in vivo function. Design and analysis of monomers and dimers. *J Biol Chem, 282*(48), 35386-35395.

Werner, E. D., Brodsky, J. L., & McCracken, A. A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proc Natl Acad Sci U S A, 93*(24), 13797-13801.

Whitesell, L., & Lindquist, S. L. (2005). HSP90 and the chaperoning of cancer. *Nature Reviews Cancer, 5*, 761.

Wigley, W. C., Fabunmi, R. P., Lee, M. G., Marino, C. R., Muallem, S., DeMartino, G. N., & Thomas, P. J. (1999). Dynamic association of proteasomal machinery with the centrosome. *J Cell Biol, 145*(3), 481-490.

Xia, B., Sheng, Q., Nakanishi, K., Ohashi, A., Wu, J., Christ, N., Liu, X., Jasin, M., Couch, F. J., & Livingston, D. M. (2006). Control of BRCA2 Cellular and Clinical Functions by a Nuclear Partner, PALB2. *Mol Cell, 22*(6), 719-729.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., & Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature, 366*(6456), 701-704.

Xu, C., Li, C. Y., & Kong, A. N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res, 28*(3), 249-268.

Xu, P., Liu, Q., Xie, Y., Shi, X., Li, Y., Peng, M., Guo, H., Sun, R., Li, J., Hong, Y., Liu, X., & Xu, G. (2018). Breast cancer susceptibility protein 1 (BRCA1) rescues neurons from cerebral ischemia/reperfusion injury through NRF2-mediated antioxidant pathway. *Redox Biology, 18*, 158-172.

Yorimitsu, T., & Klionsky, D. J. (2005). Autophagy: molecular machinery for self-eating. *Cell Death & Differentiation, 12*(2), 1542-1552.

Youker, R. T., Walsh, P., Beilharz, T., Lithgow, T., & Brodsky, J. L. (2004). Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast. *Mol Biol Cell, 15*(11), 4787-4797.

Zelko, I. N., Mariani, T. J., & Folz, R. J. (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biology and Medicine, 33*(3), 337-349.

Zhang, D. D., & Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol, 23*(22), 8137-8151.

Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., & Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol Cell Biol, 24*(24), 10941-10953.

Zhang, H., Amick, J., Chakravarti, R., Santarriaga, S., Schlanger, S., McGlone, C., Dare, M., Nix, Jay C., Scaglione, K. M., Stuehr, Dennis J., Misra, S., & Page, Richard C. (2015a). A Bipartite Interaction between Hsp70 and CHIP Regulates Ubiquitination of Chaperoned Client Proteins. *Structure, 23*(3), 472-482.

Zhang, H., Davies, K. J. A., & Forman, H. J. (2015b). Oxidative stress response and Nrf2 signaling in aging. *Free Radic Biol Med, 88*(Pt B), 314-336.

Zhou, L., Zhang, H., Davies, K. J. A., & Forman, H. J. (2018). Aging-related decline in the induction of Nrf2-regulated antioxidant genes in human bronchial epithelial cells. *Redox Biology, 14*, 35-40.

Zhou, S., Ye, W., Duan, X., Zhang, M., & Wang, J. (2013). The noncytotoxic dose of sorafenib sensitizes Bel-7402/5-FU cells to 5-FU by down-regulating 5-FU-induced Nrf2 expression. *Dig Dis Sci, 58*(6), 1615-1626.

Zhu, J., Wang, H., Chen, F., Fu, J., Xu, Y., Hou, Y., Kou, H. H., Zhai, C., Nelson, M. B., Zhang, Q., Andersen, M. E., & Pi, J. (2016). An overview of chemical inhibitors of the Nrf2-ARE signaling pathway and their potential applications in cancer therapy. *Free Radical Biology and Medicine, 99*, 544-556.

Zhuang, C., Wu, Z., Xing, C., & Miao, Z. (2017). Small molecules inhibiting Keap1-Nrf2 protein-protein interactions: a novel approach to activate Nrf2 function. *MedChemComm, 8*(2), 286-294.

Zilfou, J. T., & Lowe, S. W. (2009). Tumor suppressive functions of p53. *Cold Spring Harbor perspectives in biology, 1*(5), a001883-a001883.

Zipper, L. M., & Mulcahy, R. T. (2002). The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J Biol Chem, 277*(39), 36544-36552.

Zondler, L., Miller-Fleming, L., Repici, M., Gonçalves, S., Tenreiro, S., Rosado-Ramos, R., Betzer, C., Straatman, K. R., Jensen, P. H., Giorgini, F., & Outeiro, T. F. (2014). DJ-1 interactions with α-synuclein attenuate aggregation and cellular toxicity in models of Parkinson's disease. *Cell Death & Disease, 5*(7), e1350-e1350.

Chapter 2

2 A novel yeast model detects Nrf2 and Keap1 interactions with Hsp90

Nrf2 is the master transcriptional regulator of cellular responses against oxidative stress. It is chiefly regulated by Keap1, a substrate adaptor protein that mediates Nrf2 degradation. Nrf2 activity is also influenced by many other protein interactions that provide Keap1-independent regulation. To study Nrf2 regulation, we establish and characterize yeast models expressing human Nrf2, Keap1, and other proteins that interact with and regulate Nrf2. Yeast models have been well-established as powerful tools to study protein function and genetic and physical protein-protein interactions. In this work, we recapitulate previously described Nrf2 interactions in yeast and discover that Nrf2 interacts with the molecular chaperone Hsp90. Our work establishes yeast as a useful tool to study Nrf2 and provides novel insight into the crosstalk between the antioxidant response and the heat shock response.

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2.1 Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is the master transcriptional regulator of cellular responses against oxidative stress (Moi et al., 1994). Nrf2 is negatively regulated by Kelch-like ECH-associated protein (Keap1), a substrate adaptor protein that binds to Nrf2 in the cytoplasm to promote Nrf2 ubiquitination via the Cullin 3 (Cul3) E3 ubiquitin ligase for proteasomal degradation under basal conditions (Itoh et al., 1999; Itoh et al., 2003; McMahon et al., 2003; Nguyen et al., 2003; Kobayashi et al., 2004; Kobayashi et al., 2006). Under oxidative stress, specific stress-sensing cysteine residues in Keap1 are modified (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003; Wakabayashi et al., 2004), leading to a conformational change that impairs the interaction between Keap1 and Nrf2, thereby preventing Nrf2 ubiquitination and degradation and stabilizing Nrf2 for accumulation, nuclear translocation, and the induction of cytoprotective antioxidant genes (Itoh et al., 1999; Itoh et al., 2003; McMahon et al., 2003; Kobayashi et al., 2006).

Stability, and therefore the activity of Nrf2, is tightly regulated by two binding events to Keap1. Keap1 first recruits Nrf2 by binding to the high-affinity ETGE motif within the Neh2 domain of Nrf2. Subsequent binding at the low-affinity DLG motif within Neh2 locks Nrf2 in place by orienting the lysine residues within Neh2 in the correct position for ubiquitination (Tong et al., 2006; Tong et al., 2007). This two-site binding model has been widely accepted as the primary mechanism of Keap1-mediated Nrf2 regulation. Mutations that disrupt the Keap1-Nrf2 interaction alter Nrf2 regulation and contribute to the pathogenesis of many human diseases (Padmanabhan et al., 2006; Shibata et al., 2008). For example, gain-of-function mutations within the Keap1-binding domain of Nrf2, specifically within the DLG motif (e.g., L30F) and ETGE motif (e.g., T80R) impair its recognition by Keap1-Cul3, leading to the dysregulation and subsequent hyperactivation of Nrf2 in lung cancer (Shibata et al., 2008). In addition to Keap1, Nrf2 regulation is greatly dependent on its interactions with many other proteins.

An interesting alternate mechanism of Nrf2 regulation first proposed by Zhang et al. identified p21 (also known as $p21^{WAF1/Cip1}$) as a regulator of Nrf2 transcriptional activity (Chen et al., 2009). p21 is a cyclin-dependent kinase inhibitor with well-established roles in p53-regulated tumour suppression, including cell cycle control, DNA replication and repair, and apoptosis (Xiong et al.,

1993; Gartel & Radhakrishnan, 2005; Abbas & Dutta, 2009). The authors found that ablation of p21 results in increased cellular levels of reactive oxygen species (ROS). Reciprocal immunoprecipitation assays and pull-down experiments for p21 and Nrf2 suggest that p21 directly interacts with Nrf2 by competing with Keap1 for binding, indicating that p21 binding to Nrf2 prevents Keap1-directed Nrf2 degradation (Chen et al., 2009). Several studies have linked the overexpression of cytoplasmic p21 to decreased responsiveness to chemotherapy and radiotherapy (Liu et al., 2003) and poor prognosis in numerous cancers (Baretton et al., 1999; Bae et al., 2001; Cheung et al., 2001). The cellular and molecular mechanisms of the interaction of p21 with Nrf2 remain unclear.

Other key proteins in the Nrf2 interactome that are investigated in this study include β-transducin repeat-containing protein (βTrCP), which acts as a substrate receptor for the Skp1-Cul1-Rbx1/Roc1 ubiquitin ligase complex involved in Keap1-independent Nrf2 degradation (Rada et al., 2011; Chowdhry et al., 2013); the Cullin 3 (Cul3) ubiquitin E3 ligase, which binds Keap1 in the cytosol and upon Keap1-Nrf2 binding, polyubiquitinates Nrf2 for degradation by the 26S proteasome (Kobayashi et al., 2004; Zhang et al., 2004); and the underexplored prothymosin alpha (ProTα/PTMA), which is thought to inhibit the Keap1-Nrf2 complex by competing with Nrf2 for Keap1 binding (Karapetian et al., 2005; Khan et al., 2013).

In this work, we establish a novel approach to studying the interactions of human Nrf2 in the budding yeast *Saccharomyces cerevisiae*. Yeast and human cells share fundamental commonalities in many conserved cellular processes, making yeast a powerful model system for studying the mechanisms of important cellular processes, including those that underlie protein regulation and human disease (Hartwell, 2005; Smith & Snyder, 2006; Tenreiro & Outeiro, 2010; Di Gregorio & Duennwald, 2018). Yeast is a formidable model to identify and characterize *genetic* interactions, which can be defined as the phenomenon by which the phenotypic effects of the function of one gene modifies the phenotypic effects of another gene or genes (Mani et al., 2008). Yeast can also be used to study *physical* protein-protein interactions (PPIs) by employing the yeast two-hybrid system (Fields & Song, 1989) or split-ubiquitin system (Johnsson & Varshavsky, 1994) to identify and characterize such physical interactions that occur through specific contact and molecular docking (De Las Rivas & Fontanillo, 2010). Yeast does not express any close Nrf2 homologue, which allows us to minimize interference with endogenous Nrf2 regulation as it occurs

in mammalian cells. This feature makes yeast an optimal living test tube for studying Nrf2 interactions. Our yeast model of human Nrf2 confirms previously established genetic and physical Nrf2 interactions and allowed us to characterize a previously unexplored interaction between Nrf2 with the molecular chaperone Hsp90.

2.2 Results

2.2.1 Expression of human Nrf2 and associated proteins in yeast

Yeast growth assays were used to assess the relative growth and toxicity of select human proteins within the Nrf2 interactome (**Figure S2.1**) expressed in yeast to determine genetic interactions with Nrf2. Human Nrf2 expressed in yeast leads to toxicity, defined as an impaired growth phenotype on growth media compared to the empty vector control (**Figure 2.1A, left**). Relative growth on solid media was quantified to show statistical significance, performed as described before (Petropavlovskiy et al., 2020) (**Figure 2.1A, right**). Means derived from five biological replicates were used during analysis. These results were also confirmed quantitatively by assessing the growth rate of yeast cells grown in liquid culture (**Figure 2.1B**). Nrf2 toxicity in yeast is likely attributed to cellular quiescence but not cell death, as determined by a propidium iodide (PI) assay which showed no cell death in yeast cells expressing Nrf2 compared to the boiled positive control (**Figure S2.2**). Protein expression of Nrf2 in yeast is confirmed by western blot analysis (**Figure 2.1C**). Fluorescence microscopy also confirmed protein expression through the visualization of a YFP (yellow fluorescent protein) or DsRed (Discosoma red fluorescent protein) tag fused to the carboxy-terminus of Nrf2, which shows that Nrf2 is diffusely localized in the yeast cytoplasm and nucleus (**Figure S2.3A**). In ensuing studies, we exploit Nrf2 toxicity as a tractable phenotype for our genetic interaction studies, as done previously in well-established yeast models expressing other human proteins (Hartwell, 2005; Tenreiro & Outeiro, 2010; Di Gregorio & Duennwald, 2018).

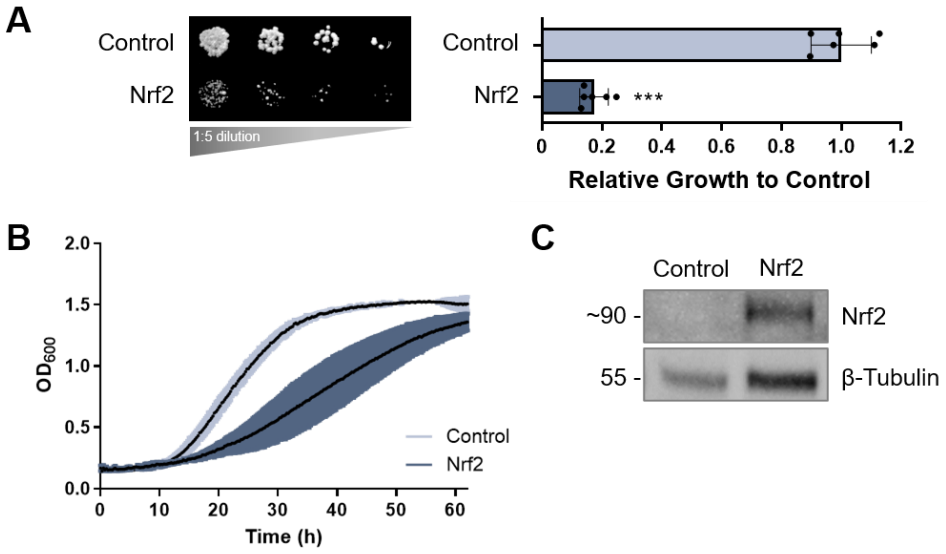

Figure 2.1: Expression of human Nrf2 in yeast. (A) Growth assay of yeast cells expressing human Nrf2 (left). Relative growth is quantified to the right of the image. Means derived from five biological replicates were used during analysis. Means were analyzed using the student's T-test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(B)** Growth curve of yeast cells expressing Nrf2 grown in liquid culture. Means derived from three biological replicates were obtained. Data are expressed as mean ± SD. **(C)** Nrf2 protein expression in yeast documented by western blot analysis, with β-tubulin serving as the internal loading control.

To assess which regions in Nrf2 give rise to the protein's toxic phenotype in yeast, three fragments of Nrf2 were examined (**Figure 2.2A**): (1) the N-terminal (NH_2) fragment, consisting of the Neh2, Neh4, Neh5, and Neh7 domains; (2) the ΔNeh2/3 variant, with deletions of the Neh2 and Neh3 domains; and (3) the C-terminal (COOH) fragment, containing the Neh6, Neh1, and Neh3 domains. Like wild-type Nrf2, the NH_2 and COOH fragments are toxic in yeast while the ΔNeh2/3 variant is not (**Figure 2.2B**). Fluorescence microscopy in shows the diffuse expression of Nrf2 ΔNeh2/3-YFP but greater localization to fluorescent foci for Nrf2 NH_2-YFP and Nrf2 COOH-YFP, which might indicate protein inclusions, particularly for the NH_2 fragment (**Figure 2.2C**). (Note that YFP-tagged yeast cells were imaged with a green fluorescent protein (GFP) filter due to the unavailability of a YFP filter at the time of imaging). The NH_2 fragment contains the crucial

Neh2 (Keap1-binding) domain of Nrf2 which has been characterized as intrinsically disordered (Tong et al., 2006). Intrinsically disordered proteins or regions are more prone to misfolding under certain conditions (Uversky, 2011); thus, the Neh2 domain is likely a plausible main driver of this observed misfolding and toxicity in living cells. However, despite its intrinsically disordered nature and increased propensity to misfold, the Neh2 domain remains a key functional domain within Nrf2.

In addition, GFP-tagged protein constructs for mammalian expression in HeLa cells recapitulates the fluorescence microscopy results observed in yeast, illustrating the formation of protein inclusions for Nrf2 NH_2-GFP (**Figure 2.2D**). Upon analyzing the cell viability of wild-type Nrf2 and its fragmented variants in HeLa cells, we find that overexpression of wild-type Nrf2 is not toxic to HeLa cells, but instead promotes increased cell viability (determined by the quantification of ATP levels which indicates the presence of metabolically active cells (Crouch et al., 1993)) compared to the untreated control; however, cell viability was impaired with the expression of Nrf2 NH_2 (**Figure 2.2E**). Experiments in the HEK293 cell line reproduced the results observed in HeLa cells (**Figure S2.4**).

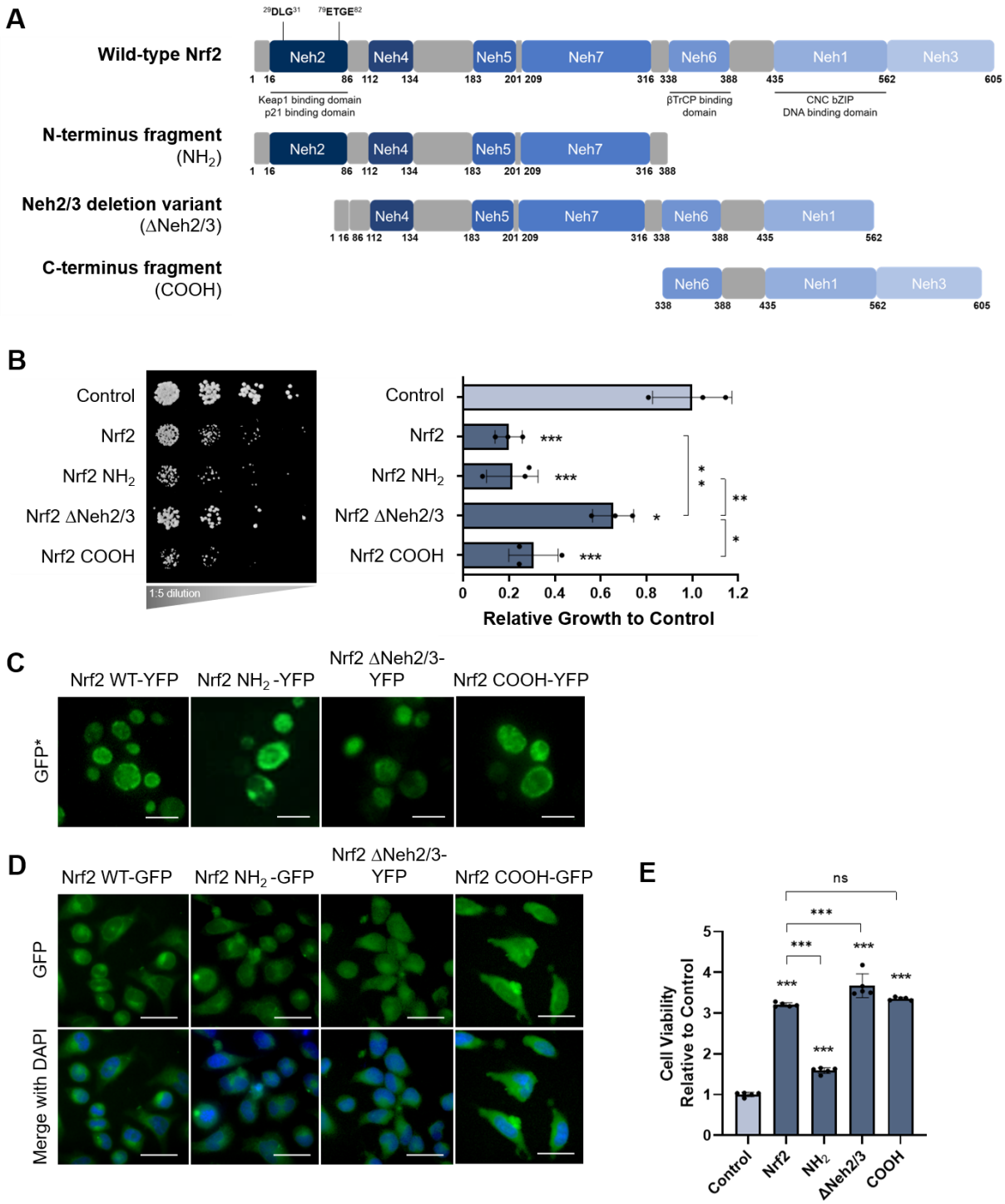

Figure 2.2: Nrf2 fragments expressed in yeast. (A) Schematic representation of the domains within full-length wild-type human Nrf2 and the fragmented variants of Nrf2: N-terminal fragment (NH_2), the Neh2/3 deletion variant (ΔNeh2/3), and the C-terminal fragment (COOH). **(B)** Growth assay of yeast cells expressing the three Nrf2 fragments. **(C)** Fluorescence microscopy of yeast

cells expressing the YFP-tagged Nrf2 fragments. Scale bars correspond to 10 μm. **(D)** Fluorescence microscopy of HeLa cells expressing the Nrf2 GFP-tagged fragments (top), merged with DAPI nuclear staining (bottom). Scale bars correspond to 25 μm. **(E)** Relative cell viability of HeLa cells transfected with wild-type Nrf2 and its fragments. (B, E) Means derived from a minimum of three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

To assess genetic Nrf2 interactions, we co-transformed yeast with well-established Nrf2-interacting proteins described in the literature, including Keap1 (Itoh et al., 1999), p21 (Chen et al., 2009), and βTrCP (Rada et al., 2011; Chowdhry et al., 2013), as well as the Keap1-interacting Cul3 (Kobayashi et al., 2004; Zhang et al., 2004) and ProTα (Karapetian et al., 2005) to serve as negative controls. As previously observed, human Nrf2 is toxic in yeast, but human Keap1 expression alone is only mildly toxic. When Keap1 is co-expressed with Nrf2, improved growth is observed relative to the expression of Nrf2 alone (**Figure 2.3A**), i.e., the expression of Keap1 results in a partial rescue of Nrf2 toxicity, suggesting a genetic interaction. This is also observed in liquid growth assays (**Figure S2.5**). It is important to note that in yeast expression studies, co-expression of a mildly toxic protein with a toxic protein adds toxicity in an additive manner if there is no interaction; however, if there is a rescue in toxicity, as observed for Keap1 co-expressed with Nrf2, then it is indicative of a genetic interaction. Thus, our yeast model confirms the well-established genetic interaction between Keap1 and Nrf2 observed in mammalian cells.

When human p21 is co-expressed with Nrf2, there is no significant rescue of Nrf2 toxicity, indicating no *genetic* p21-Nrf2 interaction in yeast (**Figure 2.3B**) although a *physical* protein-protein interaction may still exist (to be discussed). Co-expression with human βTrCP exacerbates Nrf2 toxicity, indicating a βTrCP-Nrf2 genetic interaction (**Figure 2.3C**) as suggested before (Rada et al., 2011; Chowdhry et al., 2013). Of note, this may be a result of synthetic toxicity and thus a limitation of the yeast model because GSK3 is not present in yeast to phosphorylate Nrf2 for modulation of βTrCP activity (Rada et al., 2011; Chowdhry et al., 2013). Co-expression of Nrf2 with human Cul3 does not modify the Nrf2 phenotype (**Figure 2.3D**), nor does co-expression with human ProTα (**Figure 2.3E**). This is consistent with literature describing both Cul3 and

ProTα as binding partners of Keap1 (Karapetian et al., 2005; Khan et al., 2013). Protein expression in yeast is confirmed by western blot analysis for all proteins of interest (**Figure 2.3F**) except for ProTα where no yeast-compatible commercially available antibody could be found. Fluorescence microscopy with YFP-tagged constructs also confirms protein expression in yeast for all proteins of interest, both alone and co-expressed with Nrf2 (**Figure S2.3B**).

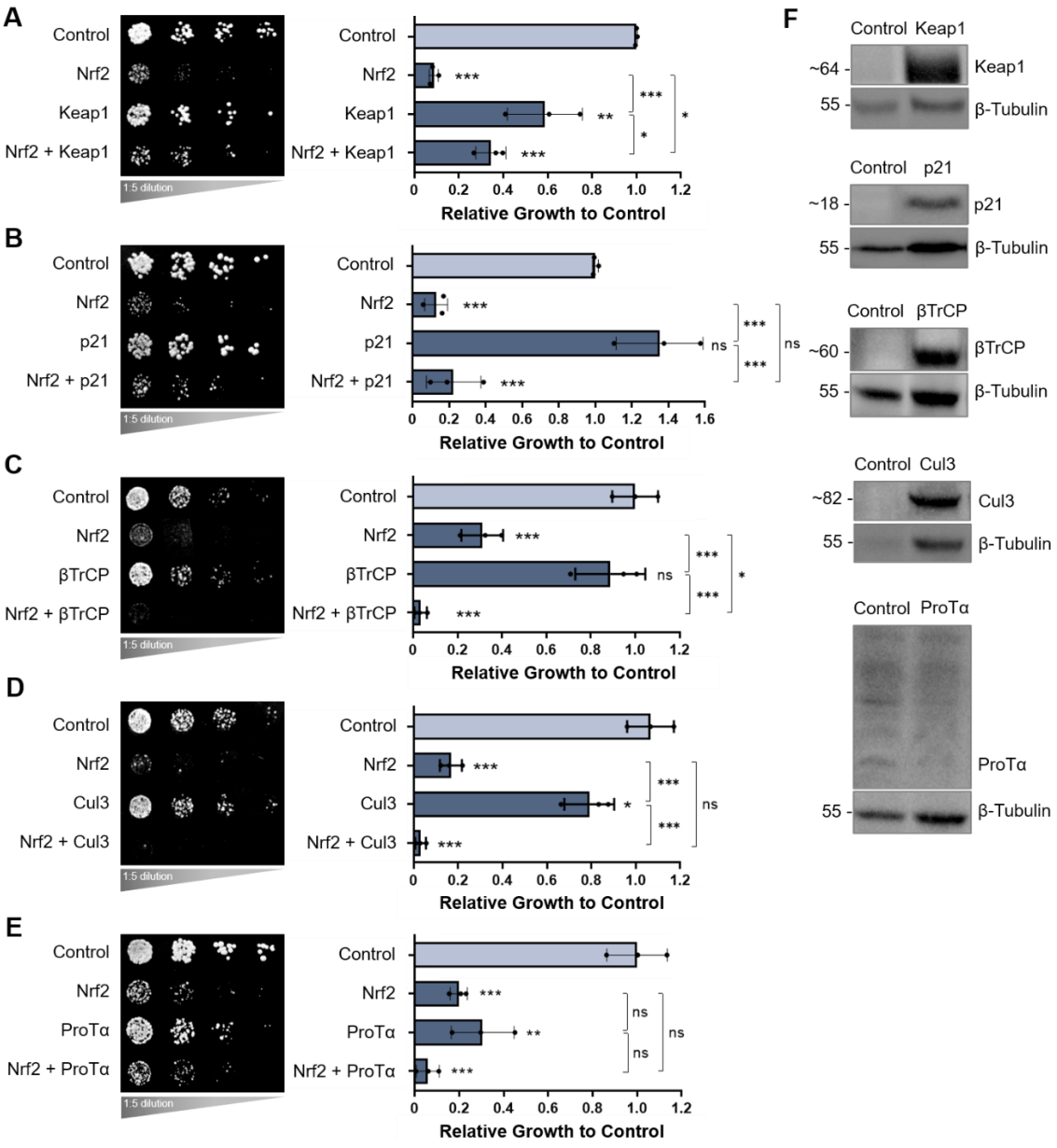

Figure 2.3: Co-expression of Nrf2 with other Nrf2-associated proteins. Growth assays of yeast cells co-expressing human Nrf2 along with the following human proteins: **(A)** Keap1, **(B)** p21, **(C)** βTrCP, **(D)** Cul3, and **(E)** ProTα. All growth assays are quantified as shown on the right with means derived from three biological replicates. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(F)** Keap1, p21, βTrCP, and Cul3 expression are shown by western blot analysis, with β-tubulin serving as the internal loading control. ProTα could not be reliably detected (see text for details).

In addition to wild-type Nrf2, we examined two variants of Nrf2 with mutations in the Neh2, Keap1-binding domain of Nrf2: an L30F mutation in the DLG motif, and a T80R mutation in the ETGE motif. Like wild-type Nrf2, Nrf2 L30F and T80R were toxic in yeast. (**Figure 2.4A**). Protein expression was confirmed by western blot analysis (**Figure 2.4B**) and fluorescence microscopy (**Figure S2.3C**), which showed no major differences between wild-type and L30F and T80R Nrf2 steady-state protein levels and subcellular localization in yeast. We then assessed genetic interactions of the L30F and T80R variants. When Keap1 is co-expressed with Nrf2 L30F or T80R, Keap1's ability to rescue Nrf2 toxicity (observed in **Figure 2.3A**) is impaired (**Figure 2.4C**). As observed for wild-type Nrf2, co-expression of p21 with the L30F and T80R variants did not affect toxicity (**Figure S2.6**). Collectively, these experiments demonstrate how simple growth assays of yeast cells expressing wild-type Nrf2, fragmented and mutated variants of Nrf2, and Nrf2-associated proteins allow the assessment of genetic interactions, such as that with Keap1 and βTrCP, that regulate Nrf2 in mammalian cells.

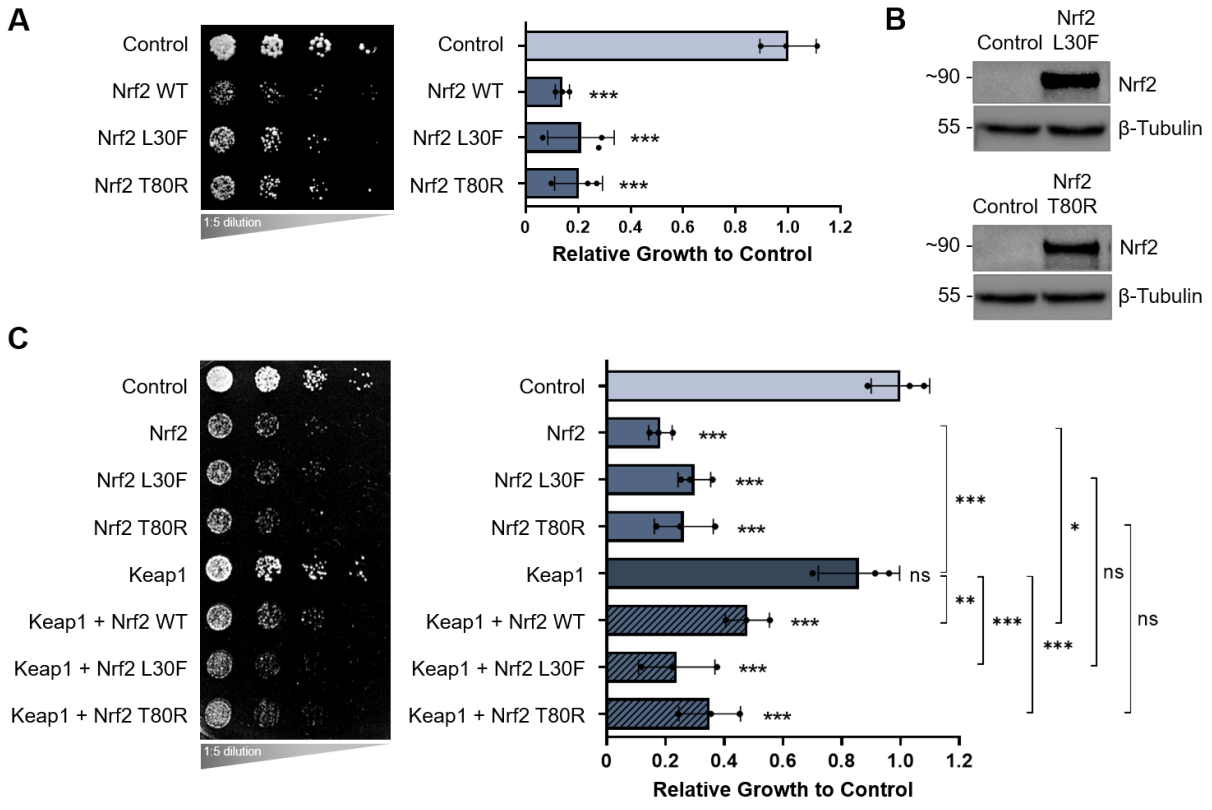

Figure 2.4: Nrf2 mutant variants expressed in yeast. (A) Growth assays of yeast cells expressing wild-type human Nrf2 and its mutant variants, L30F and T80R. **(B)** Expression of Nrf2 L30F and Nrf2 T80R in yeast documented by western blot analysis, with β-tubulin serving as the internal loading control. **(C)** Growth assays of yeast cells expressing wild-type Nrf2 and its mutant variants co-expressed with Keap1. (A, C) Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

2.2.2 The split-ubiquitin system detects protein-protein interactions of Nrf2

We next employed the split-ubiquitin system (Johnsson & Varshavsky, 1994; Stagljar et al., 1998; Müller & Johnsson, 2008), outlined schematically in **Figure 2.5A**, to assess the *physical* protein-protein interactions of Nrf2 (i.e., interactions between a 'bait' protein with a 'prey' protein). We engineered Nrf2, Keap1, and other proteins of interest fused to Nub, the amino-terminal half of a

full-length 'pseudo-ubiquitin' molecule, and to Cub-R-*URA3* (CRU), the carboxy-terminal (Cub) half of pseudo-ubiquitin fused to a *URA3* reporter containing a degron (R) for rapid degradation by cellular ubiquitin specialized proteases (Ubps). These Nub and CRU fusions were co-expressed in yeast. Nrf2 interactions can be detected by growth on the following selective growth media: (1) media lacking uracil (uracil$^-$), which selects for the presence of the *URA3* reporter—an *interaction* between the bait and prey results in degradation of the *URA3* reporter and loss of uracil synthesis, detected by impaired growth on uracil$^-$ media; (2) media containing 5-fluoroorotic (5FOA), which selects for the absence of the *URA3* reporter and associated loss of uracil synthesis (5FOA reacts with uracil to produced a toxic metabolite, 5-fluorouracil, that impairs yeast growth)—if the bait and prey *interact*, then the *URA3* reporter is degraded and growth is observed on 5FOA media (**Figure 2.5A**). Different mutant alleles of Nub—NuI, NuA, and NuG (in order from highest to lowest affinity for Cub)—are used to differentiate strong interactions (e.g., in stable complexes) from weaker ones (e.g., transient interactions), where NuI detects stable interactions and NuG only detects transient interactions (Stagljar et al., 1998).

Figure 2.5B confirms that the CRU constructs expressed in yeast grow on agar plates lacking uracil, which is a prerequisite for the split-ubiquitin assay to work. Testing the established Keap1-Nrf2 interaction utilizing the Nrf2-CRU + Keap1 NuI/NuA/NuG in yeast demonstrated no growth on uracil$^-$ plates but growth on 5FOA plates, which confirms the *physical* PPI between Keap1 and Nrf2 (**Figure 2.5C**, 2nd row). This was also observed for Nrf2-CRU + p21-NuI/NuA/NuG, indicating a *physical* interaction between p21 and Nrf2 (**Figure 2.5C**, 3rd row). Phosphoglycerate kinase 1 (Pgk1), an enzyme involved in gluconeogenesis with no reported Nrf2 interactions, was used as a negative specificity control and indeed did not interact with Nrf2 in the split-ubiquitin assay (**Figure 2.5C**, 1st row). Note that the low expression of the Nrf2-split-ubiquitin fusion proteins did not result in cellular toxicity compared to the high expression plasmids used in our genetic interaction studies. We then assessed PPIs of all combinations of Nrf2, Keap1, p21, βTrCP, Cul3, and ProTα (data not shown), summarized in **Figure 2.5D**, with '+' indicating an interaction and '−' indicating no interaction, which confirms the physical interactions between Nrf2 with Keap1, p21, and βTrCP.

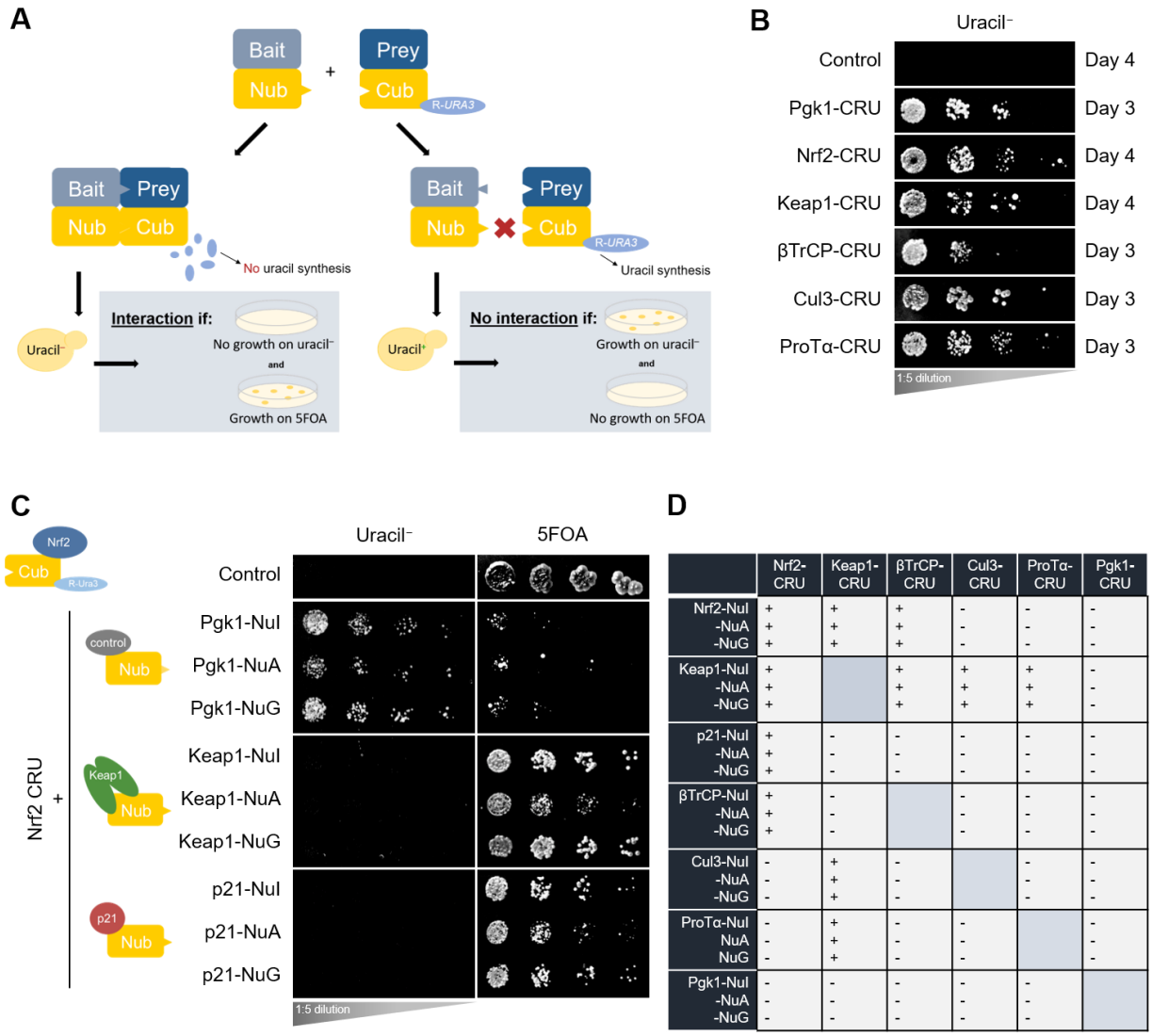

Figure 2.5: The yeast split-ubiquitin system for studying physical Nrf2 protein-protein interactions. (A) Schematic representation of the split-ubiquitin system. If the bait and prey proteins interact, then the following growth conditions are met: no growth on uracil⁻ and growth on 5FOA. **(B)** Confirmation of the CRU constructs showing that yeast cells expressing the CRU fusions grow on uracil⁻ plates. **(C)** Split-ubiquitin assays for the Nrf2-CRU + Keap1-Nub and Nrf2-CRU + p21-Nub combinations. Pgk1 served as a negative specificity control. Three biological replicates were performed. **(D)** Summary of tested protein-protein interactions within the Nrf2-interactome as detected by the split-ubiquitin assay; '+' indicates an interaction and '-' indicates no interaction.

2.2.3 Interaction between Nrf2 and Keap1 with Hsp90

Since Nrf2 contains disordered regions (Itoh et al., 2004) and certain fragmented variants of Nrf2 form inclusions in yeast (**Figure 2.2C**) and mammalian cells (**Figure 2.2D**), we used the split-ubiquitin assay to probe for interactions with molecular chaperones which help to fold, stabilize, and degrade disordered and misfolded proteins (Frydman, 2001; Bukau et al., 2006). Interestingly, our split-ubiquitin data indicate that both Nrf2 and Keap1 *physically* interact with the molecular chaperone heat shock protein 90 (Hsp90) (**Figure 2.6A** and **Figure 2.6B**, respectively). Furthermore, we had previously shown that the co-expression of Keap1 with Nrf2 partially rescued Nrf2 toxicity (**Figure 2.3A**); however, treatment with 2.5 μM radicicol, a small molecule inhibitor of Hsp90, impaired the ability of Keap1 to rescue Nrf2 toxicity (**Figure 2.6C**), further suggesting a *genetic* interaction between Keap1 and Nrf2 with Hsp90. Of note, the interaction between Keap1 and Hsp90 has previously been described (Taipale et al., 2012; Prince et al., 2015) but is not well explored. The interaction between Nrf2 and Hsp90, to our knowledge, has not been previously described.

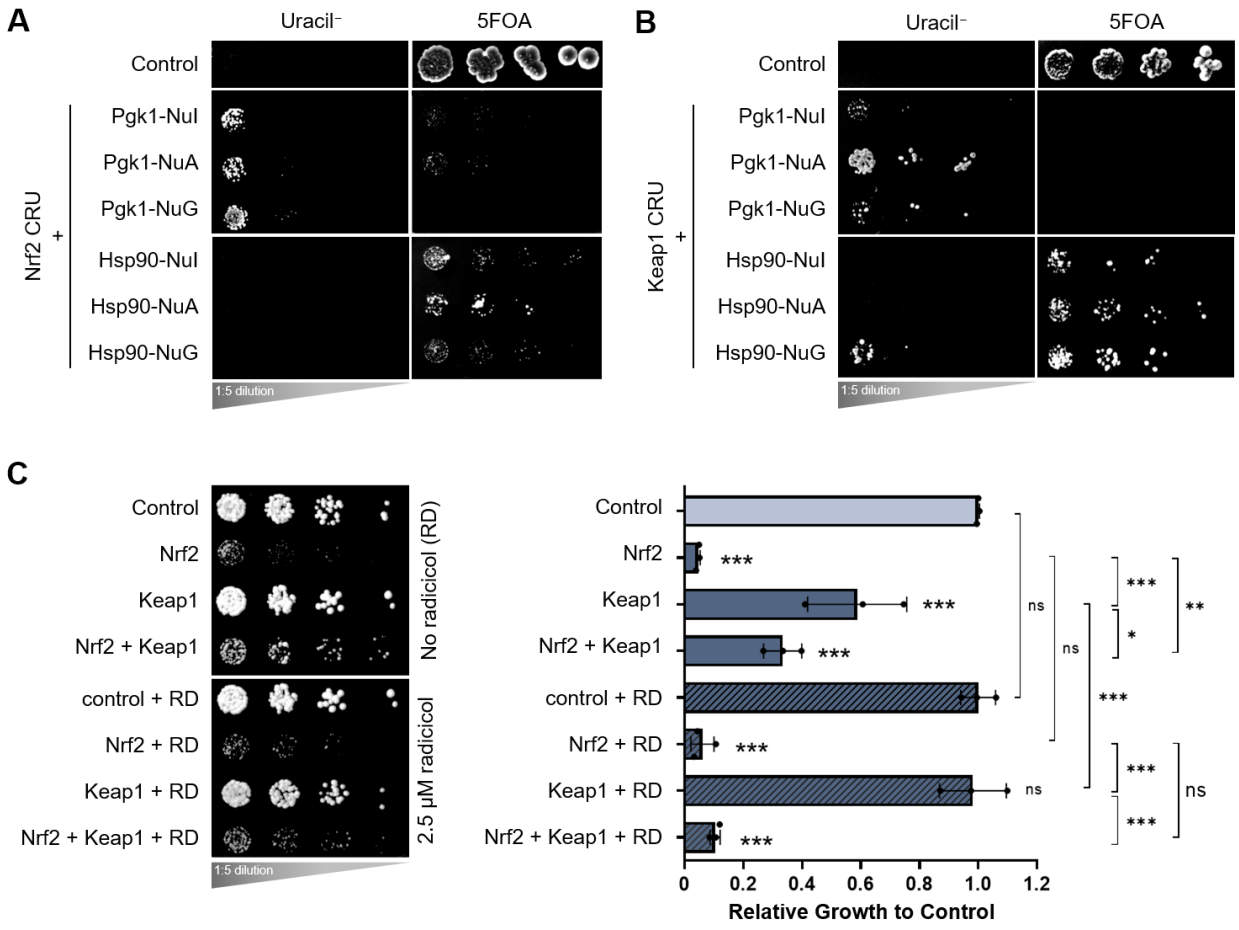

Figure 2.6: Interaction between Nrf2 and Keap1 with Hsp90. (A-B) Split-ubiquitin assays of yeast cells co-expressing the indicated Nub and CRU fusion proteins for (A) Nrf2 and (B) Keap1, in combination with Hsp90. **(C)** Growth assay of yeast cells co-expressing Nrf2 and Keap1 in the absence of radicicol (DMSO solvent control) and presence of 2.5 µM radicicol. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

To further investigate this interesting link between Nrf2 and Keap1 with Hsp90, Nrf2 and Keap1 were expressed in deletions strains for yeast Hsp90 (Δ*hsp82* and Δ*hsc82*) and two yeast Hsp90 co-chaperones (Δ*aha1* and Δ*sti1*). Deletion of *HSP82*, *HSC82*, or co-chaperone *STI1* exacerbate Nrf2 toxicity compared to wild-type cells, whereas the *AHA1* deletion had no effect (**Figure 2.7A**). These deletion strains did not significantly alter Keap1 expression (**Figure S2.7**). Nrf2 toxicity was not, however, altered in cells overexpressing Hsp90 and its co-chaperones (**Figure 2.7B**). Western blot analysis confirms the expression of Hsp82 and Hsc82 in yeast cells for our overexpression studies (**Figure 2.7C**).

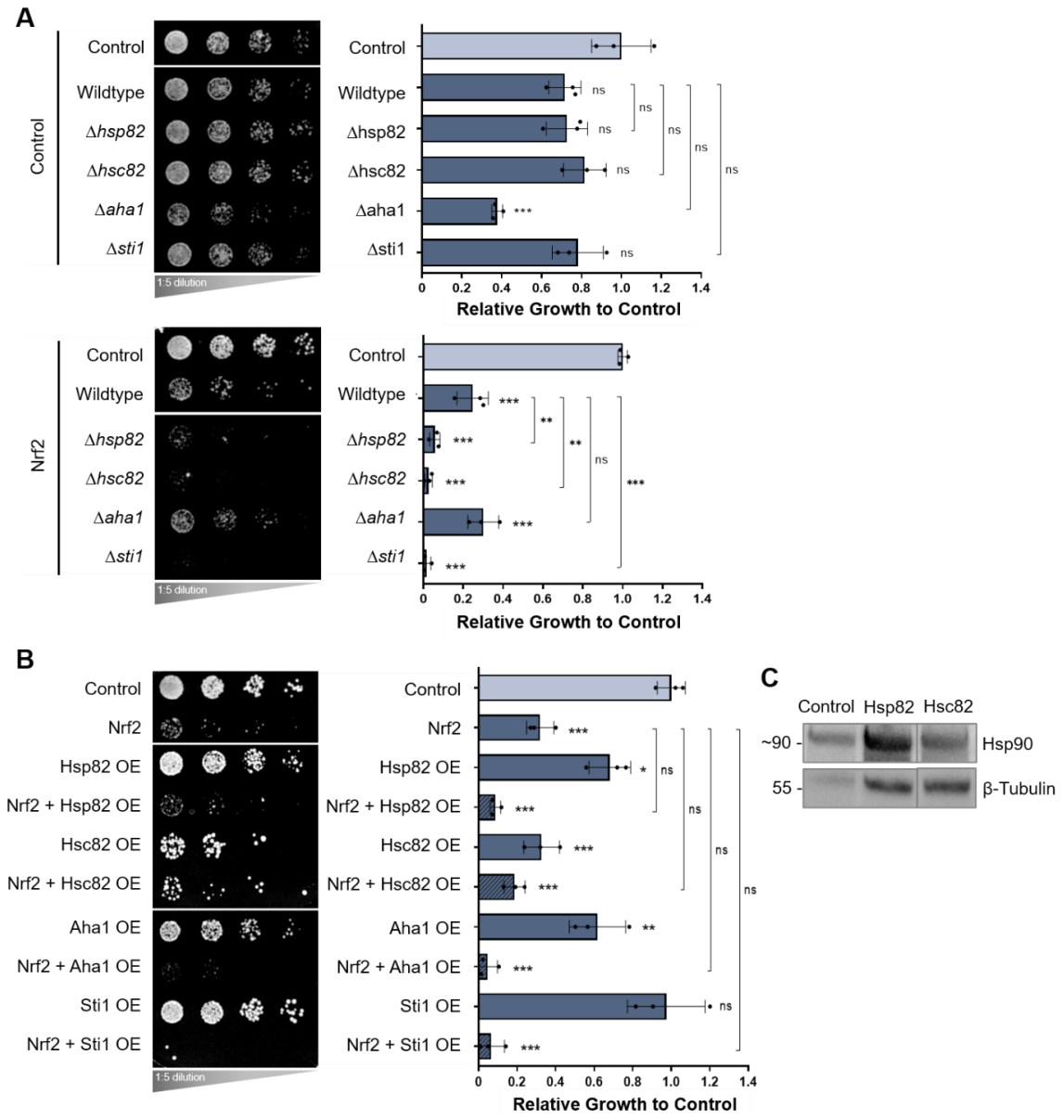

Figure 2.7: Expression of Nrf2 in yeast Hsp90 and co-chaperone deletion strains and overexpression constructs. (A) Growth assays of yeast cells expressing Nrf2 in deletions strains for yeast Hsp90 (Δ*hsp82* and Δ*hsc82*) and two yeast Hsp90 co-chaperones (Δ*aha1* and Δ*sti1*). **(B)** Growth assays of yeast cells expressing Nrf2 and overexpressing (OE) Hsp90 or its co-chaperones. (A-B) Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(C)** Hsp82 and Hsc82 protein expression in yeast is shown by western blot analysis, with β-tubulin serving as the internal loading control.

Finally, we begin to assess the interaction between Hsp90 and Nrf2 and Keap1 in mammalian cells. **Figure 2.8A** shows both endogenous and transfected Nrf2-GFP together with endogenous Hsp90 detected by immunofluorescence microscopy in HeLa cells. Similarly, **Figure 2.8B** shows endogenous and transfected Keap1-GFP with endogenous Hsp90. Co-localization with Hsp90 is observed for both Nrf2 and Keap1. We then assessed the effect of Hsp90 inhibition on cells overexpressing Nrf2 and Keap1. GFP-tagged Nrf2- and Keap1-transfected HeLa cells were treated with 15 μM of the Hsp90 inhibitor radicicol for 6 h, which induced the formation of Nrf2 and Keap1 inclusions in the nucleus (**Figure 2.8C**). Radicicol treatment also increased the cell viability (detected by increased ATP levels (Crouch et al., 1993)) of cells expressing Nrf2 and Keap1 when compared to untreated control cells (**Figure 2.8D**).

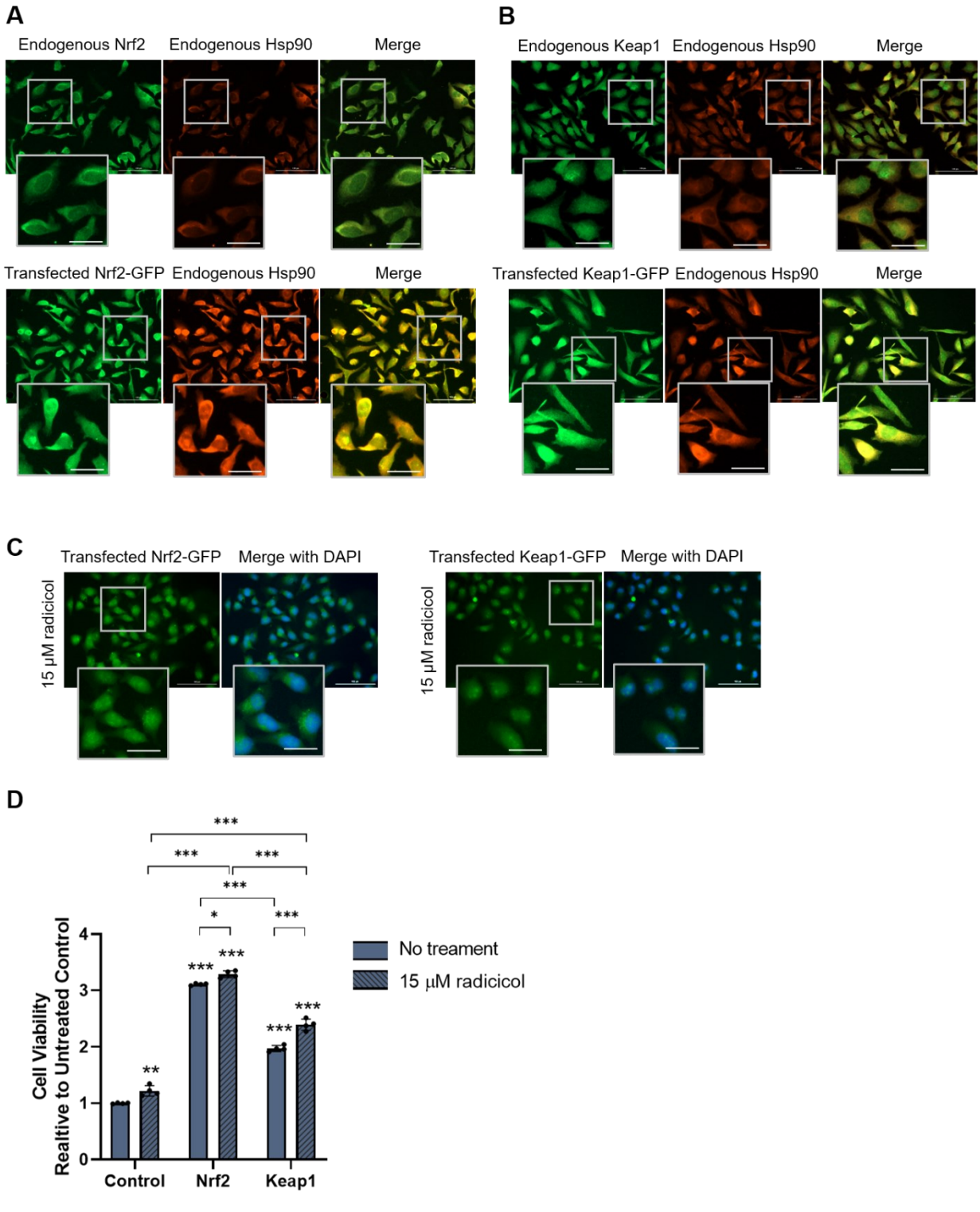
A
Endogenous Nrf2
Endogenous Hsp90
Merge
Transfected Nrf2-GFP
Endogenous Hsp90
Merge
B
Endogenous Keap1
Endogenous Hsp90
Merge
Transfected Keap1-GFP
Endogenous Hsp90
Merge
C
Transfected Nrf2-GFP
Merge with DAPI
15 μM radicicol
Transfected Keap1-GFP
Merge with DAPI
15 μM radicicol
D
Cell Viability
Realtive to Untreated Control
Control
Nrf2
Keap1
No treament
15 μM radicicol

Figure 2.8: Nrf2 and Keap1 expressed in HeLa cells with Hsp90 detection. (A) Immunofluorescence microscopy for endogenous Nrf2 and Hsp90 (top) and fluorescence microscopy for transfected Nrf2-GFP and endogenous Hsp90 detected by immunofluorescence (bottom). **(B)** Immunofluorescence microscopy for endogenous Keap1 and Hsp90 (top) and fluorescence microscopy for transfected Keap1-GFP and endogenous Hsp90 detected by immunofluorescence (bottom). **(C)** Fluorescence microscopy of HeLa cells transfected with Nrf2-GFP and Keap1-GFP treated with 15 μM radicicol for 6 h. (A-C) All unlabeled scale bars correspond to 25 μm. **(D)** Viability assays for HeLa cells expressing Nrf2-GFP or Keap1-GFP treated with 15 μM radicicol. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

2.3 Discussion

We establish the yeast *Saccharomyces cerevisiae* as a living test tube to identify and characterize genetic and physical Nrf2 interactions. Importantly for our studies, yeast does not express any close Nrf2 homologue which allows for the advantage of minimizing interference with endogenous Nrf2 regulation as it occurs in mammalian cells. Since yeast genes do not contain the antioxidant response element (ARE) within their promotor which is required for Nrf2-mediated transcriptional activation (Itoh et al., 1997), Nrf2 is likely non-functional in yeast, allowing for protein interactions to be examined in isolation. We note that this is also a limitation in that endogenous factors that may have otherwise influenced the interaction or mechanism are not present. Nrf2 contains long disordered regions (Tong et al., 2006) and thus may misfold easily in yeast, where many of its interacting proteins are not expressed and it is not degraded. This misfolding may be the cause of Nrf2 toxicity in yeast, as documented for other misfolded proteins before (Outeiro & Lindquist, 2003; Duennwald, 2011; Fushimi et al., 2011). Also, yeast does not endogenously express proteins that, under normal conditions, lead to the rapid degradation of Nrf2 in mammalian cells, leading to high expression levels of Nrf2 in yeast, which can contribute to its misfolding and the ensuing toxicity. Here, we exploit Nrf2 toxicity in yeast as a tractable phenotype to study genetic Nrf2 interactions.

We examined Nrf2 fragments to assess which region(s) of the protein contribute to its toxicity and found that the N-terminal NH_2 fragment was the most toxic in both yeast and mammalian cells. The NH_2 fragment also formed protein inclusions in both yeast and mammalian cells. The Neh2 domain within the N-terminus of Nrf2 has been structurally characterized and is highly intrinsically disordered with little secondary structure (Tong et al., 2006) and thus, a plausible main driver of this misfolding process and toxicity in living cells. Additionally, while overexpression of wild-type Nrf2, the ΔNeh2/3 variant, and C-terminal fragment in HeLa cells conferred increased cell viability—likely through increased antioxidant capacity—the NH_2 fragment did not to the same degree. We speculate that this may be due to the absence of the Neh1 domain which is required for DNA/ARE-binding to activate the oxidative stress response.

Since this work focuses on characterizing a new model to study Nrf2 interactions, it is important to confirm already known interactions to determine the validity of the model. Notably, we were able to recapitulate key Nrf2 interactions with Keap1 and other documented proteins using yeast growth assays and the split-ubiquitin system. Keap1 partially rescues wild-type Nrf2 toxicity in yeast but does not rescue toxicity for the Neh2 domain mutants, L30F and T80R, indicating that the impairments in the Keap1-Nrf2-interaction that are associated with mutations in this Keap1-binding domain previously described by Shibata et al. (Shibata et al., 2008), is reflected in our yeast model. We provide evidence for other previously documented Nrf2 interactions, notably the physical interaction between Nrf2 and p21, which may have strong implications in cancer. Of note, the yeast system does not detect a genetic interaction between p21 and Nrf2, possibly because yeast cells lack other cellular factors or mechanisms that characterize this interaction in mammalian cells. We furthermore document the genetic and physical interactions between both Nrf2 and Keap1 with the molecular chaperone Hsp90. This interaction was also observed in cultured mammalian cells, as treatment with Hsp90-inhibitor radicicol altered Nrf2 and Keap1 localization patterns in HeLa cells.

Taipale et al. found that ubiquitin E3 ligases with Kelch domains (e.g., Keap1) interact with Hsp90 in a high-throughput study (Taipale et al., 2012) and Prince et al. have since confirmed the interaction between Keap1 and Hsp90 (Prince et al., 2015) which is further confirmed by our model. To our knowledge, the Nrf2-Hsp90 interaction detected in our studies has not been previously described. Heat shock proteins and molecular chaperones such as Hsp90 have

protective roles in the refolding of proteins damaged or misfolded by cell stress and stabilizing newly synthesized proteins to ensure their correct folding (Schopf et al., 2017). Hsp90 might bind and stabilize the mostly intrinsically disordered protein Nrf2 to allow it to effectively function as a transcription factor. Further work must be done to determine the mechanism and functional outcome of the Nrf2-Hsp90 and Keap1-Hsp90 interactions.

Taken together, we show that genetic interaction assays and the split-ubiquitin system in yeast are powerful tools to study known Nrf2 interactions and to identify previously unknown interactions. Due to its intrinsically disordered structure and numerous binding partners, Nrf2 can in some cases be challenging to study in mammalian systems. Our yeast model presents a useful and effective complementary tool to explore Nrf2 regulation and function and may serve as a platform to screen for small molecules that modulate Nrf2 interactions and activity, which has potential therapeutic value. Our work also provides evidence for the interaction of Nrf2 and Keap1 with the molecular chaperone Hsp90 and may thus indicate an important nexus between two cellular stress response pathways, i.e., the antioxidant response and the heat shock response, with possible implications in normal cellular stress regulation and cancer.

2.4 Materials and Methods

2.4.1 Plasmids

All plasmids for yeast growth assays, fluorescence microscopy, western blots, and mammalian cell expression were created using the Gateway cloning technology developed by Invitrogen (Katzen, 2007) according to the manufacturer's protocol. The yeast and mammalian plasmids used in this study are listed in **Table 2.1** and **Table 2.2** respectively. All plasmids for split-ubiquitin assays were created using traditional restriction digest and ligation-based cloning and are listed in **Table 2.3**. Note that the p21-CRU construct was not viable in *E. coli* during the plasmid generation process and was excluded from the study.

Table 2.1: Yeast expression plasmids created using Gateway cloning.

Template	Destination Vector(s)
Nrf2 wild-type	pAG423Gal-ccdB pAG425Gal-ccdB
Nrf2 NH_2	pAG423Gal-ccdB
Nrf2 ΔNeh2/3	pAG423Gal-ccdB
Nrf2 COOH	pAG423Gal-ccdB
Nrf2 L30F	pAG425Gal-ccdB
Nrf2 T80R	pAG425Gal-ccdB
Keap1	pAG423Gal-ccdB
p21	pAG423Gal-ccdB
βTrCP	pAG423Gal-ccdB
Cul3	pAG423Gal-ccdB
ProTα	pAG423Gal-ccdB
Hsp82	pAG423Gal-ccdB
Hsc82	pAG423Gal-ccdB
Hsp82 overexpression	pAG423Gal-ccdB
Hsc82 overexpression	pAG423Gal-ccdB
Aha1 overexpression	pAG423Gal-ccdB
Sti1 overexpression	pAG423Gal-ccdB

Table 2.2: Mammalian expression plasmids created using Gateway cloning.

Template	Destination Vector
Nrf2 wild-type	pcDNA3.1-ccdB
Nrf2 NH_2	pcDNA3.1-ccdB
Nrf2 ΔNeh2/3	pcDNA3.1-ccdB
Nrf2 COOH	pcDNA3.1-ccdB
Nrf2 L30F	pcDNA3.1-ccdB
Nrf2 T80R	pcDNA3.1-ccdB
Keap1	pcDNA3.1-ccdB

Table 2.3: Yeast split-ubiquitin plasmids created using restriction digest and ligation-based cloning.

Template	Destination Vectors			
Nrf2	p415-Cub-R-*URA3* CUP	p414-NuI CUP	p414-NuA CUP	p414-NuG CUP
Keap1	p415-Cub-R-*URA3* CUP	p414-NuI CUP	p414-NuA CUP	p414-NuG CUP
p21	N/A (see text)	p414-NuI CUP	p414-NuA CUP	p414-NuG CUP
βTrCP	p415-Cub-R-*URA3* CUP	p414-NuI CUP	p414-NuA CUP	p414-NuG CUP
Cul3	p415-Cub-R-*URA3* CUP	p414-NuI CUP	p414-NuA CUP	p414-NuG CUP
ProTα	p415-Cub-R-*URA3* CUP	p414-NuI CUP	p414-NuA CUP	p414-NuG CUP
Hsp90	p415-Cub-R-*URA3* CUP	p414-NuI CUP	p414-NuA CUP	p414-NuG CUP
Pgk1	p415-Cub-R-*URA3* CUP	p414-NuI CUP	p414-NuA CUP	p414-NuG CUP

2.4.2 Yeast strains, culture conditions, and growth assays

For assessment of relative yeast growth and protein toxicity, yeast strains derived from W303 (MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15) (Thomas & Rothstein, 1989) were used. Yeast deletion strains were obtained from the *Saccharomyces* Genome Deletion Project (Thomas & Rothstein, 1989). Yeast cells were transformed using the standard lithium acetate/salmon sperm carrier DNA/PEG method for the incorporation of yeast plasmids (Gietz & Schiestl, 2007). Transformed yeast cells were grown overnight in synthetic selective media to maintain these plasmid(s). Growth assays and split-ubiquitin assays were performed by spotting 5X serial dilutions of OD_{600} = 0.2 on selective agar plates. To induce protein expression for liquid growth assays, fluorescence microscopy, and western blots, overnight cultures were washed twice with water and resuspended in media containing 2% galactose and incubated overnight. Liquid growth assays were performed using the Bioscreen C Pro Automated Microbiology Growth Curve Analysis System (Growth Curves USA).

2.4.3 Spotting assay growth quantification

Quantification was carried out as described before (Petropavlovskiy et al., 2020). In brief, yeast agar plates were imaged in black and white using the Gel Doc XR+ System (Bio-Rad). Images

were pre-processed using Image Lab Software (Bio-Rad) to remove colour and background data. Images were then imported into ImageJ (NIH) and white pixel count was measured and summed for dilutions 1-3 for each condition. Data was quantified relative to the empty vector control on the same respective plate and imported into Prism 8 (GraphPad Software) where scatter dot plots with bars were generated. Statistical analysis was performed as outlined in **Section 2.4.9**.

2.4.4 Yeast fluorescence microscopy

For assessment of fluorescently-tagged protein expression and localization, yeast strains derived from BY 4741 (MAT α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) (Brachmann et al., 1998) were used. Yeast expression plasmids were tagged with either YFP or DsRed. Cells were transferred to a glass microscope slide and coverslip and imaged using either the Olympus BX-51 Bright Field/Fluorescence Microscope at 60X and captured using an equipped CCD camera (Spot Pursuit) or the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) at 20X and captured using Gen5 Software (BioTek).

2.4.5 Electrophoresis and western blot analysis

Proteins were extracted from yeast cells using the alkaline lysis method (Kushnirov, 2000). 30 µl of lysate was resolved on an SDS-PAGE gel. The membrane was blocked with 5% BSA in PBST and incubated with primary antibody overnight (refer to **Table 2.4**). The membrane was incubated with an HRP-conjugated secondary antibody for 1 h at room temperature, either anti-rabbit (Abcam, ab6721), anti-mouse (Abcam, ab6728) or anti-rat (Abcam, ab97057) as required. Western blots were visualized using the Clarity Western ECL Substrate kit (Bio-Rad, 1705061) and images were taken using the ChemiDoc Imaging System (Bio-Rad).

Table 2.4: Antibodies for western blot analyses.

Antigen	Supplier	Product Code	Species	Concentration
Nrf2	Abcam	ab62352	Rabbit	1:1000
Keap1	Proteintech	10503-2-AP	Rabbit	1:1000
p21/p21$^{CiP/Waf}$	Cell Signaling	2947S	Rabbit	1:1000
βTrCP	Santa Cruz	sc-390629	Mouse	1:50
Cul3	Bethyl Laboratories	A301-109A	Rabbit	1:2000
ProTα/PTMA	(1) Invitrogen	PA5-75828	Rabbit	1:500
	(2) LifeSpan BioSciences	LS-C162288	Rabbit	1:1000
Hsp90	Abcam	ab13492	Mouse	1:1000
β-Tubulin	Abcam	ab6160	Rat	1:5000

2.4.6 Mammalian cell culture conditions and transfections

The HeLa and HEK293 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, 41966-029), supplemented with 10% FBS (Wisent, 080-150) and 1X penicillin-streptomycin (Corning, 30-001-CI). Cells were cultured at 37°C with 5% CO_2. For transfections, cells were seeded in a 6-well plate at $1.0x10^6$ cells per well and grown to approximately 80% confluency. Cells were transfected using Lipofectamine LTX with PLUS Reagent (Thermo Fisher Scientific, A12621) according to the manufacturer's protocol in Opti-MEM I Reduced Serum Medium (Gibco, 31985-062). The transfected cells were incubated at 37°C for 6 h followed by incubation in DMEM for 18 h at 37°C. Cells were then split into the appropriate plates for subsequent experiments.

2.4.7 Immunofluorescence microscopy

Transfected HeLa cells were seeded onto 15 mm circular glass coverslips (Matsunami, C015001) in a 12-well plate at $1x10^5$ cells per well to ensure approximately 80% confluency the following day. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked with 20% goat head serum in PBB (0.5% BSA in PBS), and incubated with one of the following primary antibodies overnight at 4°C at a concentration of 1:100: mouse anti-Nrf2

(Abcam, ab62352), mouse anti-Keap1 (Proteintech, 10503-2-AP), or rabbit anti-Hsp90 (Proteintech, 13171-1-AP). The coverslips were incubated with the following Alexa Fluor-conjugated secondary antibody for 1 h at room temperature at a concentration of 1:1500: goat anti-mouse (Thermo Fisher Scientific, A-11094)., or goat anti-rabbit (Thermo Fisher Scientific, A11036). Coverslips were then mounted onto glass microscope slides with SlowFade Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, S36938) and cured at room temperature for 24 h. Cells were imaged using the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) using a 20X objective lens.

2.4.8 Cell viability assays

Transfected HeLa or HEK293 cells were seeded into 96-well solid white microplates (Greiner, M0187-32EA) at $4x10^4$ cells per well and incubated for 16 h. Following treatment, cell viability was assessed using the CellTiter-Glo 2.0 Cell Viability Assay (Promega, G9242) according to the manufacturer's protocol. Luminescence was measured using the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek).

2.4.9 Statistical analysis

Statistical analyses were conducted using Prism 8 (GraphPad Software). Statistical significance was obtained by performing a one-way ANOVA with Tukey post hoc for comparison between groups, or the Student's t-test for comparison between two groups (with a minimum of 3 biological replicates). Error bars represent standard deviation. P-values less than 0.05 were considered statistically significant. Significance levels are indicated using asterisks, where * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$. Shapiro-Wilk tests were performed for all data sets to ensure normality.

2.5 Supplemental Figures

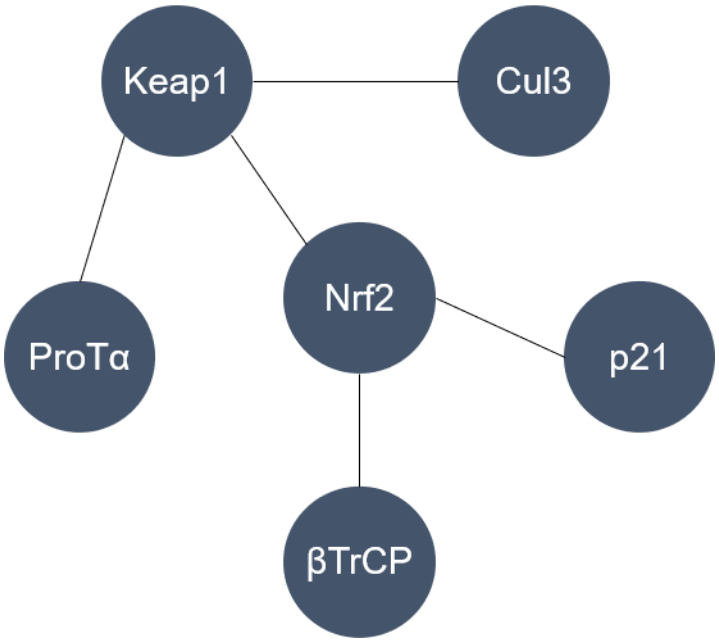

Figure S2.1: Nrf2 protein interactions. Select proteins in the Nrf2 interactome were observed in this study.

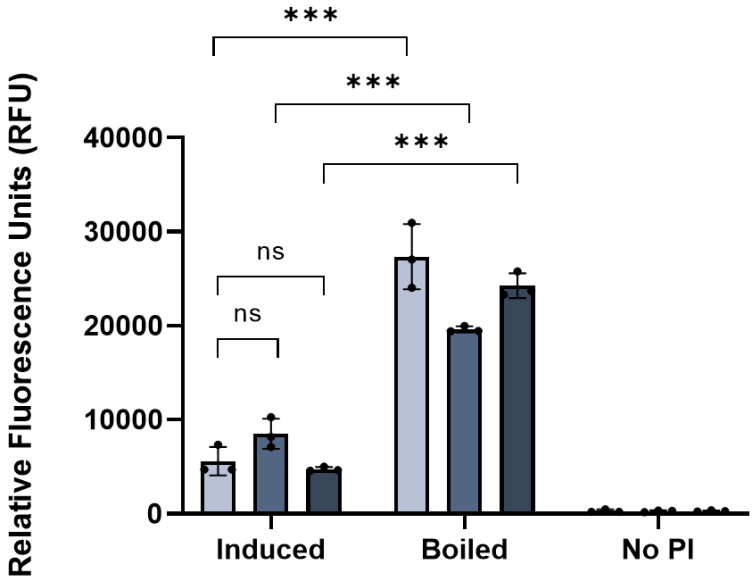

Figure S2.2: Propidium iodide assay for Nrf2 and Keap1 expressed in yeast. Induced expression of Nrf2 and Keap1 in yeast was monitored by propidium iodide (PI) assays. Boiled cells served as a positive control for cell death. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

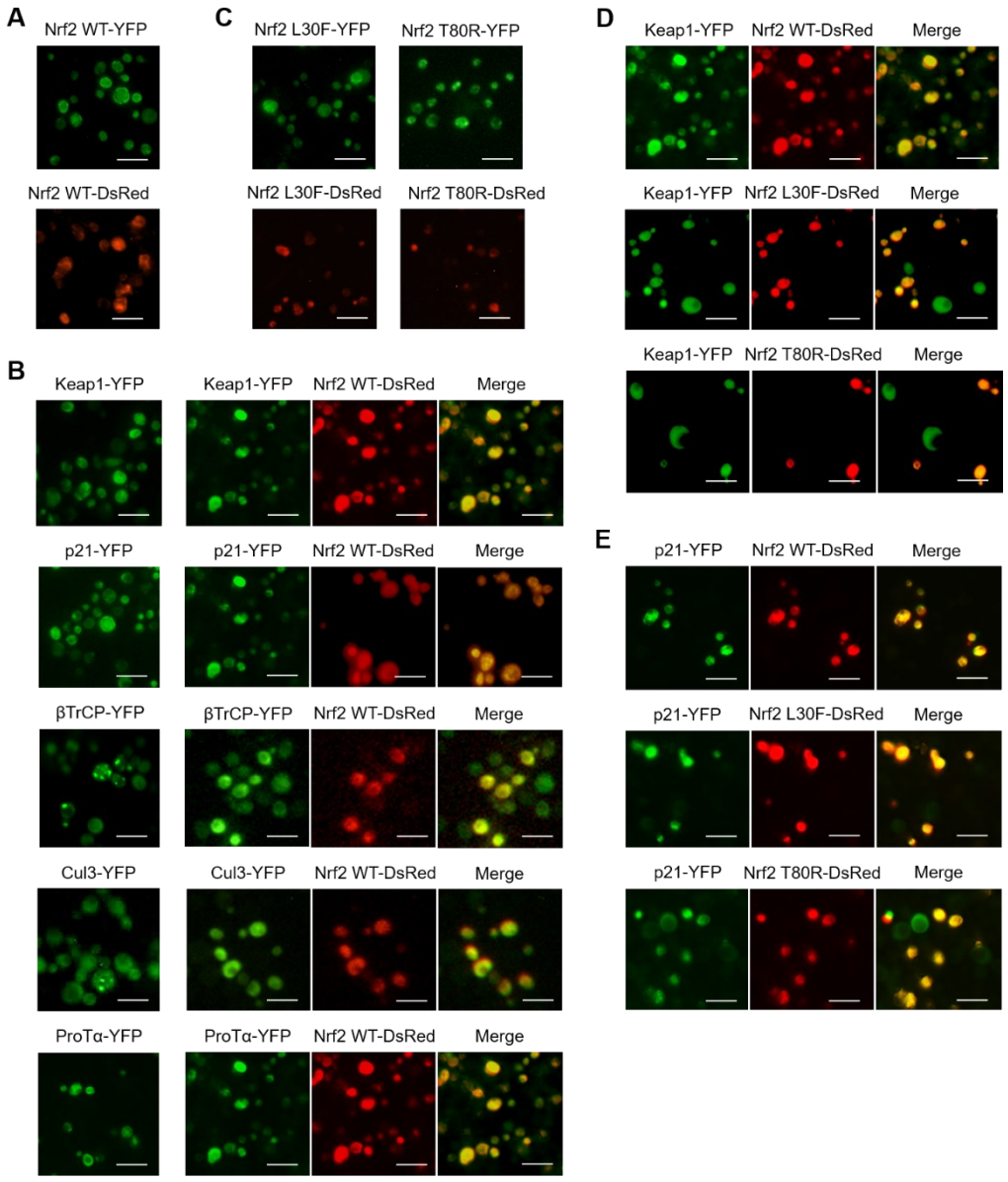

Figure S2.3: Fluorescence microscopy for all proteins of interest expressed in yeast. **(A)** Fluorescence microscopy of yeast cells expressing YFP- and DsRed-tagged wild-type Nrf2. **(B)** Fluorescence microscopy of yeast cells expressing YFP-tagged Keap1, p21, βTrCP, Cul3, and ProTα alone and co-expressed with Nrf2-DsRed. **(C)** Fluorescence microscopy of yeast cells expressing YFP- and DsRed-tagged Nrf2 mutant variants, L30F and T80R. **(D)** Fluorescence microscopy of yeast cells expressing Keap1-YFP co-expressed with DsRed-tagged wild-type or mutant Nrf2. **(E)** Fluorescence microscopy of yeast cells expressing p21-YFP co-expressed with DsRed-tagged wild-type or mutant Nrf2. All scale bars correspond to 10 μm.

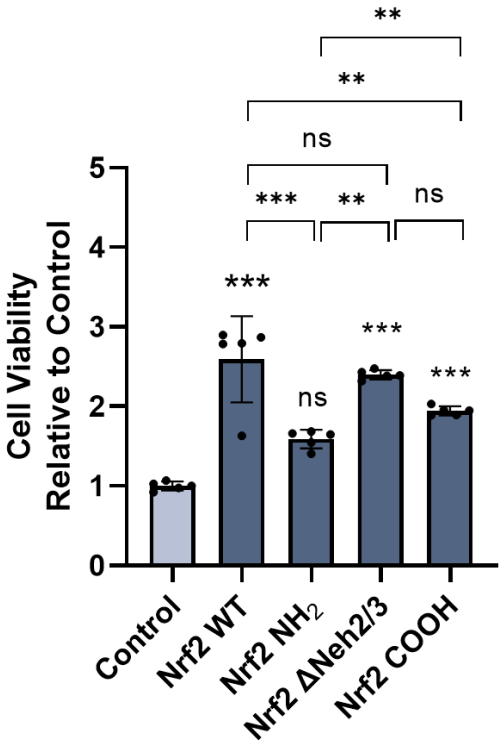

Figure S2.4: Cell viability assay for wild-type Nrf2 and its fragmented variants expressed in HEK293 cells. Results recapitulate those observed in HeLa cells. Means derived from five biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

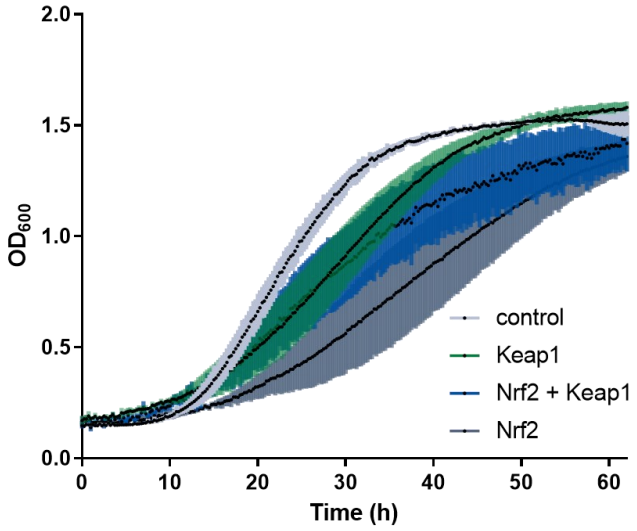

Figure S2.5: Liquid growth curve for the co-expression of Nrf2 and Keap1 in yeast. Results recapitulate those observed on solid growth media. Means derived from three biological replicates were used during analysis. Data are expressed as mean (shown in black) ± SD (shown in colour).

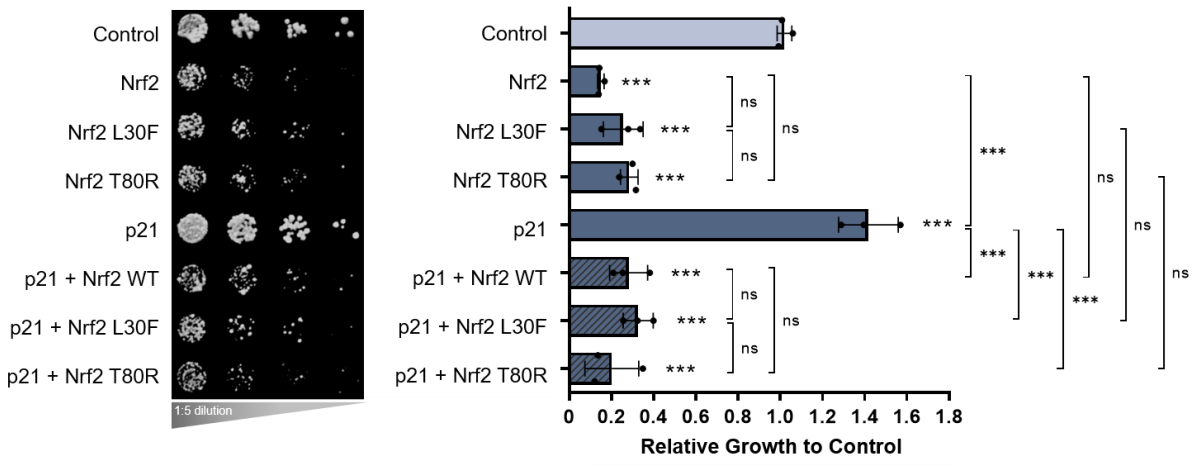

Figure S2.6: p21 co-expressed with Nrf2 mutant variants in yeast. Growth assays were performed for yeast cells co-expressing wild-type Nrf2 or its mutant variants L30F and T80R with p21. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

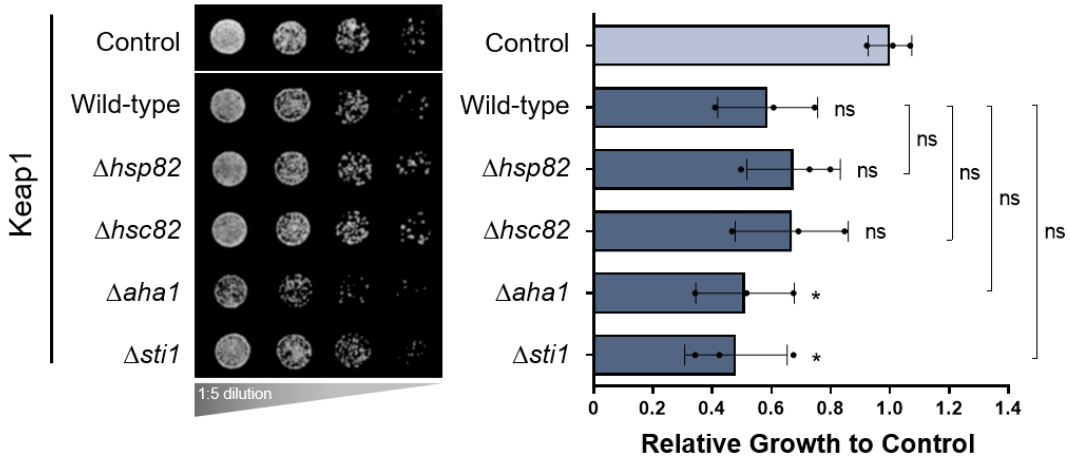

Figure S2.7: Keap1 expressed in yeast Hsp90 deletion strains. Growth assays of yeast cells expressing Keap1 in deletions strains for yeast Hsp90 (Δ*hsp82* and Δ*hsc82*) and its co-chaperones (Δ*aha1* and Δ*sti1*). Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

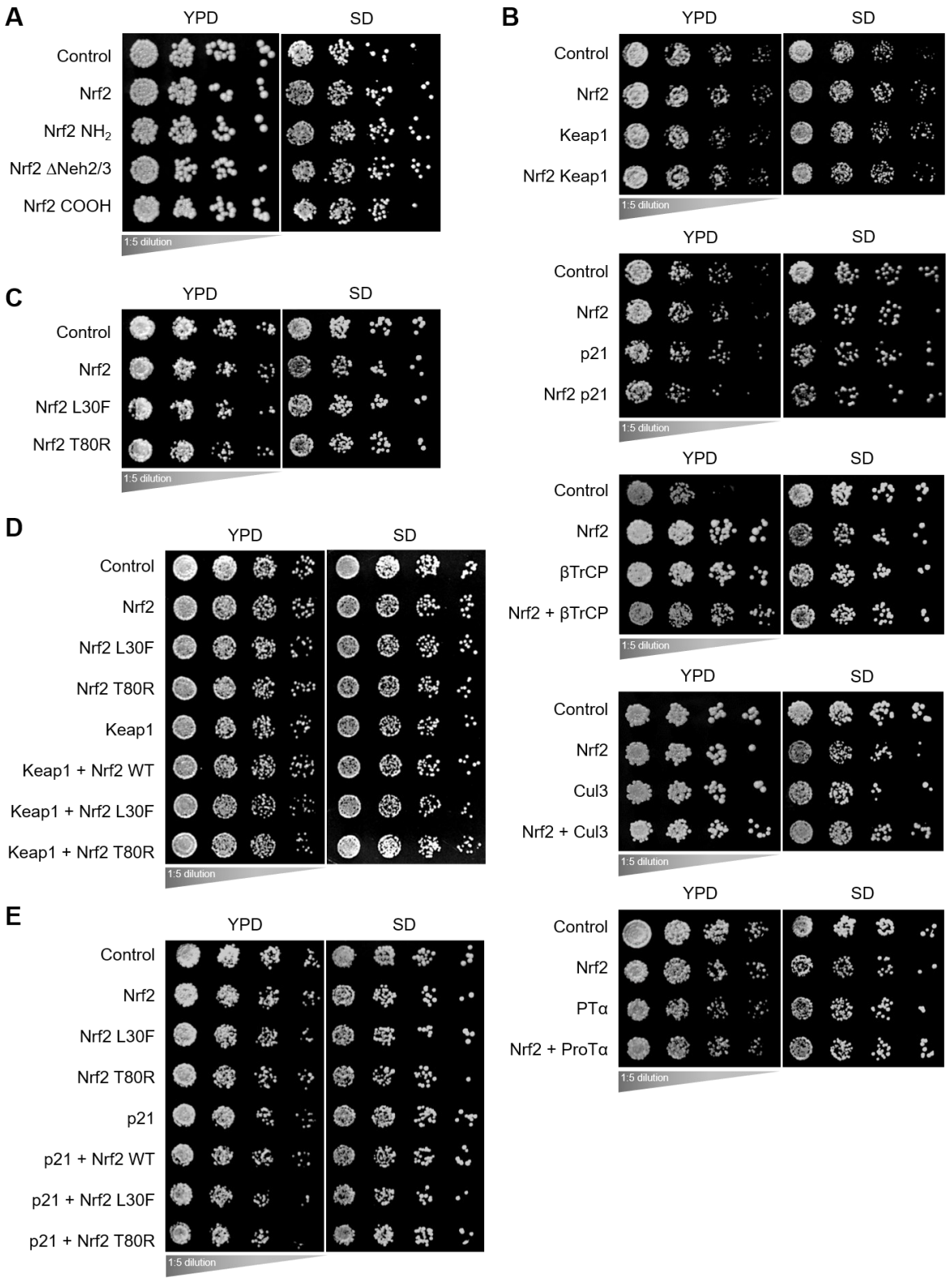

Figure S2.8: (A-E) Control plates for growth assay interaction studies. Yeast peptone dextrose (YPD) and selective dextrose (SD) control plates are shown.

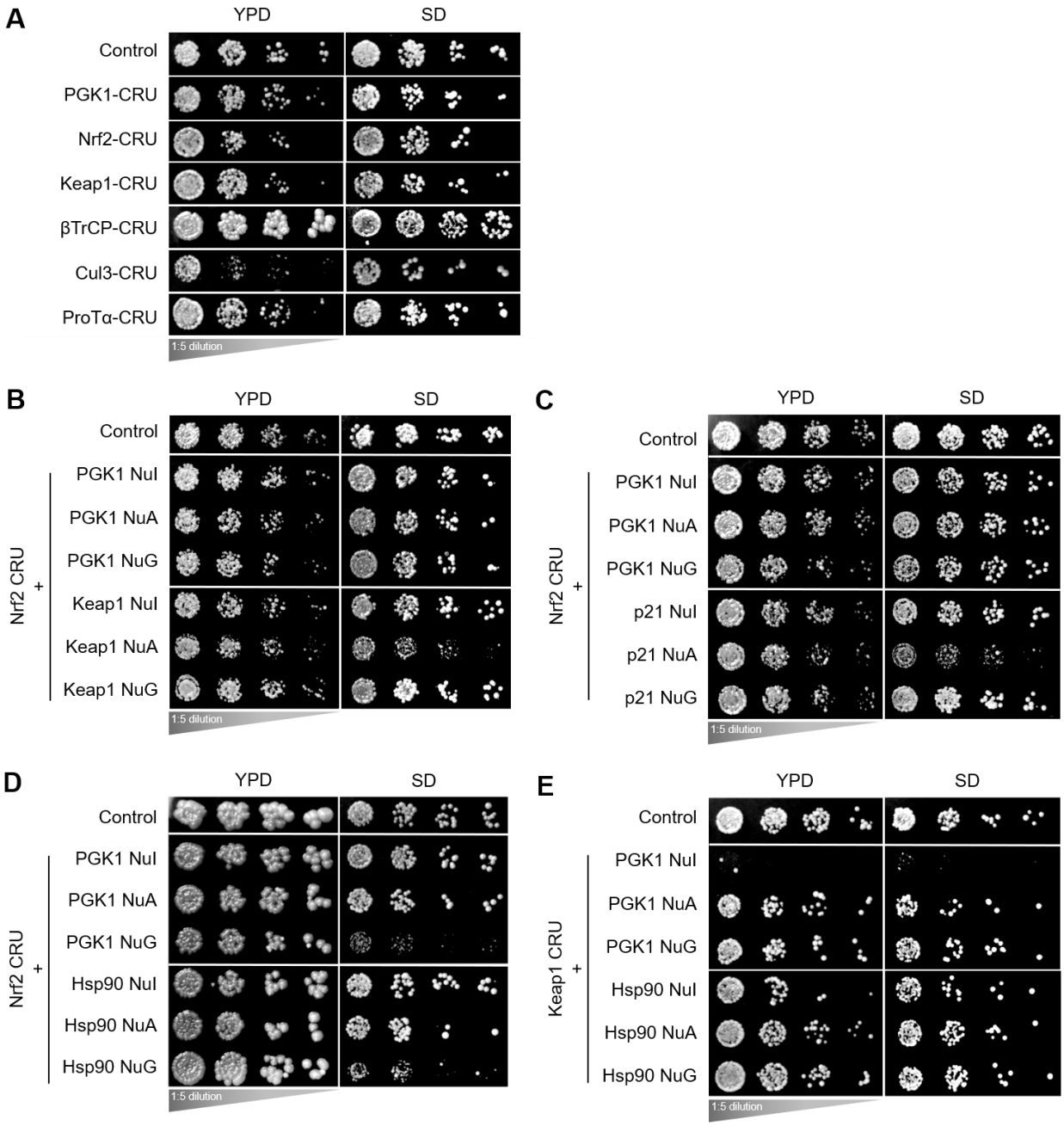

Figure S2.9: (A-E) Control plates for split-ubiquitin interaction studies. Yeast peptone dextrose (YPD) and selective dextrose (SD) plates are shown.

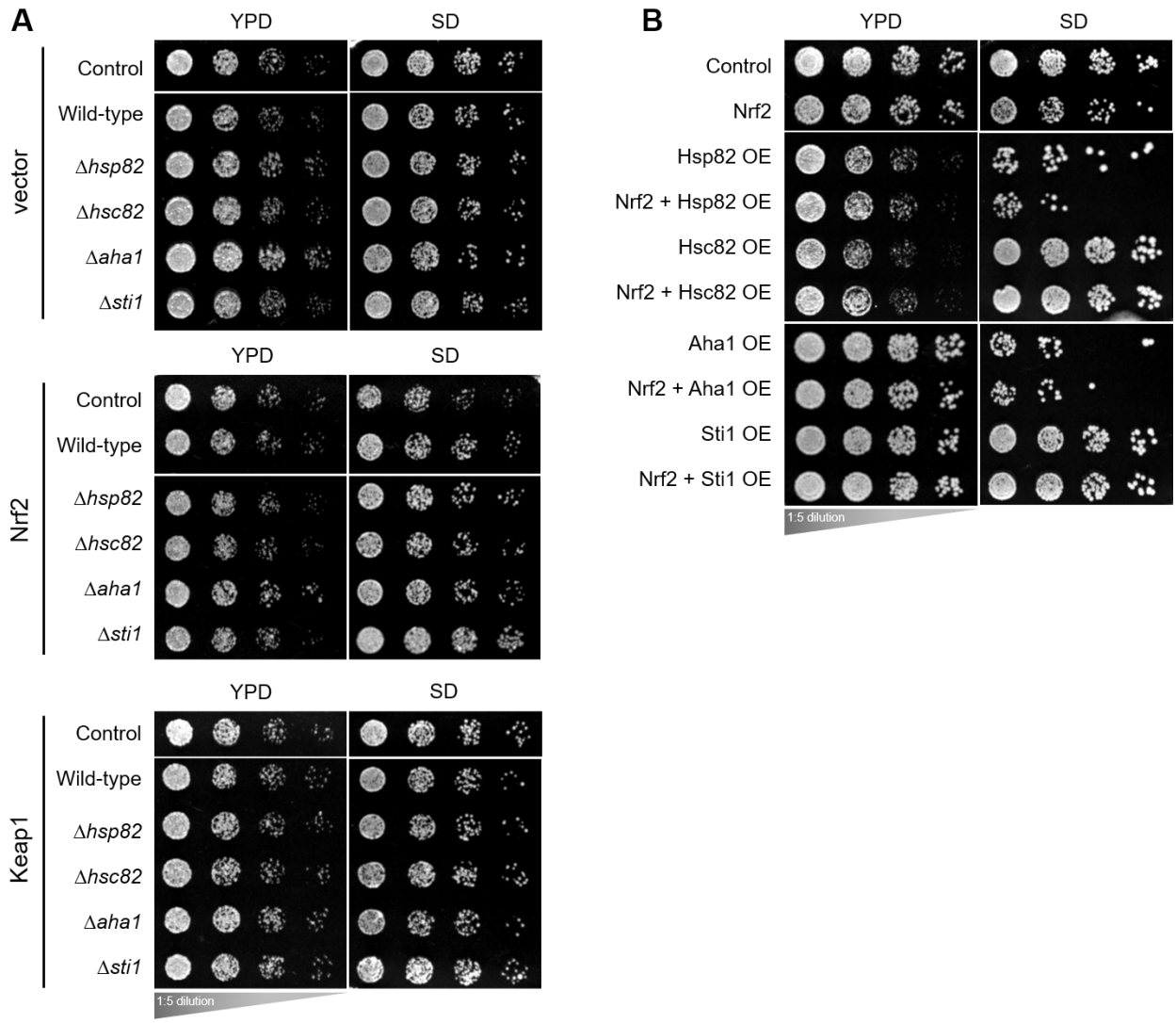

Figure S2.10: (A-B) Control plates for Hsp90 deletion and overexpression studies. Yeast peptone dextrose (YPD) and selective dextrose (SD) plates are shown.

2.6 References

Abbas, T., & Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer, 9*(6), 400-414.

Bae, D. S., Cho, S. B., Kim, Y. J., Whang, J. D., Song, S. Y., Park, C. S., Kim, D. S., & Lee, J. H. (2001). Aberrant expression of cyclin D1 is associated with poor prognosis in early stage cervical cancer of the uterus. *Gynecol Oncol, 81*(3), 341-347.

Baretton, G. B., Klenk, U., Diebold, J., Schmeller, N., & Löhrs, U. (1999). Proliferation- and apoptosis-associated factors in advanced prostatic carcinomas before and after androgen deprivation therapy: prognostic significance of p21/WAF1/CIP1 expression. *British Journal Of Cancer, 80*, 546.

Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., & Boeke, J. D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast, 14*(2), 115-132.

Bukau, B., Weissman, J., & Horwich, A. (2006). Molecular Chaperones and Protein Quality Control. *Cell, 125*(3), 443-451.

Chen, W., Sun, Z., Wang, X. J., Jiang, T., Huang, Z., Fang, D., & Zhang, D. D. (2009). Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol Cell, 34*(6), 663-673.

Cheung, T. H., Lo, K. W. K., Yu, M. M. Y., Yim, S. F., Poon, C. S., Chung, T. K. H., & Wong, Y. F. (2001). Aberrant expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in cervical carcinoma. *Cancer Letters, 172*(1), 93-98.

Chowdhry, S., Zhang, Y., McMahon, M., Sutherland, C., Cuadrado, A., & Hayes, J. D. (2013). Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene, 32*(32), 3765-3781.

Crouch, S. P., Kozlowski, R., Slater, K. J., & Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J Immunol Methods, 160*(1), 81-88.

De Las Rivas, J., & Fontanillo, C. (2010). Protein-protein interactions essentials: key concepts to building and analyzing interactome networks. *PLoS computational biology, 6*(6), e1000807-e1000807.

Di Gregorio, S. E., & Duennwald, M. L. (2018). ALS Yeast Models—Past Success Stories and New Opportunities. *Frontiers in Molecular Neuroscience, 11*(394).

Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., & Talalay, P. (2002). Direct evidence that sulfhydryl groups of Keap1 are

the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences, 99*(18), 11908.

Duennwald, M. L. (2011). Polyglutamine misfolding in yeast: Toxic and protective aggregation. *Prion, 5*(4), 285-290.

Fields, S., & Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature, 340*(6230), 245-246.

Frydman, J. (2001). Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annu Rev Biochem, 70*, 603-647.

Fushimi, K., Long, C., Jayaram, N., Chen, X., Li, L., & Wu, J. Y. (2011). Expression of human FUS/TLS in yeast leads to protein aggregation and cytotoxicity, recapitulating key features of FUS proteinopathy. *Protein Cell, 2*(2), 141-149.

Gartel, A. L., & Radhakrishnan, S. K. (2005). Lost in Transcription: p21 Repression, Mechanisms, and Consequences. *Cancer Res, 65*(10), 3980.

Gietz, R. D., & Schiestl, R. H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols, 2*, 31.

Hartwell, L. H. (2005). Yeast and Cancer. *Biosci Rep, 24*(4-5), 523-544.

Itoh, K., Tong, K. I., & Yamamoto, M. (2004). Molecular mechanism activating nrf2–keap1 pathway in regulation of adaptive response to electrophiles. *Free Radical Biology and Medicine, 36*(10), 1208-1213.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., & Yamamoto, M. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev, 13*(1), 76-86.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T., & Yamamoto, M. (2003). Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells, 8*(4), 379-391.

Johnsson, N., & Varshavsky, A. (1994). Split ubiquitin as a sensor of protein interactions in vivo. *Proc Natl Acad Sci U S A, 91*(22), 10340-10344.

Karapetian, R. N., Evstafieva, A. G., Abaeva, I. S., Chichkova, N. V., Filonov, G. S., Rubtsov, Y. P., Sukhacheva, E. A., Melnikov, S. V., Schneider, U., Wanker, E. E., & Vartapetian, A. B. (2005). Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Mol Cell Biol, 25*(3), 1089-1099.

Katzen, F. (2007). Gateway((R)) recombinational cloning: a biological operating system. *Expert Opin Drug Discov, 2*(4), 571-589.

Khan, H., Cino, E. A., Brickenden, A., Fan, J., Yang, D., & Choy, W.-Y. (2013). Fuzzy Complex Formation between the Intrinsically Disordered Prothymosin α and the Kelch Domain of Keap1 Involved in the Oxidative Stress Response. *Journal of Molecular Biology, 425*(6), 1011-1027.

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., & Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol, 24*(16), 7130-7139.

Kobayashi, A., Kang, M. I., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., & Yamamoto, M. (2006). Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol Cell Biol, 26*(1), 221-229.

Kushnirov, V. V. (2000). Rapid and reliable protein extraction from yeast. *Yeast, 16*(9), 857-860.

Liu, S., Bishop, W. R., & Liu, M. (2003). Differential effects of cell cycle regulatory protein p21(WAF1/Cip1) on apoptosis and sensitivity to cancer chemotherapy. *Drug Resist Updat, 6*(4), 183-195.

Mani, R., St.Onge, R. P., Hartman, J. L., Giaever, G., & Roth, F. P. (2008). Defining genetic interaction. *Proceedings of the National Academy of Sciences, 105*(9), 3461.

McMahon, M., Itoh, K., Yamamoto, M., & Hayes, J. D. (2003). Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem, 278*(24), 21592-21600.

Moi, P., Chan, K., Asunis, I., Cao, A., & Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Sciences, 91*(21), 9926-9930.

Müller, J., & Johnsson, N. (2008). Split-Ubiquitin and the Split-Protein Sensors: Chessman for the Endgame. *ChemBioChem, 9*(13), 2029-2038.

Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S., & Pickett, C. B. (2003). Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem, 278*(7), 4536-4541.

Outeiro, T. F., & Lindquist, S. (2003). Yeast cells provide insight into alpha-synuclein biology and pathobiology. *Science, 302*(5651), 1772-1775.

Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsuji, M., Kang, M. I., Kobayashi, A., Yokoyama, S., & Yamamoto, M. (2006). Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol Cell, 21*(5), 689-700.

Petropavlovskiy, A. A., Tauro, M. G., Lajoie, P., & Duennwald, M. L. (2020). A Quantitative Imaging-Based Protocol for Yeast Growth and Survival on Agar Plates. *STAR Protocols*, 100182.

Prince, T. L., Kijima, T., Tatokoro, M., Lee, S., Tsutsumi, S., Yim, K., Rivas, C., Alarcon, S., Schwartz, H., Khamit-Kush, K., Scroggins, B. T., Beebe, K., Trepel, J. B., & Neckers, L. (2015). Client Proteins and Small Molecule Inhibitors Display Distinct Binding Preferences for Constitutive and Stress-Induced HSP90 Isoforms and Their Conformationally Restricted Mutants. *PLoS ONE, 10*(10), e0141786.

Rada, P., Rojo, A. I., Chowdhry, S., McMahon, M., Hayes, J. D., & Cuadrado, A. (2011). SCF/β-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Mol Cell Biol, 31*(6), 1121-1133.

Schopf, F. H., Biebl, M. M., & Buchner, J. (2017). The HSP90 chaperone machinery. *Nature Reviews Molecular Cell Biology, 18*(6), 345-360.

Shibata, T., Ohta, T., Tong, K. I., Kokubu, A., Odogawa, R., Tsuta, K., Asamura, H., Yamamoto, M., & Hirohashi, S. (2008). Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci U S A, 105*(36), 13568-13573.

Smith, M. G., & Snyder, M. (2006). Yeast as a model for human disease. *Curr Protoc Hum Genet, Chapter 15*, Unit 15.16.

Stagljar, I., Korostensky, C., Johnsson, N., & te Heesen, S. (1998). A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. *Proc Natl Acad Sci U S A, 95*(9), 5187-5192.

Taipale, M., Krykbaeva, I., Koeva, M., Kayatekin, C., Westover, K. D., Karras, G. I., & Lindquist, S. (2012). Quantitative Analysis of Hsp90-Client Interactions Reveals Principles of Substrate Recognition. *Cell, 150*(5), 987-1001.

Tenreiro, S., & Outeiro, T. F. (2010). Simple is good: yeast models of neurodegeneration. *FEMS Yeast Res, 10*(8), 970-979.

Thomas, B. J., & Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. *Cell, 56*(4), 619-630.

Tong, K. I., Katoh, Y., Kusunoki, H., Itoh, K., Tanaka, T., & Yamamoto, M. (2006). Keap1 Recruits Neh2 through Binding to ETGE and DLG Motifs: Characterization of the Two-Site Molecular Recognition Model. *Mol Cell Biol, 26*(8), 2887-2900.

Tong, K. I., Padmanabhan, B., Kobayashi, A., Shang, C., Hirotsu, Y., Yokoyama, S., & Yamamoto, M. (2007). Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol Cell Biol, 27*(21), 7511-7521.

Uversky, V. N. (2011). Intrinsically disordered proteins may escape unwanted interactions via functional misfolding. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1814*(5), 693-712.

Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., & Talalay, P. (2004). Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci U S A, 101*(7), 2040-2045.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., & Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature, 366*(6456), 701-704.

Zhang, D. D., & Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol, 23*(22), 8137-8151.

Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., & Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol Cell Biol, 24*(24), 10941-10953.

Chapter 3

3 Oxidative stress-induced misfolding and inclusion formation of Nrf2 and Keap1

Cells that experience high levels of oxidative stress respond with the induction of antioxidant proteins through the activation of the transcription factor Nrf2. Nrf2 is negatively regulated by Keap1 which binds to Nrf2 to facilitate its ubiquitination and ensuing proteasomal degradation under basal conditions. Upon oxidative stress, stress-sensing cysteines in Keap1 are modified, leading to a conformational change in Keap1 that stabilizes Nrf2 for accumulation, nuclear translocation, and activation of the oxidative stress response. Here, we study Nrf2 and Keap1 in yeast, mammalian cells, and purified proteins and find that both Nrf2 and Keap1 are susceptible to protein misfolding and inclusion formation upon oxidative stress. We argue that the disordered regions in Nrf2 and the high cysteine content of Keap1 contribute to their misfolding. Our work reveals previously unexplored aspects of Nrf2 and Keap1 regulation by oxidation-induced protein misfolding.

A version of this chapter has been submitted for publication.

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3.1 Introduction

Oxidative stress is regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Moi et al., 1994). Nrf2 regulates the expression of a multitude of antioxidant genes and is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1) (Itoh et al., 1999), a substrate adaptor protein that binds to Nrf2 in the cytoplasm to promote its ubiquitination and ensuing degradation by the proteasome (Itoh et al., 1999; Itoh et al., 2003; McMahon et al., 2003; Nguyen et al., 2003; Kobayashi et al., 2006). During oxidative stress, specific stress-sensing cysteine residues in Keap1 become oxidized (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003; Wakabayashi et al., 2004), resulting in a conformational change in Keap1 that leads to dissociation of the Keap1-Nrf2 complex. This in turn leads to Nrf2 stabilization, nuclear translocation, and ultimately, activation of cytoprotective antioxidant genes (Yamamoto et al., 2018; Baird & Yamamoto, 2020). Induction of the Keap1-Nrf2 antioxidant pathway is fundamental to protecting cells against oxidative stress and mutations that disrupt Keap1-Nrf2 binding compromise Nrf2 regulation and contribute to disease pathogenesis (Padmanabhan et al., 2006; Shibata et al., 2008). Thus, the transcriptional activity of Nrf2 is tightly regulated by its interaction with Keap1 (Tong et al., 2006; Tong et al., 2007).

Nrf2 contains seven conserved regions that are referred to as the Nrf2-ECH homology (Neh) domains. Notably, the Neh2 domain that mediates Nrf2's binding with Keap1 is shown to be intrinsically disordered (Tong et al., 2006). Intrinsically disordered proteins (IDPs) or proteins that contain long intrinsically disordered regions (IDRs) lack a fixed three-dimensional structure and are susceptible to protein misfolding and inclusion formation in cells due to their structural heterogeneity and flexible nature; however, this also allows for enhanced binding capacity and multifunctionality (Dunker et al., 2001; Dyson & Wright, 2005; Uversky, 2019). This may explain Nrf2's ability to bind to a vast array of different proteins. We use the term 'protein misfolding' here to indicate proteins that have acquired a non-native, aberrant conformation, often in the form of inclusions or aggregates. Misfolded proteins often lose their normal function (i.e., loss of protein function) and tend to aggregate and form inclusions that can have deleterious effects on the cell (i.e., toxic gain of function) (Dobson, 2003). Examples of disease-associated IDPs include α-Synuclein in Parkinson's disease (Wise-Scira et al., 2013), while in cancer, many key tumour suppressors contain long IDRs, such as p53 (Xue et al., 2013). Interestingly, oxidative stress can

affect the structural flexibility of IDPs/IDRs (Wise-Scira et al., 2013) and may thus, also modulate the folding or contribute to the misfolding of intrinsically disordered Nrf2, which may impair its interactions with other proteins as well as its transcriptional activity.

Protein misfolding may also expose hydrophobic or oxidation-prone cysteine residues to the surface of the protein, rendering them targets for oxidation by reactive oxygen species (ROS) and other oxidants (Stadtman, 1993). Oxidation products of cysteines include disulfide bonds and mixed disulfide bonds. Oxidation can also lead to alteration of non-covalent interactions within proteins, fragmentation of peptide chains, cross-linking of proteins, and/or oxidation of specific side chains, ultimately leading to protein destabilization and misfolding (Dean et al., 1985; Davies, 1987; Davies et al., 1987). Cysteine residues are particularly susceptible to aberrant oxidation by ROS due to the presence of their nucleophilic thiol groups (Di Simplicio et al., 2003). Keap1, which contains a very high percentage of cysteine residues, may thus be particularly susceptible to oxidation and inclusion formation.

In this study, we examine two aspects of protein oxidation: the misfolding of the intrinsically disordered Nrf2, and the misfolding of the cysteine-rich Keap1, under oxidative stress conditions. Using yeast, cultured mammalian cells, and purified proteins, we find that both Nrf2 and Keap1 misfold and form aberrant cytoplasmic and possibly nuclear protein inclusions upon exposure to high levels of oxidative stress. Our results suggest a previously unexplored mechanism by which the Keap1-Nrf2 interaction may be altered by oxidative stress as it pertains to protein misfolding and inclusion formation.

3.2 Results

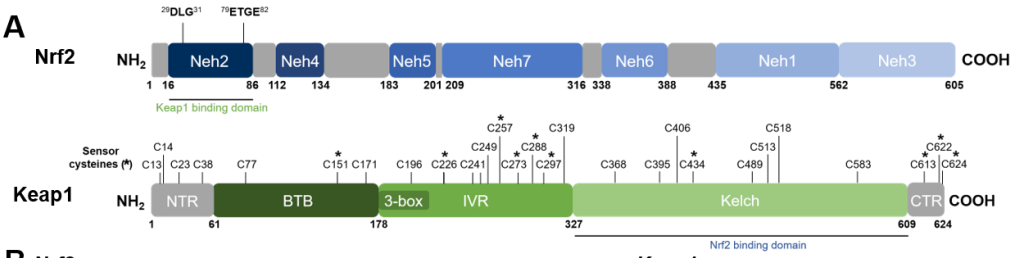
3.2.1 Nrf2 is intrinsically disordered and Keap1's high cysteine content is evolutionarily conserved

Figure 3.1A schematically illustrates the functional domains of human Nrf2 and Keap1. Nrf2 contains seven conserved Neh domains and six cysteine residues, whereas Keap1 contains three functional domains and an abundant 27 cysteine residues. The key oxidative stress-sensing sensor cysteines in Keap1 are indicated with an asterisk (*) (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003; Wakabayashi et al., 2004; McMahon et al., 2010).

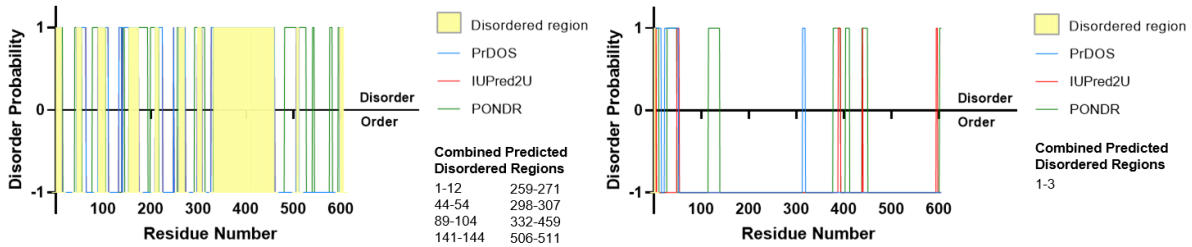
Disordered profile plots for Nrf2 and Keap1 predict the location of intrinsically disordered regions as predicted by three independent algorithms (PrDOS, IUPred2A, and PONDR) (Ishida & Kinoshita, 2007; Xue et al., 2010; Mészáros et al., 2018) (**Figure 3.1B**). (See **Figure S3.1** for the individual algorithm predictions). Intrinsically disordered regions are highlighted in yellow. In our analyses, an amino acid residue is denoted as 'disordered' if it is predicted as 'disordered' by all three individual algorithms, i.e., a prediction value of >0.5 = +1 (disorder) and prediction value of <0.5 = -1 (order); thus, a combined valued of +3 was denoted as 'disordered'. From these results, Nrf2 is predicted to contain 11 intrinsically disordered regions with an overall combined percent disordered score of 39.34%. In comparison, Keap1 is mostly ordered with a single predicted disordered region and an overall combined percent disordered score of 0.48%, which is also corroborated by structural studies of the folded Kelch domain of Keap1 (Li et al., 2004).

Next, we looked at the amino acid composition of Nrf2 and Keap1 and calculated the percentage of cysteine content across 15 metazoan species (**Figure S3.2**) from humans to zebrafish (**Figure 3.1C**). The total cysteine content in human Nrf2 is 0.99%, which is below the average for the human proteome of 2.3% (Miseta & Csutora, 2000), while the average for Nrf2 across all 15 species is 1.11%. Intriguingly, human Keap1 contains an abundant 4.33% cysteine content, which is highly above average; the average for Keap1 across all species is 4.07%. To determine if cysteine residues are evolutionarily conserved across these 15 species, we performed a protein sequence alignment using MEGA X (Kumar et al., 2018) and found that all six cysteine residues in Nrf2 (highlighted in yellow) are either completely or highly conserved across species (i.e., perfectly aligned in 13-15 species) (**Figure 3.1D, top**). For Keap1, 24 of the 27 cysteine residues in human Keap1 are either completely or highly conserved (i.e., perfectly aligned in 12-15 species) (**Figure 3.1D, bottom**). Interestingly, all sensor cysteines within Keap1 (indicated with an asterisk (*)) are either completely or almost completely conserved (i.e., perfectly aligned in 14-15 species).

A



B



Combined Predicted Disordered Regions

1-12	259-271
44-54	298-307
89-104	332-459
141-144	506-511
154-174	597-605
209-216	

Number of residues: 605
Number disordered regions: 11
Number residues disordered: 238
Longest disordered region: 128
Overall percent disordered: 39.33884%

Combined Predicted Disordered Regions
1-3

Number of residues: 624
Number disordered regions: 1
Number residues disordered: 3
Longest disordered region: 3
Overall percent disordered: 0.48077%

C

Nrf2

Species	Specific Name	% Cys
Human	*Homo sapiens*	0.99174
Chimpanzee	*Pan troglodytes*	1.16667
Orangutan	*Pongo abelii*	0.99010
Rhesus macaque	*Macaca mulatta*	0.99010
Marmoset	*Callithrix jacchus*	0.99010
Galago	*Otolemur garnettii*	1.15512
Mouse	*Mus musculus*	1.17253
Rat	*Rattus norvegicus*	1.15894
Golden hamster	*Mesocricetus auratus*	1.16861
Rabbit	*Oryctolagus cuniculus*	0.98847
Cow	*Bos taurus*	1.32670
Bat	*Myotis lucifugus*	0.98847
Elephant	*Loxodonta africana*	1.54639
Chicken	*Gallus gallus*	1.15894
Zebrafish	*Danio rerio*	0.85324
Average % Cys content in Nrf2		**1.10889**

Keap1

Species	Specific Name	% Cys
Human	*Homo sapiens*	4.32692
Chimpanzee	*Pan troglodytes*	4.32692
Orangutan	*Pongo abelii*	4.16667
Rhesus macaque	*Macaca mulatta*	4.00641
Marmoset	*Callithrix jacchus*	4.16667
Galago	*Otolemur garnettii*	4.16667
Mouse	*Mus musculus*	4.00641
Rat	*Rattus norvegicus*	4.00641
Golden hamster	*Mesocricetus auratus*	4.00641
Rabbit	*Oryctolagus cuniculus*	4.00641
Cow	*Bos taurus*	3.84615
Bat	*Myotis lucifugus*	4.00641
Elephant	*Loxodonta africana*	4.00641
Chicken	*Gallus gallus*	4.02010
Zebrafish	*Danio rerio*	3.99334
Average % Cys content in Keap1		**4.07089**

D

Residue # in Human Nrf2	119	199	242	325	422	514
Human	C	C	C	C	C	C
Chimpanzee	C	C	C	C	C	C
Orangutan	C	C	C	C	C	C
Rhesus macaque	C	C	C	C	C	C
Marmoset	C	C	C	C	C	C
Galago	C	C	C	C	C	C
Mouse	C	C	C	C	C	C
Rat	C	C	C	C	C	C
Golden hamster	C	C	C	C	C	C
Rabbit	C	C	C	C	C	C
Cow	C	C	C	C	C	C
Bat	C	C	C	C	C	C
Elephant	C	C	C	C	C	C
Chicken	C	C	C	W	R	C
Zebrafish	C	C	C	G	L	C

Residue # in Huaman Keap1	13	14	23	38	77	151	171	196	226	241	249	257	273	288	297	319	368	395	406	434	489	513	518	583	613	622	624
Key sensor cysteines (*)						*			*			*	*	*	*					*					*	*	*
Human	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Chimpanzee	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Orangutan	C	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Rhesus macaque	C	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	R	C	C	C	C	C	C
Marmoset	C	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Galago	R	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Mouse	S	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Rat	S	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Golden hamster	S	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Rabbit	C	S	R	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Cow	H	T	R	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Bat	H	T	R	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Elephant	H	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Chicken	-	-	-	C	C	C	C	C	C	C	C	C	C	C	C	G	C	C	C	C	A	C	C	C	C	C	C
Zebrafish	-	-	R	F	C	C	C	C	C	C	C	A	I	C	C	H	C	C	L	S	R	V	Y	C	C	E	M

Figure 3.1: Protein disorder analyses and cysteine analyses for Nrf2 and Keap1. (A) Domain maps for Nrf2 and Keap1 showing the location of all cysteine residues. Key oxidative stress-sensing cysteines in Keap1 are marked with an asterisk (*). **(B)** Disordered profile plots for Nrf2 and Keap1 predicting the location of intrinsically disordered regions within each protein as predicted by three independent algorithms (PrDOS, IUPred2A, and PONDR). The intrinsically disordered regions predicted by all three algorithms are highlighted in yellow. **(C)** The percentage of cysteine content is calculated for 15 species from human to zebrafish. **(D)** Protein sequence alignment for cysteine residues in Nrf2 and Keap1 across 15 species. All cysteines are highlighted in yellow. Sensor cysteines within Keap1 are marked with an asterisk (*).

3.2.2 Oxidative stress and Nrf2 and Keap1 expression in yeast

We previously established yeast as a useful tool to study Nrf2 interactions (Ngo et al., 2020, *submitted*). Here, we use growth assays to assess if Nrf2 expression in yeast is affected by the absence of certain oxidative stress genes. Human Nrf2 expressed in yeast causes 'toxicity', defined as an impaired growth phenotype on growth media compared to the empty vector control. Nrf2 was expressed in wild-type yeast and yeast strains deleted for an array of oxidative stress genes. Only significant data is shown; for the complete list of deletion strains, refer to **Figure S3.3**. Yeast AP-1 (Yap1) (Moye-Rowley et al., 1989) is a bZIP transcription factor and essential regulator of the H_2O_2 adaptive response in yeast (Schnell et al., 1992; Kuge & Jones, 1994). Yeast Yap1 oxidant-sensing and the mammalian Keap1-Nrf2 pathway share parallels, including shared target genes (e.g., *GPX2*), although the biochemical details differ (Simaan et al., 2019). The deletion of *YAP1* decreases Nrf2 toxicity (**Figure 3.2A**). Growth is quantified to the right as done previously (Petropavlovskiy et al., 2020). Moreover, Keap1 expression in wild-type yeast is not toxic, but Keap1 toxicity is induced by deletion of the antioxidant genes *BTN1*, *SOD1*, and *TSA2* (**Figure 3.2B**). For the full panel of growth assays, refer to **Figure S3.4** and **Figure S3.5**. For growth assay control plates, refer to **Figure S3.6**.

Yeast cells expressing YFP-tagged wild-type Nrf2 or mutants of Nrf2, L30F and T80R, which have a reduced or impaired capacity to interact with Keap1 (Shibata et al., 2008), were treated with 300 μM hydrogen peroxide (H_2O_2) for 3 h to elicit oxidative stress. A change in Nrf2 localization

patterns was observed, as Nrf2-YFP was no longer diffusely spread throughout the yeast cytoplasm and nucleus but formed fluorescent foci (**Figure 3.2C**). Moreover, when yeast cells expressing Keap1-YFP were treated with 300 µM H_2O_2, Keap1-YFP formed protein inclusions (**Figure 3.2D**). The optimal treatment dose and duration were determined by measuring cell viability in non-transfected HeLa cells to achieve a moderate, dose-dependent response to hydrogen peroxide treatment (**Figure S3.7**).

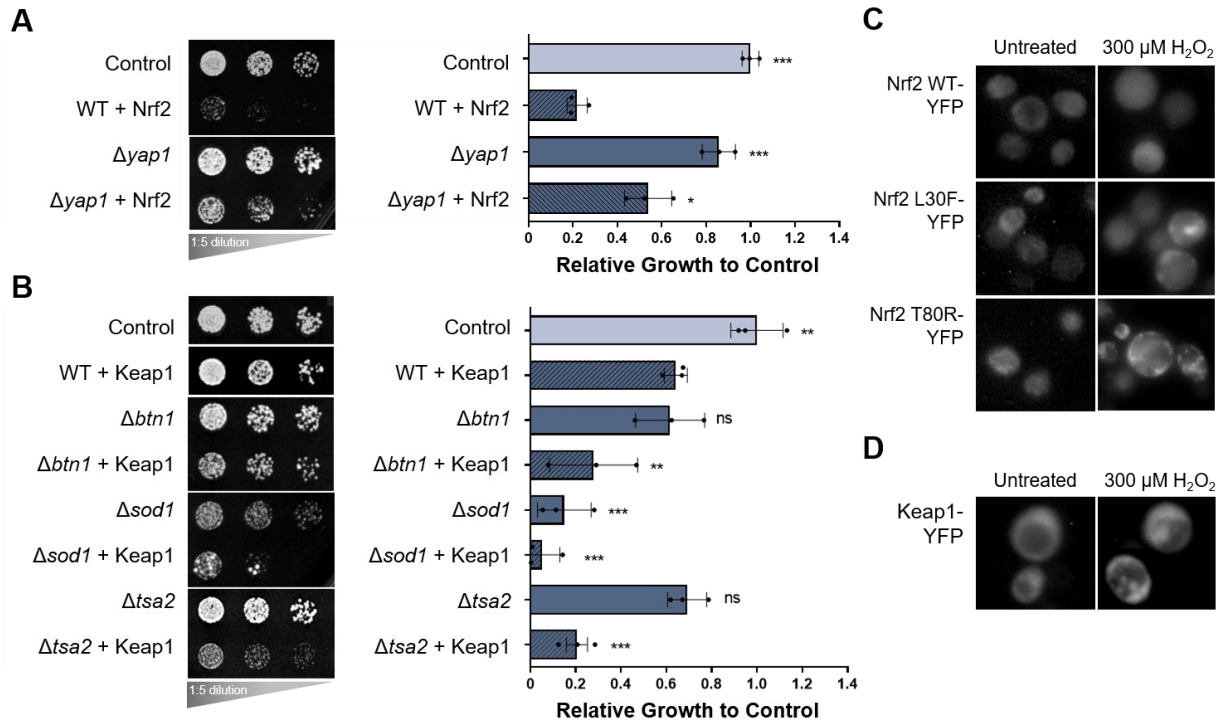


Figure 3.2: Oxidative stress and Nrf2 and Keap1 in yeast. (A) Human Nrf2 and **(B)** human Keap1 transformed into yeast deletion strains for various oxidative stress genes grown on agar plates. Growth is quantified relative to the empty vector control. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(C)** Yeast expressing YFP-tagged Nrf2 and two Nrf2 mutants treated with 300 µM H_2O_2. (D) Yeast expressing Keap1-YFP treated with 300 µM H_2O_2.

3.2.3 Nrf2 forms protein inclusions under oxidative stress conditions in HeLa cells

Figure 3.3A documents the intramolecular localization of wild-type Nrf2 and two Nrf2 mutants, L30F and T80R, in HeLa cells. HeLa cells were transfected with wild-type or mutant GFP-tagged Nrf2 and treated with 100 or 300 μM H_2O_2 for 3 h. The dose and duration were optimized in non-transfected HeLa cells as shown in **Figure S3.7**. Fluorescence microscopy revealed the formation of cytosolic and possibly nuclear protein inclusions of wild-type and mutant Nrf2 in both untreated and treated cells, at endogenous expression levels and even more so when Nrf2 was overexpressed by transient transfection. The percentage of cells with inclusions increased in a hydrogen peroxide dose-dependent manner (**Figure 3.3B**). Nrf2 T80R shows a significantly higher percentage of cells with inclusions compared to wild-type (**Figure 3.3C**). In comparison, no stress-induced protein inclusions were observed for control HeLa cells transfected with GFP alone (**Figure S3.8**). Untreated cells expressing wild-type Nrf2 and Nrf2 L30F demonstrated increased cell viability (determined by the quantification of ATP levels which indicates the presence of metabolically active cells (Crouch et al., 1993)) relative to the untreated control; however, cell viability decreased in all Nrf2-expressing cells upon treatment with 300 μM H_2O_2. These results are recapitulated in the HEK293 cell line (**Figure S3.9**).

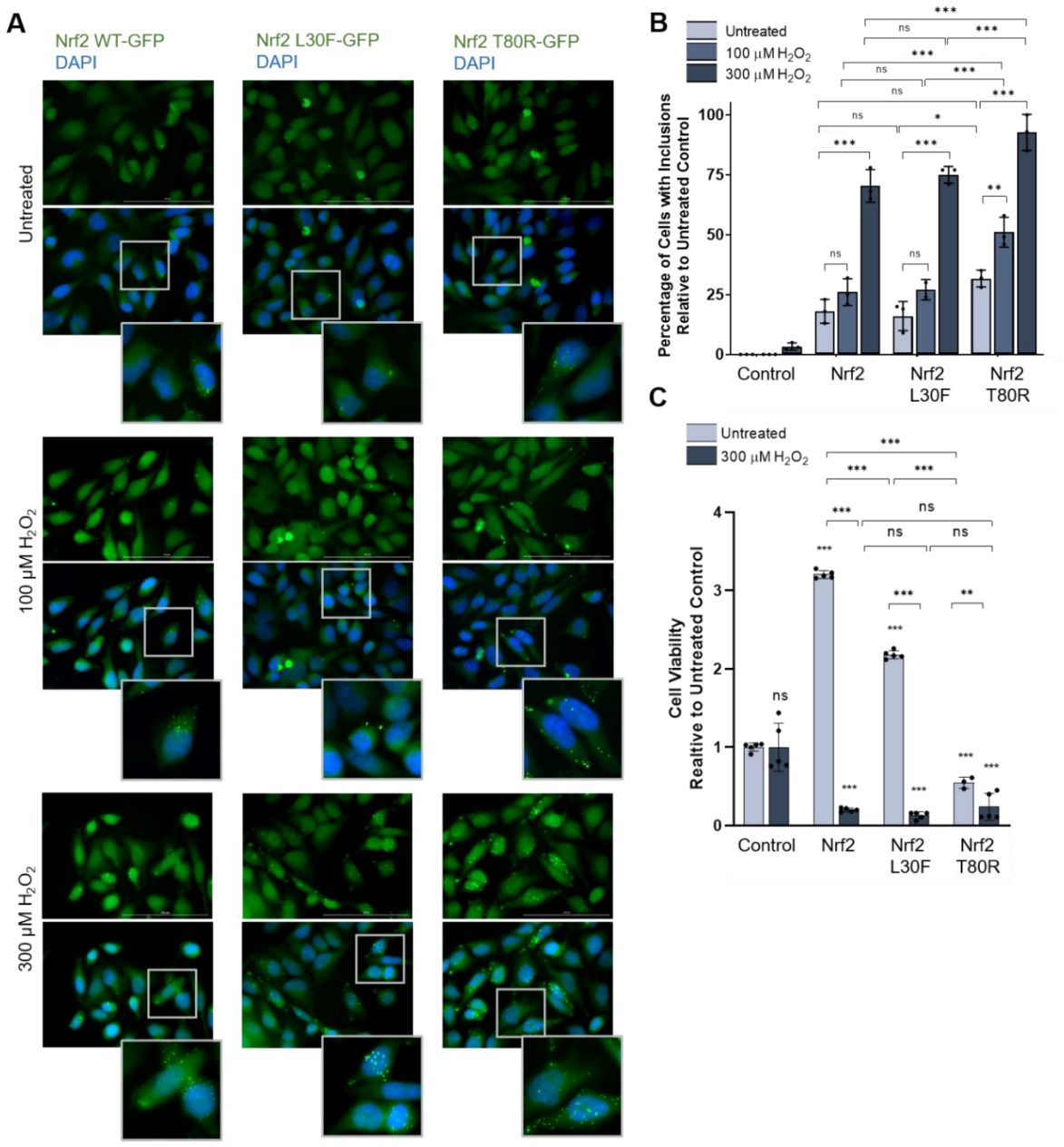


Figure 3.3: Nrf2 forms inclusions upon exposure to oxidative stress in vitro. (A) HeLa cells transfected with GFP-tagged Nrf2 and two Nrf2 mutants and treated with 100 or 300 μM H_2O_2 for 3 h, visualized by fluorescence microscopy. **(B)** Quantification of Nrf2-expressing cells with inclusions following hydrogen peroxide treatment observed in (A). **(C)** Cell viability assays for Nrf2-expressing cells treated with 300 μM H_2O_2 for 3 h. (B, C) Means derived from a minimum of three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.2.4 Nrf2 inclusion formation is oxidative stress-specific and prevented by certain antioxidants

To determine whether oxidative-stress induced Nrf2 inclusion formation is an artifact of overexpression by transient transfection, un-transfected HeLa cells were treated with 300 μM H_2O_2 for 3 h and observed by immunofluorescence for endogenous Nrf2. The localization patterns for endogenous Nrf2 are similar to that of transfected Nrf2 with hydrogen peroxide treatment (**Figure 3.4A**), confirming that this observed effect is likely, not due to Nrf2 overexpression. To determine if Nrf2 inclusion formation is oxidative stress-specific, HeLa cells expressing Nrf2-GFP were treated with 50 μM MG132, a proteasome inhibitor that elicits general protein misfolding stress. Compared to cells treated with 300 μM H_2O_2 which formed some Nrf2 inclusions, treatment with MG132 did not result in the formation of cytosolic Nrf2 inclusions (**Figure 3.4B**).

Furthermore, we explored if treatment with antioxidants, such as N-acetylcysteine (NAC) and vitamin C (ascorbic acid), prevents Nrf2 inclusion formation. Transfected cells were pre-treated with 3 μM NAC or 100 μM vitamin C for 24 h and subsequently treated with 300 μM H_2O_2 for 3 h. A reduction in Nrf2 inclusion formation was observed for pre-treatment with NAC but not vitamin C (**Figure 3.4C**).

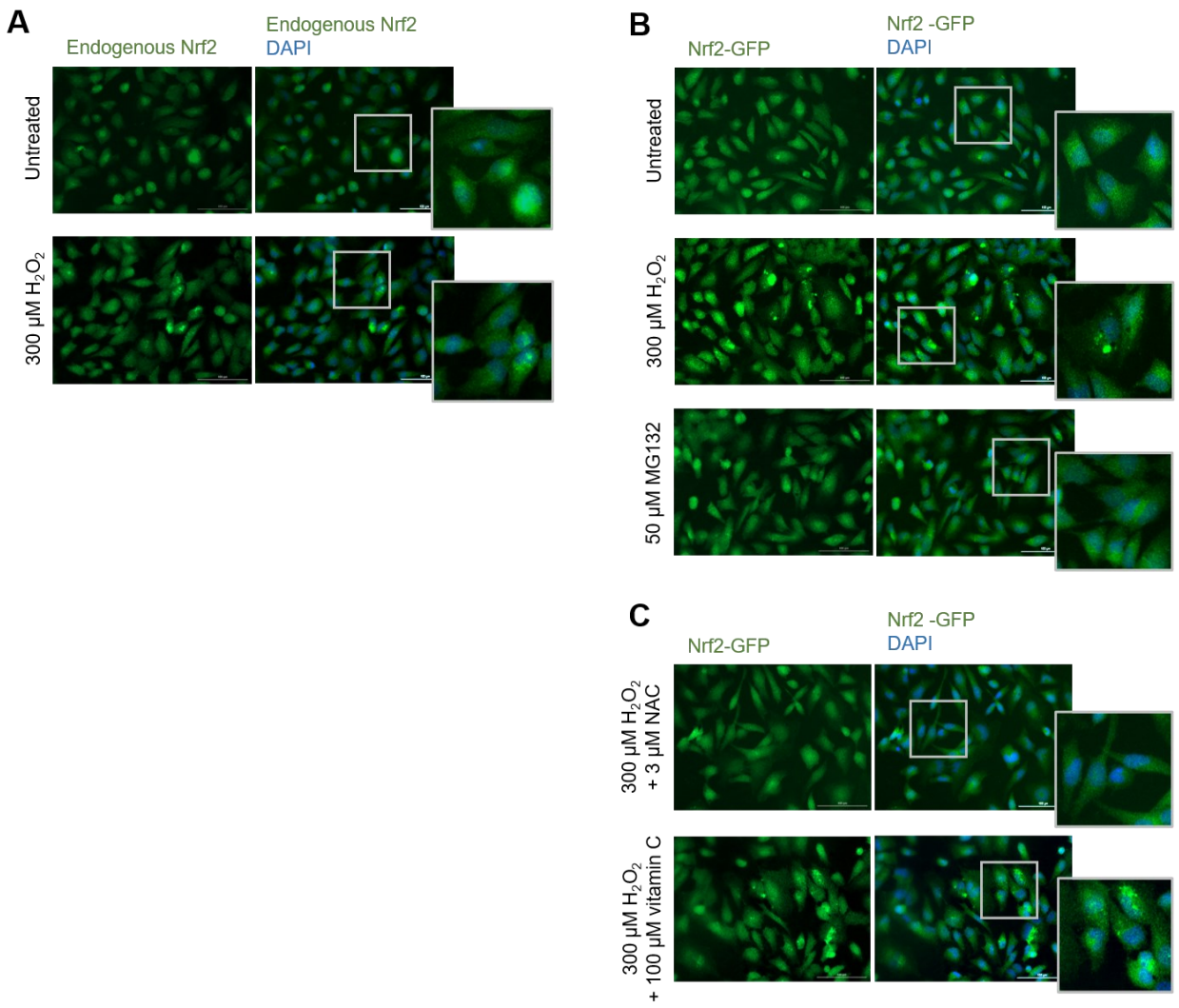


Figure 3.4: Further analyses of the oxidative stress-induced protein misfolding of Nrf2. **(A)** Endogenous Nrf2 in un-transfected HeLa cells treated with 300 μM H_2O_2 for 3 h, visualized by immunofluorescence microscopy. **(B)** Transfected Nrf2-GFP in HeLa cells treated with 50 μM MG132 for 6 h, visualized by fluorescence microscopy. **(C)** Transfected Keap1-GFP in HeLa cells pretreated with 3 μM NAC and or 100 μM vitamin C for 24 h followed by treatment with 300 μM H_2O_2 for 3 h, visualized by fluorescence microscopy.

3.2.5 Keap1 forms protein inclusions under oxidative stress conditions in HeLa cells

HeLa cells were transfected with GFP-tagged Keap1 and treated with 100 or 300 μM H_2O_2 for 3 h. Fluorescence microscopy revealed the formation of cytosolic and possibly nuclear protein inclusions in both untreated and treated cells (**Figure 3.5A**). Confocal microscopy was used to visualize these inclusions at a higher resolution (**Figure 3.5B**) and 3-dimensional stacking reveals that Keap1 inclusions are situated around the nucleus rather than within it (**Figure 3.5C**), thus, confirming these inclusions are cytosolic. Quantification of the percentage of cells containing inclusions reveals that the oxidative stress-induced Keap1 inclusions form in a hydrogen peroxide dose-dependent manner (**Figure 3.5D**). Cells expressing Keap1 demonstrated increased cell viability with hydrogen peroxide treatment (determined by the quantification of ATP levels (Crouch et al., 1993)) (**Figure 3.5E**). Results are recapitulated in the HEK293 cell line (**Figure S3.9**).

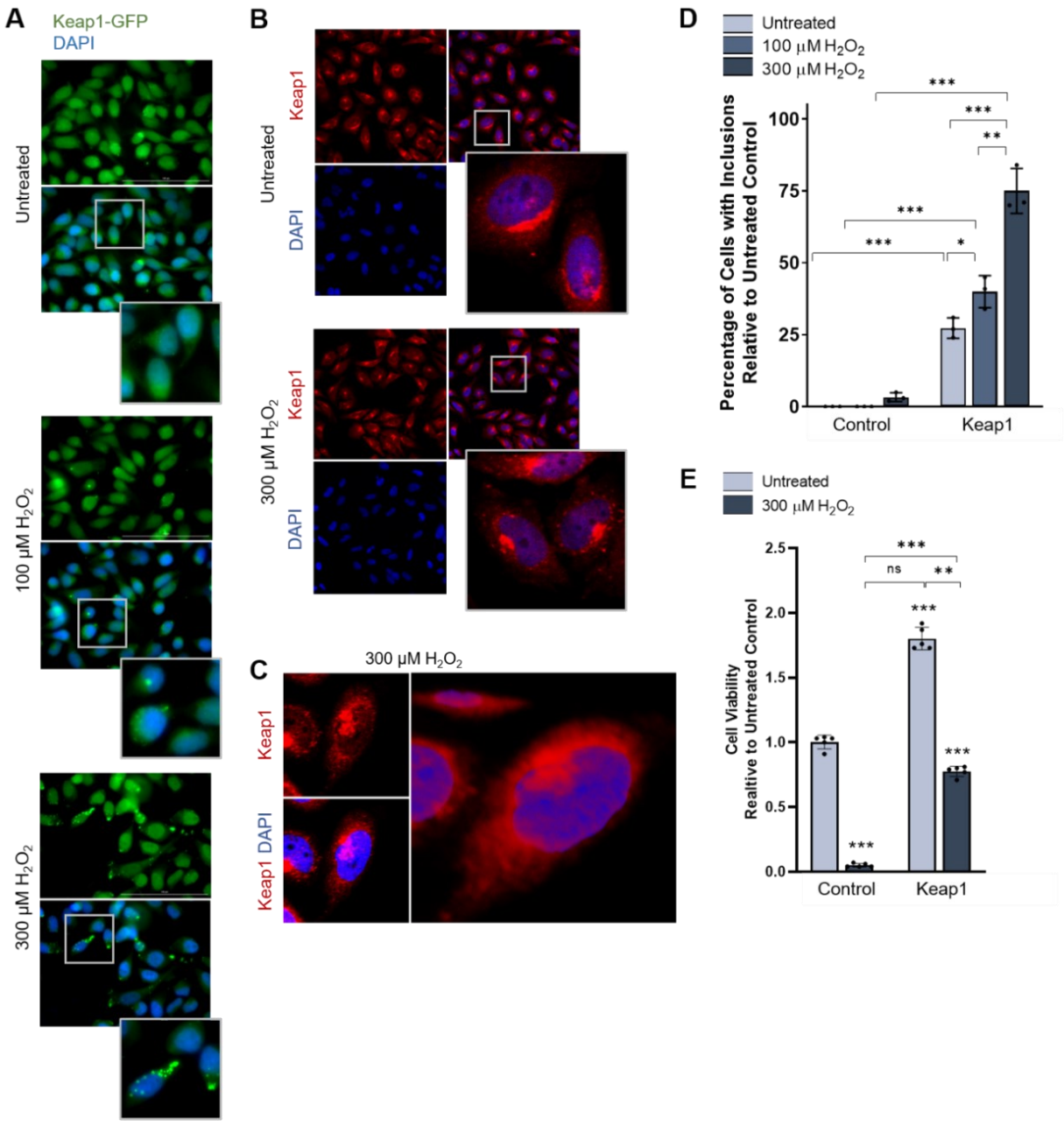


Figure 3.5: Keap1 forms inclusions upon exposure to oxidative stress in vitro. (A) HeLa cells transfected with Keap1-GFP and treated with 100 or 300 μM H_2O_2 for 3 h, visualized by fluorescence microscopy. **(B)** Confocal microscopy for Keap1-expressing cells treated with 300 μM H_2O_2 for 3 h, visualized by immunofluorescence. **(C)** Confocal microscopy with 3-D stacking for Keap1-expressing cells treated with hydrogen peroxide, visualized by immunofluorescence and demonstrating that Keap1 inclusions are cytosolic. **(D)** Quantification of Keap1-expressing cells with inclusions following hydrogen peroxide treatment in (A). **(E)** Cell viability assay for

Keap1-expressing cells treated with 300 μM H_2O_2 for 3 h. (D, E) Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.2.6 Keap1 inclusion formation is oxidative stress-specific and cannot be prevented by tested antioxidants

Again, to determine whether oxidative stress-induced Keap1 inclusion formation is an artifact of overexpression by transient transfection, un-transfected HeLa cells were treated with 300 μM H_2O_2 for 3 h and observed using immunofluorescence microscopy for endogenous Keap1. Endogenous Keap1 also formed inclusions upon treatment with hydrogen peroxide (**Figure 3.6A**), confirming that this observed effect is not due to Keap1 overexpression. To determine if Keap1 inclusion formation is oxidative stress-specific, HeLa cells expressing Keap1-GFP were treated with 50 μM MG132 to elicit general protein misfolding stress. Compared to cells treated with 300 μM H_2O_2 which formed Keap1 inclusions, treatment with MG132 did not result in the formation of inclusions (**Figure 3.6B**).

Finally, we determined if NAC and vitamin C could prevent Keap1 inclusion formation upon oxidative stress. Transfected cells were pre-treated with 3 μM NAC or 100 μM vitamin C for 24 h and subsequently treated with 300 μM H_2O_2 for 3 h; however, no significant reduction in Keap1 inclusions was observed (**Figure 3.6C**).

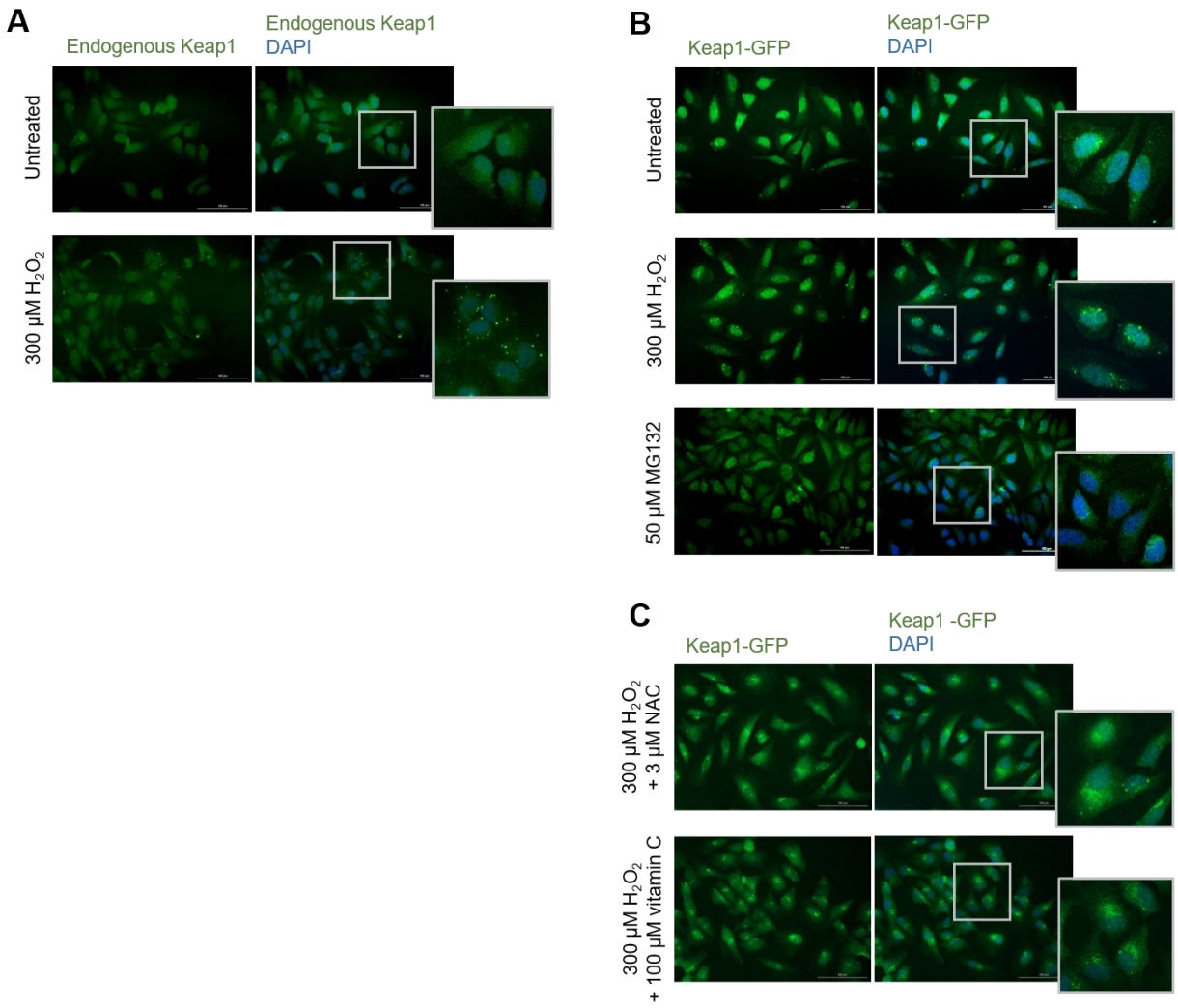


Figure 3.6: Further analyses of Keap1 oxidative stress-induced protein misfolding. **(A)** Endogenous Keap1 in un-transfected HeLa cells treated with 300 µM H_2O_2 for 3 h, visualized by immunofluorescence microscopy. **(B)** Transfected Keap1-GFP in HeLa cells treated with 50 µM MG132 for 6 h, visualized by fluorescence microscopy. **(C)** Transfected Keap1-GFP in HeLa cells pretreated with 3 µM NAC or 100 µM vitamin C for 24 h followed by treatment with 300 µM H_2O_2 for 3 h, visualized by fluorescence microscopy.

3.2.7 Oxidative stress-induced Keap1 inclusion formation in breast cancer cell lines

To ensure that Keap1 stress-induced inclusion formation is not a HeLa cell-specific phenomenon, we treated two human breast cancer cell lines, 21MT-1 and SKBR3, with 300 µM H_2O_2 for 3 h and performed immunofluorescence microscopy for endogenous Keap1. Upon oxidative stress, both cell lines showed Keap1 inclusions (**Figure 3.7A** and **Figure 3.7B**), quantified to be statistically significant from the untreated control (**Figure 3.7C**).

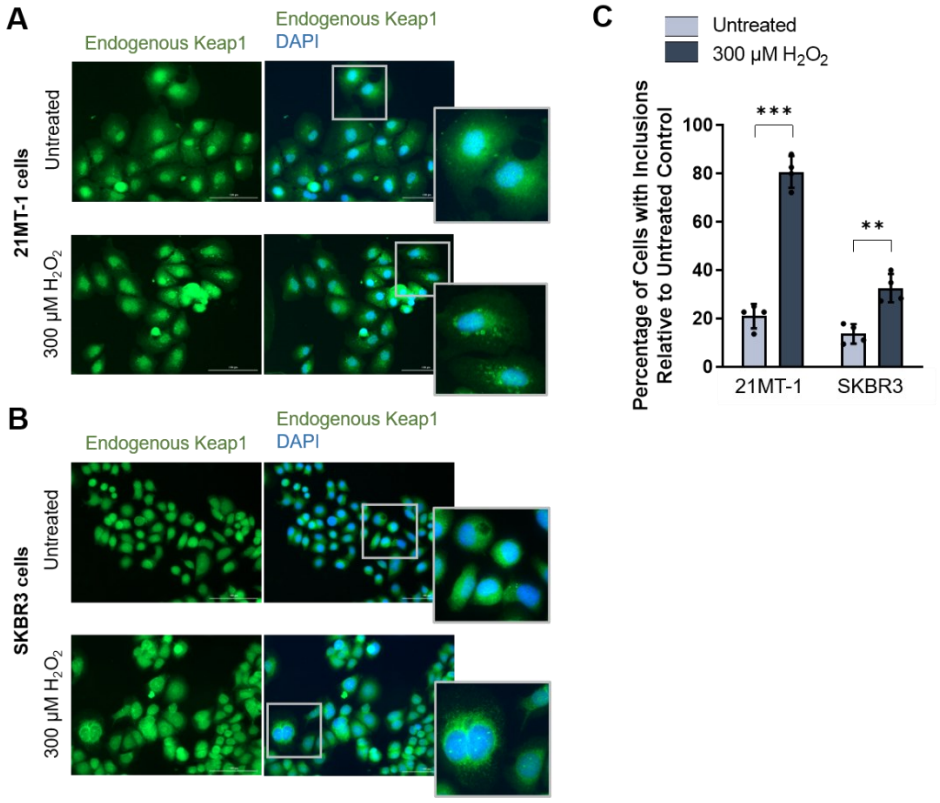


Figure 3.7: Keap1 forms oxidative stress-induced inclusions in breast cancer cell lines. (A-B) Endogenous Keap1 expression in two breast cancer cell lines, 21MT-1 and SKBR3, treated with 300 µM H_2O_2 for 3 h, visualized by immunofluorescence microscopy. **(C)** Quantification of Keap1 inclusions following hydrogen peroxide treatment. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.2.8 Purified proteins for Nrf2 and Keap1 form inclusions upon exposure to oxidative stress

Purified proteins were used to biochemically assess Nrf2 and Keap1 misfolding and inclusion formation (or aggregation) using two methods: (1) traditional SDS-PAGE with Coomassie blue gel staining, and (2) semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) with western bot analysis. As shown in **Figure 3.8A**, purified Nrf2 was incubated with 600 μM H_2O_2 in the absence and presence of reducing agent β-mercaptoethanol (βME). Upon treatment with hydrogen peroxide, Nrf2 forms a dense high molecular weight smear, indicating the formation of aggregated, higher molecular weight species. With the addition of 5% βME, this structure collapses, indicating that this high molecular weight protein species is, to some degree, dependent upon disulfide bonds. Furthermore, analysis of purified Neh5 domain of Nrf2, which harbours one of the six cysteines in the protein, revealed the formation of an aggregated higher molecular weight species with hydrogen peroxide treatment that also collapses with the addition of βME (**Figure 3.8B**).

Similarly, purified Keap1 protein was treated with 600 μM H_2O_2 in the presence and absence of βME. Upon treatment with hydrogen peroxide, Keap1 forms a dense high molecular weight smear, indicating the formation of an aggregated higher molecular weight species. With the addition of 5% βME, this structure collapses (**Figure 3.8C)**, indicating that this high molecular weight protein species is also, at least in part, dependent on the formation of disulfide bonds. Purified protein for Keap1's Kelch domain was then used to determine if this domain alone, which contains eight cysteine residues, would misfold under oxidative stress conditions. Unlike full-length Keap1, treatment of the Kelch domain alone with 600 μM H_2O_2 did not produce a high molecular weight species (**Figure 3.8D**).

Finally, fractionation assays were used to determine the soluble and aggregated fractions for purified Nrf2 and Keap1 (**Figure 3.8E**). The 'total' purified protein sample was centrifuged and divided into the soluble 'supernatant' fraction and the aggregated 'pellet' fraction and resolved using traditional SDS-PAGE with Coomassie blue gel staining. Both Nrf2 and Keap1 contain soluble and aggregated protein fractions in the supernatant and pellet, respectively.

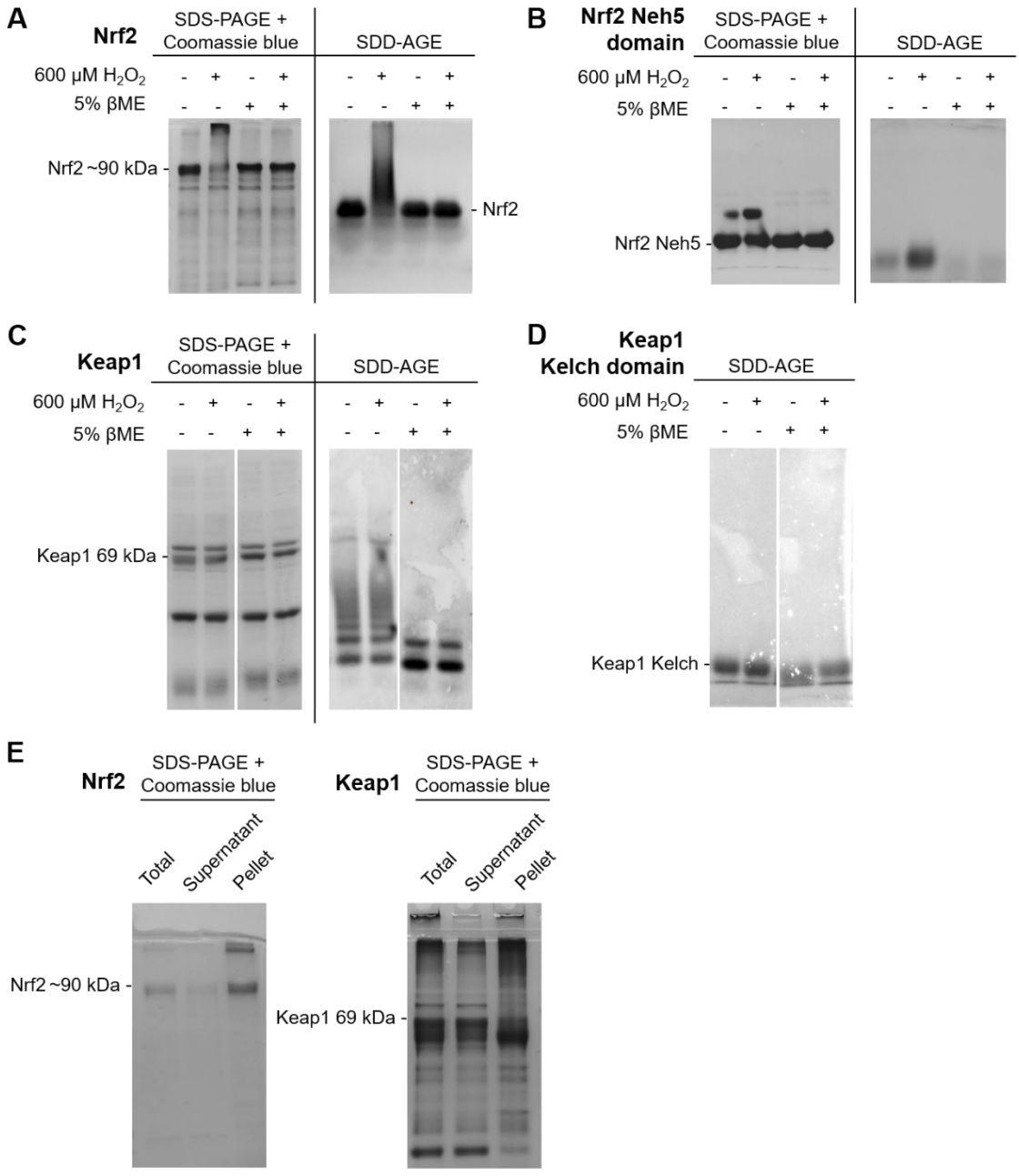


Figure 3.8: Nrf2 and Keap1 purified proteins aggregate upon exposure to oxidative stress *in vitro*. Purified protein treated with 600 µM H_2O_2 ± βME for **(A)** Nrf2, **(B)** Nrf2's Neh5 domain, **(C)** Keap1, and **(D)** Keap1's Kelch domain. **(E)** Fractionation assay for purified Nrf2 and Keap1 protein. All purified proteins are resolved using SDS-PAGE with Coomassie Blue gel staining and/or SDD-AGE, as indicated.

3.3 Discussion

In this work, we demonstrate that two key proteins of the antioxidant pathway, Nrf2 and Keap1, form intracellular inclusions upon exposure to high levels of oxidative stress. We find that at least in part, the aberrant formation of disulfide bonds causes the misfolding and inclusion formation of both proteins. Nrf2's intrinsically disordered nature may also contribute to its propensity to misfold.

We observe that in both yeast and mammalian cells, treatment of cells expressing Nrf2 with hydrogen peroxide results in the formation of protein inclusions in a dose-dependent manner. Interestingly, protein inclusion formation was exacerbated for the Nrf2 mutants, L30F and T80R, wherein Keap1-binding to the Neh2 domain in Nrf2 at the low- and high-affinity motifs, respectively, is impaired, resulting in loss of Keap1-mediated degradation (Tong et al., 2006; Shibata et al., 2008). Oxidatively damaged Nrf2 accumulates in the cell as inclusions, most notably for the Nrf2 T80R mutant which escapes all Keap1-mediated degradation. This could explain the significantly high levels of Nrf2 T08R inclusions. On the other hand, Nrf2 L30F demonstrates impaired binding at the low-affinity motif but may still retain some Nrf2-binding via the high-affinity motif. Nonetheless, ubiquitination is unlikely without the intact low-affinity binding site (Tong et al., 2007). Future work will further investigate the consequence of these mutants and how impaired Keap1-dependent Nrf2 regulation affects stress-induced inclusion formation.

The Neh2 domain of Nrf2 has previously been characterized as intrinsically disordered (Tong et al., 2006) and our data suggest that other regions within Nrf2 are also highly intrinsically disordered. While this structural flexibility could allow Nrf2 to bind to a large number of different proteins (Nam & Keum, 2019), it may also render Nrf2 susceptible to aberrant protein misfolding. Intrinsically disordered proteins tend to misfold under certain conditions (Uversky, 2011) which appears to also be the case for Nrf2 during high levels of oxidative stress. This misfolding and inclusion formation of Nrf2 could be an *adaptive* mechanism of Nrf2 regulation (i.e., the 'functional misfolding' of IDPs (Uversky, 2011)) which allows Nrf2 to escape Keap1 degradation, resulting in ARE activation) or a *maladaptive* one (i.e., misfolded Nrf2 cannot activate ARE-containing genes in response to oxidative stress). To determine this, future work will assess the functional outcome of this stress-induced Nrf2 misfolding and its consequences on Nrf2 regulation and activity.

Moreover, wildtype Nrf2 contains only six cysteine residues but our *in vitro* experiments document that at least some of the cysteines in Nrf2 are oxidized and are central to the formation of high molecular weight protein species. It is plausible that oxidation-induced inclusion formation impairs the transcriptional activity of Nrf2, as He et al. have found that some cysteine residues of Nrf2 play important roles in oxidant sensing, Keap1-dependent ubiquitination and degradation of Nrf2, and in the transcriptional activation of ARE-containing Nrf2 target genes (He & Ma, 2009). In contrast, Keap1 is not an IDP and its misfolding may be mostly dependent on the presence of reactive cysteine residues. Keap1 contains 27 cysteines, 24 of which were found to be highly or completely conserved. Interestingly, except for the chicken and zebrafish, all key sensor cysteines within Keap1 (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003; Wakabayashi et al., 2004; McMahon et al., 2010) were completely or almost completely conserved, which demonstrates their importance for Keap1 function. Cystines are one of the least abundant amino acids in mammals, comprising an average of 2.3% for the human proteome (Miseta & Csutora, 2000). Yet we find that human Keap1 contains 4.33% cysteine content, almost double the average for the human proteome (Miseta & Csutora, 2000). This high content of cysteine residues in Keap1 may render it more susceptible to oxidation, for example by aberrant disulfide bond formation. Indeed, we find that Keap1 forms inclusions and high molecular weight protein species upon treatment with hydrogen peroxide in yeast, mammalian cells, and purified proteins. Of note, our *in vitro* data argue that the cysteines within the stably folded Kelch domain of Keap1 are not susceptible to oxidation and misfolding for the Kelch domain alone, which is consistent with the notion that not all cysteine residues within Keap1 are equally reactive. Future work must determine which cysteines in Keap1 are most susceptible to oxidation and inclusion formation (e.g., by cysteine mutation analyses), and if the full-length protein is required for this misfolding to occur.

Under normal conditions, Keap1 is constantly shuttling between the cytosol and the nucleus via importin α7 (also known as karyopherin α6, KPNA6), and the nuclear import of Keap1 represses the Nrf2 antioxidant response (Sun et al., 2011); however, our results show that misfolded Keap1 inclusions are cytosolic and cannot enter the nucleus. Misfolded Keap1 may render the protein inactive, impairing Keap1-mediated degradation of both cytosolic and nuclear Nrf2. We speculate that Keap1, upon forming aberrant intra- and/or inter-molecular disulfide bonds, cannot bind to Nrf2 to target it for degradation, thus allowing free Nrf2 to activate the antioxidant response. It is plausible that this Keap1 inactivation is irreversible and that even newly synthesized Keap1 cannot

escape this oxidation-based inactivation under high levels of oxidative stress, thus causing a long-lasting constitutive activation of Nrf2. This mechanism may be predominant in cancer, where ROS levels are high due to high metabolic activity and genetic instability (Kumari et al., 2018). However, whether misfolded Nrf2 is still functional remains to be explored and will be subsequently investigated.

It is important to mention the work by Taguchi et al. which proposes that oxidative stress causes Keap1 misfolding and its sequestration into inclusion bodies that are removed by p62/SQSTM1 (Taguchi et al., 2012). This is a mechanism that is not mutually exclusive to the one proposed in this study; however, it is also important to note that the inclusion bodies observed by Taguchi et al. seem to differ in morphology compared to the inclusions observed in this study and that the end product of the p62-Keap1 interaction is Keap1 degradation by autophagy, which is not observed here. In addition, purified Keap1 protein misfolds and forms inclusions upon oxidative stress treatment in a test tube scenario where p62 is absent. Importantly, future work must still be done to further establish this mechanism and how it differs from p62-mediated autophagy.

Taken together, we employed yeast models, cultured mammalian cells, and purified proteins to assess the oxidative stress-induced inclusion formation of Nrf2 and Keap1. We argue that the intrinsically disordered nature of Nrf2 exposes its cysteine residues to ROS and thus makes it more prone to misfolding, while the more structured Keap1's unusually high content of cysteine residues makes the protein more susceptible to misfolding by aberrant disulfide bond formation. Our work provides new insight into previously unexplored aspects of Nrf2 regulation by oxidation-dependent protein inclusion formation, and future work will seek to explore the functional outcome of this oxidation event in normal cells and cancer.

3.4 Materials and Methods

3.4.1 Prediction of intrinsically disordered regions

Three independent algorithms, PrDOS, IUPred2A, and PONDR (Ishida & Kinoshita, 2007; Xue et al., 2010; Mészáros et al., 2018) were used to predict intrinsically disordered regions using protein sequences obtained from UniProt ("The Universal Protein Resource (UniProt)," 2008). Using the scores obtained from each algorithm, each amino acid residue within the protein was

assigned a numerical value of '+1' or '-1', where >0.5 = +1 (disordered) and values <0.5 = -1 (ordered). The scores were summed for each residue. Residues with a combined score of +3 across all three algorithms were considered 'disordered'.

3.4.2 Protein sequence alignment

MEGA X (Kumar et al., 2018) was used to perform protein sequence alignments using protein sequences obtained from UniProt ("The Universal Protein Resource (UniProt)," 2008).

3.4.3 Yeast growth assays and microscopy

For assessment of relative growth, wild-type yeast and deletion strains obtained from the *Saccharomyces* Genome Deletion Project (Thomas & Rothstein, 1989) were used. Yeast cells were transformed using the standard lithium acetate/salmon sperm carrier DNA/PEG method for the incorporation of yeast plasmids (Gietz & Schiestl, 2007). Transformed yeast cells were grown overnight in synthetic selective media to maintain these plasmids. Growth assays were performed by spotting 5X serial dilutions of OD_{600} = 0.2 on agar plates and incubated at 30°C. Plates were imaged using the Bio-Rad ChemiDoc (Bio-Rad). Growth was quantified as previously described (Petropavlovskiy et al., 2020). For assessment of protein expression using fluorescence microscopy, yeast was transformed with YFP-tagged plasmids. Cells were transferred to a glass microscope slide and coverslip and imaged using the Olympus BX-51 Bright Field/Fluorescence Microscope at 60X and captured using an equipped CCD camera (Spot Pursuit).

3.4.4 Cell lines and culture conditions

The HeLa and HEK293 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, 41966-029), supplemented with 10% FBS (Wisent, 080-150) and 1X penicillin-streptomycin (Corning, 30-001-CI). Cells were cultured at 37°C with 5% CO_2. For transfections, cells were seeded in a 6-well plate at $1.0x10^6$ cells per well and grown to approximately 80% confluency. Cells were transfected with Lipofectamine LTX with PLUS Reagent (Thermo Fisher Scientific, A12621) according to the manufacturer's protocol in Opti-MEM I Reduced Serum Medium (Gibco, 31985-062). Transfected cells were incubated at 37°C for 6 h, followed by a wash

in 1X PBS, and incubated in DMEM for 18 h at 37°C. Cells were then split into the appropriate plates for subsequent experiments.

3.4.5 Fluorescence and immunofluorescence microscopy

Transfected HeLa cells were seeded on a 15 mm circular glass coverslip (Matsunami, C015001) in a 12-well plate at $1x10^5$ to ensure approximately 80% confluency the following day. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked with 20% goat head serum in PBB (0.5% BSA in PBS), and incubated with one of the following primary antibodies overnight at 4°C at a concentration of 1:100: mouse anti-Nrf2 (Abcam, ab62352) or mouse anti-Keap1 (Proteintech, 10503-2-AP). The coverslips were incubated with the following Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature at a concentration of 1:1500: goat anti-mouse (Thermo Fisher Scientific, A-11094). Coverslips were mounted onto glass microscope slides with SlowFade Gold Antifade Reagent with DAPI (Thermo Fisher Scientific, S36938) and cured at room temperature for 24 h. Cells were imaged using the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) using a 20X objective lens.

3.4.6 Cell viability assays

Transfected HeLa cells were seeded into 96-well solid white microplates (Greiner, M0187-32EA) at $4x10^4$ cells per well and incubated for 16 h. Following treatment, cell viability was assessed using the CellTiter-Glo 2.0 Cell Viability Assay (Promega, G9242) according to the manufacturer's protocol. Luminescence was measured using the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek).

3.4.7 SDS-PAGE and Coomassie blue gel staining

Purified protein (10 µg) was resolved on a 10% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue (0.1% Coomassie Brilliant Blue R-250, 50% methanol [v/v], 10% glacial acetic acid [v/v], 40% H_2O) for 30 minutes and de-stained overnight using a destining solution (50% methanol [v/v], 10% glacial acetic acid [v/v], 40% H_2O) with gentle agitation. Blots were imaged using the ChemiDoc Imaging System (Bio-Rad).

3.4.8 SDD-AGE (semi-denaturing detergent agarose gel electrophoresis)

Purified protein (10 μg) was resolved on a 1.8% agaraose-2% SDS gel and run at 80 V for approximately 1.5 h at room temperature in 1X TAE-0.1% SDS running buffer. The gel was transferred at room temperature to PVDF by an overnight wet transfer by gravity according to the manufacturer's protocol (Whatman TurboBlotter Transfer System). The membrane was blocked with 5% skim milk in PBST (phosphate-buffered saline, 1% Tween-20) and incubated with one of the following primary antibodies overnight at 4°C at a concentration of 1:1000: mouse anti-Nrf2 (Abcam, ab62352) or mouse anti-Keap1 (Proteintech, 10503-2-AP). The membrane was incubated with the following Alexa Fluor 680-conjugated antibody for 1 h at room temperature at a concentration of 1:1500: goat anti-mouse (Thermo Fisher Scientific, A-21057). Blots were imaged using the ChemiDoc Imaging System (Bio-Rad).

3.4.9 Combined SDD-AGE and fractionation assay

For partitioning into soluble and insoluble fractions, purified protein was first aliquoted into a chilled microcentrifuge tube which represents the 'total' fraction. A second aliquot was centrifuged at 10 000xg for 10 min at 4°C. The supernatant was transferred into a chilled microcentrifuge tube which represented the soluble 'supernatant' fraction. The pellet was resuspended in dialysis buffer (from the preceding protein purification process) and represents the insoluble 'pellet' fraction. Equal volumes of each fraction equivalent to 10 μg of the total fraction were resolved by SDD-AGE (see **Section 3.4.8.**).

3.4.10 Statistical analysis

Statistical analyses were conducted using Prism 8 (GraphPad Software). Statistical significance was obtained by performing a one-way ANOVA with Tukey post hoc for comparison between groups. Error bars represent standard deviation. P-values less than 0.05 were considered statistically significant. Significance levels are indicated using asterisks, where * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$. Shapiro-Wilk tests were performed for all data sets to ensure normality.

3.5 Supplementary Figures

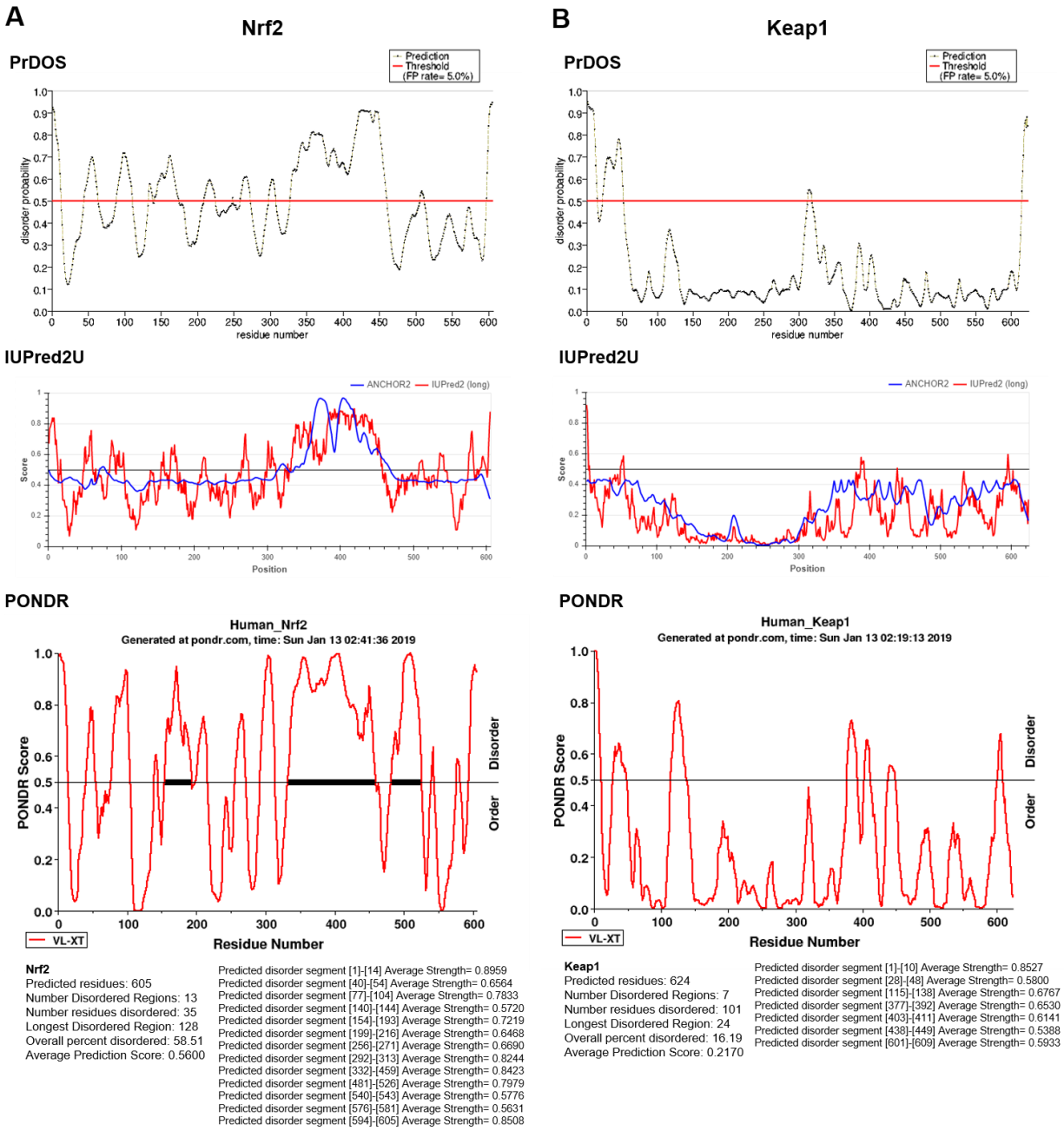


Figure S3.1: Individual disordered profile plots for PrDOS, IUPred2U, and PONDR. Disorder probability prediction graphs for Nrf2 and Keap1 are shown for all three prediction algorithms. A score of >0.5 predicts 'disorder' and a score of <0.5 predicts 'order'.

	Species	Specific Name	UniProt Code
Nrf2	Human	*Homo sapiens*	Q16236
	Chimpanzee	*Pan troglodytes*	H2RAX5
	Orangutan	*Pongo abelii*	H2P7Y6
	Rhesus macaque	*Macaca mulatta*	F7GPD8
	Marmoset	*Callithrix jacchus*	F7CLI8
	Galago	*Otolemur garnettii*	H0Y129
	Mouse	*Mus musculus*	Q60795
	Rat	*Rattus norvegicus*	O54968
	Golden hamster	*Mesocricetus auratus*	A0A1U7QFW3
	Rabbit	*Oryctolagus cuniculus*	G1SEJ1
	Cow	*Bos taurus*	Q5NUA6
	Bat	*Myotis lucifugus*	G1P184
	Elephant	*Loxodonta africana*	G3TGN3
	Chicken	*Gallus gallus*	F1P315
	Zebrafish	*Danio rerio*	Q7ZVI2
Keap1	Human	*Homo sapiens*	Q14145
	Chimpanzee	*Pan troglodytes*	H2QFB9
	Orangutan	*Pongo abelii*	Q5R774
	Rhesus macaque	*Macaca mulatta*	G7NL03
	Marmoset	*Callithrix jacchus*	F7HDW0
	Galago	*Otolemur garnettii*	H0X799
	Mouse	*Mus musculus*	Q9Z2X8
	Golden hamster	*Mesocricetus auratus*	A0A1U7R3C2
	Rat	*Rattus norvegicus*	P57790
	Rabbit	*Oryctolagus cuniculus*	G1SFF4
	Cow	*Bos taurus*	A7MBG4
	Bat	*Myotis lucifugus*	G1PRL8
	Elephant	*Loxodonta africana*	G3TJS6
	Chicken	*Gallus gallus*	Q5ZL67
	Zebrafish	*Danio rerio*	E7FB56

Figure S3.2: The 15 species observed in cysteine analysis studies. The species name, specific name, and UniProt code are shown.

Gene	Protein	**Function** (UniProt Consortium)	UniProt Code
BTN2	Protein BTN2	V-SNARE binding protein that facilitates specific protein retrieval from a late endosome to the Golgi. Modulates the rate of arginine uptake. Involved in pH homeostasis.	P53286
CTA1	Peroxisomal catalase A	Occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide.	P15202
GLR1	Glutathione reductase	Maintains high levels of reduced glutathione in the cytosol.	P41921
SOD1	Superoxide dismutase [Cu-Zn]	Destroys radicals which are normally produced within the cells and which are toxic to biological systems.	P00445
SOD2	Superoxide dismutase	Destroys radicals which are normally produced within the cells and which are toxic to biological systems.	S4VPL7
UBI4	Polyubiquitin	Becomes conjugated to proteins, marking them for selective degradation via the ubiquitin-26S proteasome system.	P0CG63
YAP1	AP-1-like transcription factor YAP1	Transcription activator involved in oxidative stress response and redox homeostasis. Regulates the transcription of genes encoding antioxidant enzymes and components of thiol-reducing pathways.	P19880
SNO4	Probable glutathione-independent glyoxalase SNO4	Catalyzes the conversion of methylglyoxal (MG) to D-lactate in a single glutathione (GSH)-independent step. May play a role in detoxifying endogenously produced glyoxals. Involved in protection against reactive oxygen species (ROS).	Q04902
SRX1	Sulfiredoxin	Contributes to oxidative stress resistance by reducing cysteine-sulfinic acid formed under exposure to oxidants in the peroxiredoxin TSA1. May catalyze the reduction in a multi-step process by acting both as a specific phosphotransferase and as thioltransferase.	P36077
TSA1	Peroxiredoxin TSA1	Thiol-specific peroxidase catalyzing the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively.	P34760
TSA2	Peroxiredoxin TSA2	Thiol-specific peroxidase catalyzing the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively.	Q04120
TRX1	Thioredoxin-1	Participates as a hydrogen donor in redox reactions through the reversible oxidation of its active center dithiol to a disulfide, accompanied by the transfer of 2 electrons and 2 protons.	P22217
TRX2	Thioredoxin-2	Participates as a hydrogen donor in redox reactions through the reversible oxidation of its active center dithiol to a disulfide, accompanied by the transfer of 2 electrons and 2 protons.	P22803

Figure S3.3: Yeast oxidative stress gene deletion strains used in this study. The gene name, protein name, function (obtained from UniProt), and UniProt Code are shown.

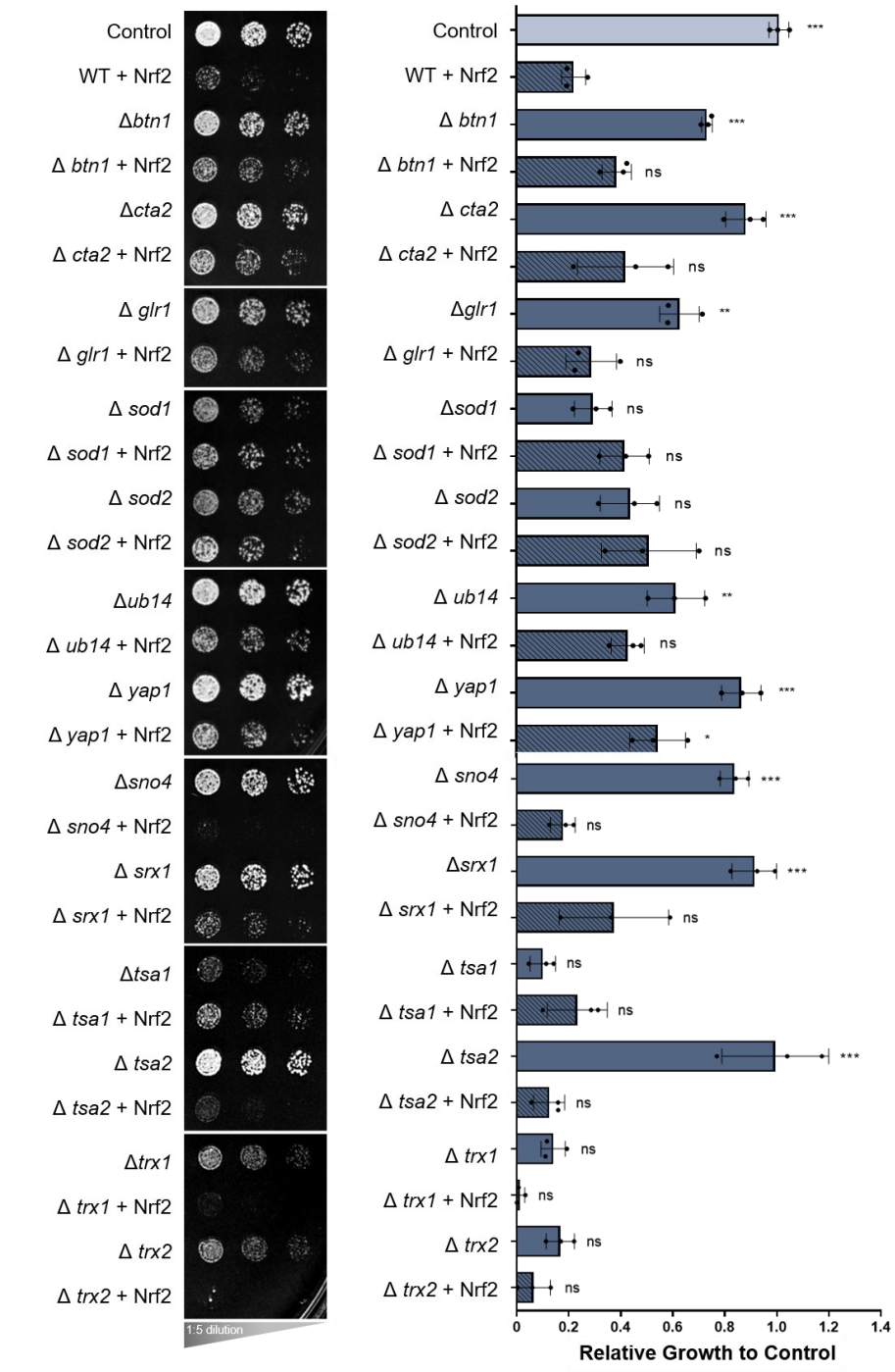


Figure S3.4: Full panel of growth assays for Nrf2 expression in yeast oxidative stress deletion strains. Growth was quantified relative to control. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

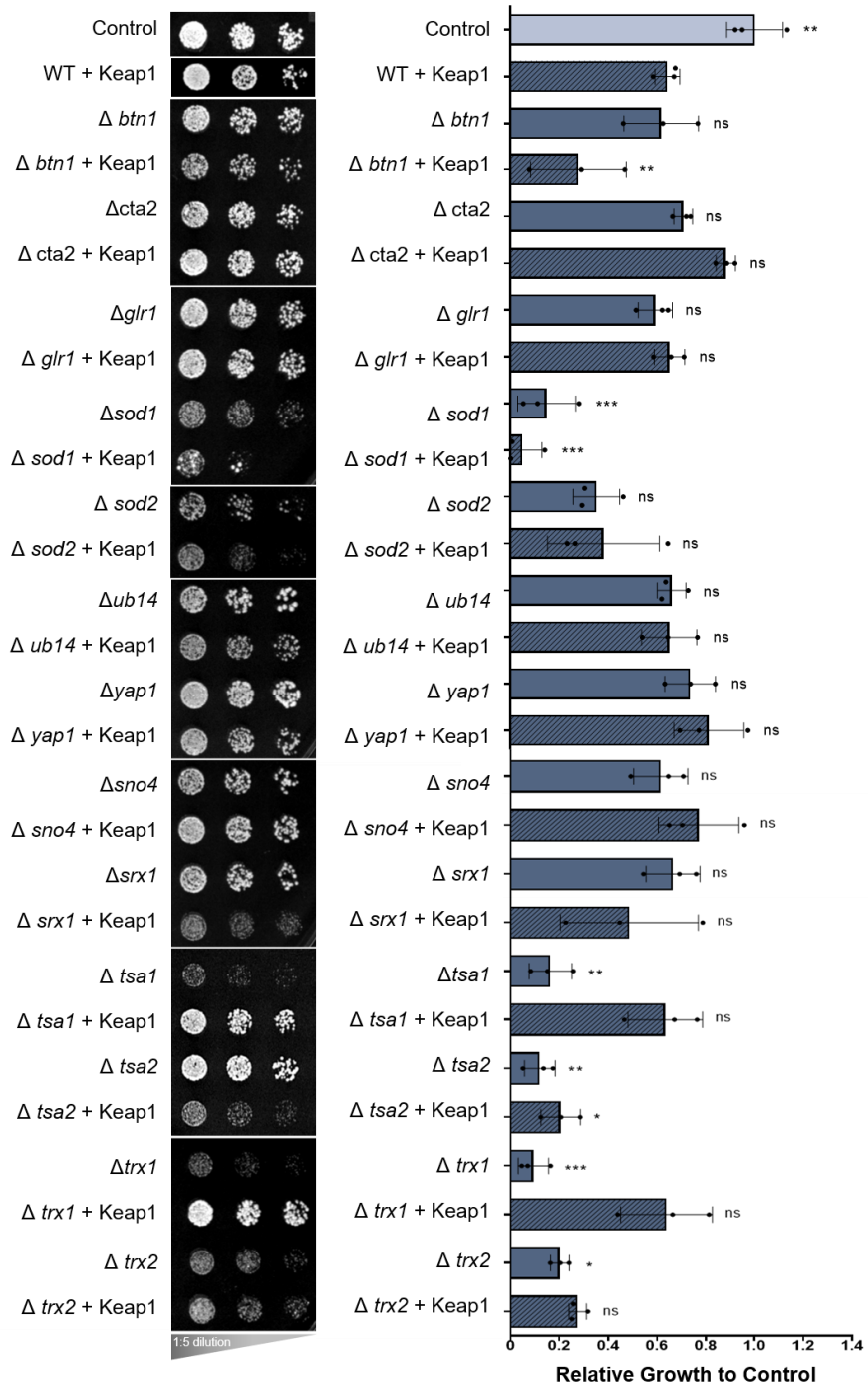


Figure S3.5: Full panel of growth assays for Keap1 expression in yeast oxidative stress deletion strains. Growth was quantified relative to control. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

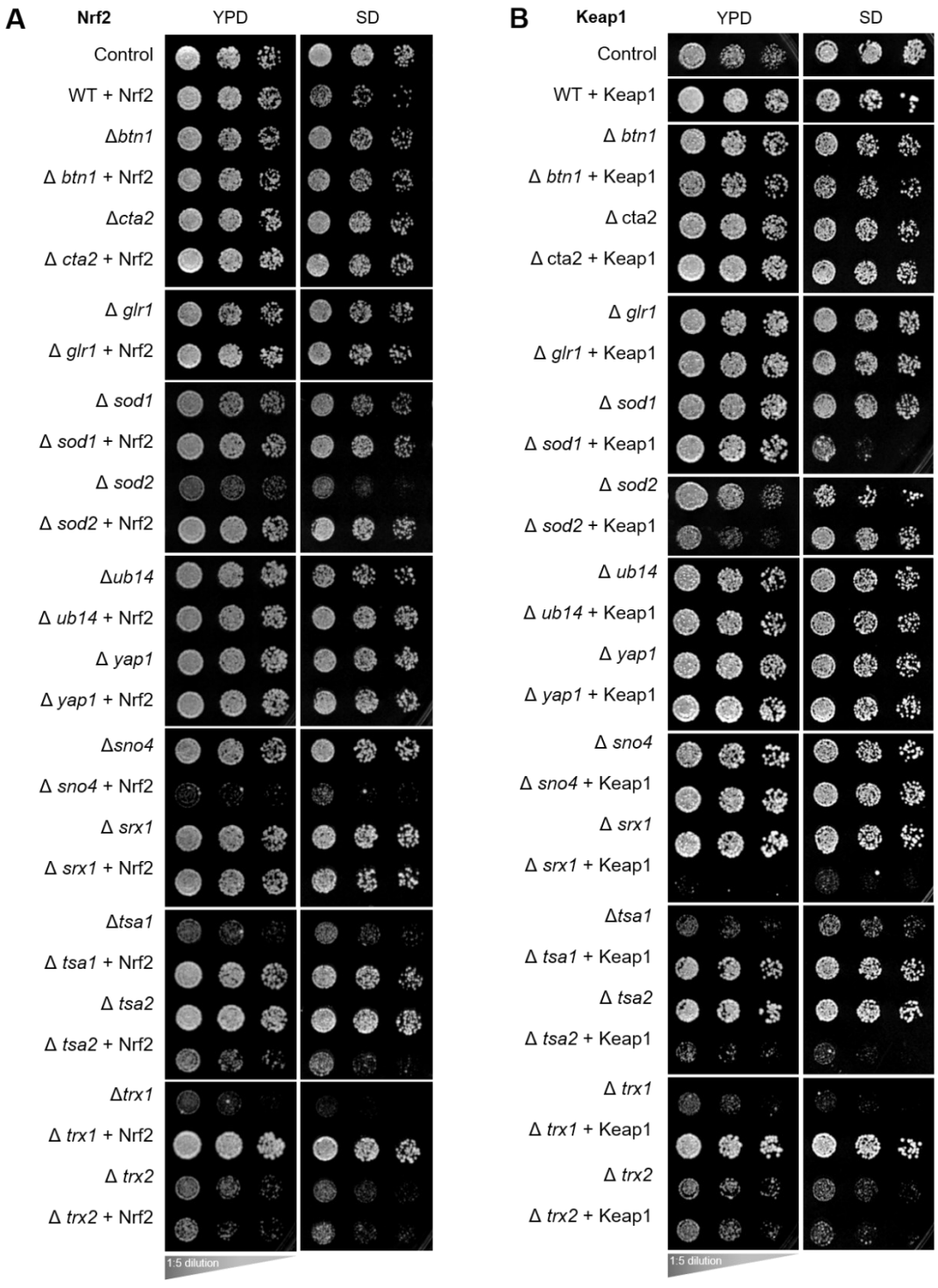


Figure S3.6: Growth assay control plates for all yeast oxidative stress deletion studies. Yeast peptone dextrose (YPD) and selective dextrose (SD) control plates are shown for **(A)** Nrf2 and **(B)** Keap1 experiments. Note that some deletions strains harbour an inherent toxic growth phenotype observed even on control media.

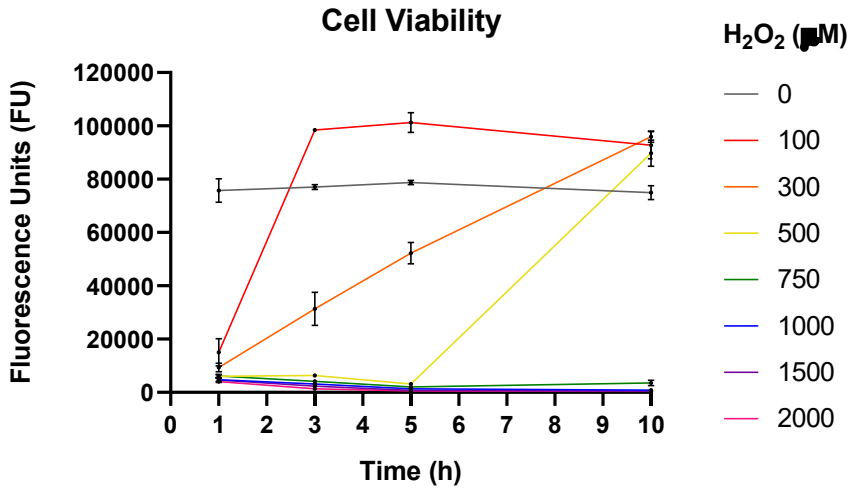


Figure S3.7: Optimization of hydrogen peroxide treatment concentration and duration. Non-transfected HeLa cells were treated with various concentrations of hydrogen peroxide for 1, 3, 5, and 10 h and cell viability was assessed (measured by ATP levels).

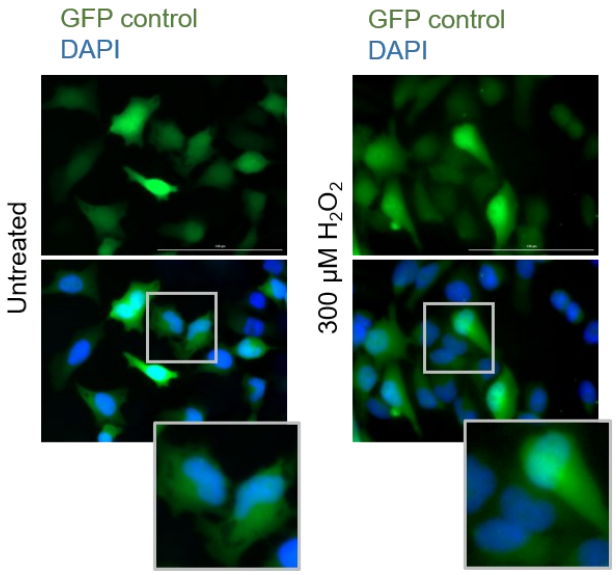


Figure S3.8: GFP controls for protein oxidation studies in HeLa cells. Cells were transfected with a pcDNA3.1-GFP empty vector for mammalian expression.

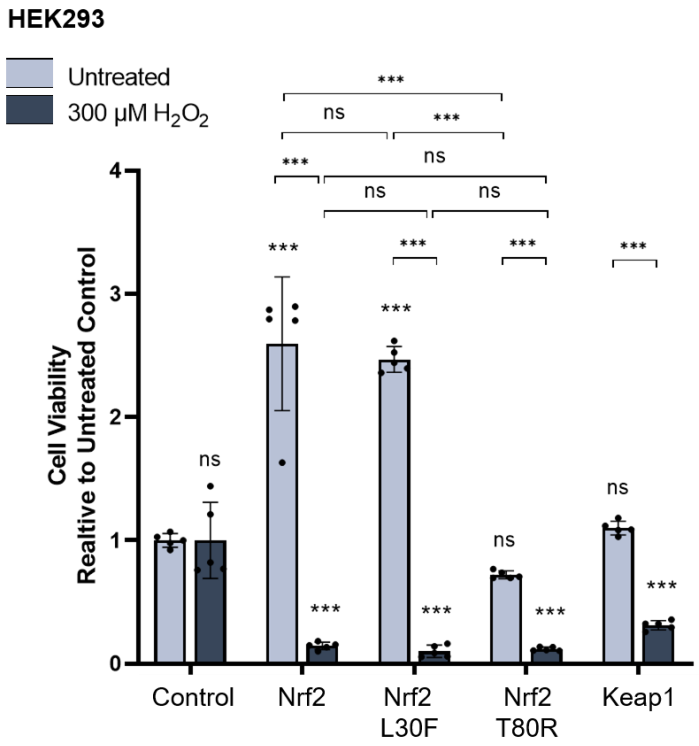


Figure S3.9: Cell viability following hydrogen peroxide treatment in HEK293 cells. Transfected HEK293 cells were treated with 300 µM H_2O_2 for 3 h and cell viability was assessed (measured by ATP levels). Results recapitulate those observed in HeLa cells. Means derived from five biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.6 References

Baird, L., & Yamamoto, M. (2020). The Molecular Mechanisms Regulating the KEAP1-NRF2 Pathway. *Mol Cell Biol, 40*(13).

Crouch, S. P., Kozlowski, R., Slater, K. J., & Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J Immunol Methods, 160*(1), 81-88.

Davies, K. J. (1987). Protein damage and degradation by oxygen radicals. I. general aspects. *J Biol Chem, 262*(20), 9895-9901.

Davies, K. J., Delsignore, M. E., & Lin, S. W. (1987). Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *J Biol Chem, 262*(20), 9902-9907.

Dean, R. T., Roberts, C. R., & Jessup, W. (1985). Fragmentation of extracellular and intracellular polypeptides by free radicals. *Prog Clin Biol Res, 180*, 341-350.

Di Simplicio, P., Franconi, F., Frosalí, S., & Di Giuseppe, D. (2003). Thiolation and nitrosation of cysteines in biological fluids and cells. *Amino Acids, 25*(3), 323-339.

Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., & Talalay, P. (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences, 99*(18), 11908.

Dobson, C. M. (2003). Protein folding and misfolding. *Nature, 426*(6968), 884-890.

Dunker, A. K., Lawson, J. D., Brown, C. J., Williams, R. M., Romero, P., Oh, J. S., Oldfield, C. J., Campen, A. M., Ratliff, C. M., Hipps, K. W., Ausio, J., Nissen, M. S., Reeves, R., Kang, C., Kissinger, C. R., Bailey, R. W., Griswold, M. D., Chiu, W., Garner, E. C., & Obradovic, Z. (2001). Intrinsically disordered protein. *Journal of Molecular Graphics and Modelling, 19*(1), 26-59.

Dyson, H. J., & Wright, P. E. (2005). Intrinsically unstructured proteins and their functions. *Nat Rev Mol Cell Biol, 6*(3), 197-208.

Gietz, R. D., & Schiestl, R. H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols, 2*, 31.

He, X., & Ma, Q. (2009). NRF2 cysteine residues are critical for oxidant/electrophile-sensing, Kelch-like ECH-associated protein-1-dependent ubiquitination-proteasomal degradation, and transcription activation. *Mol Pharmacol, 76*(6), 1265-1278.

Ishida, T., & Kinoshita, K. (2007). PrDOS: prediction of disordered protein regions from amino acid sequence. *Nucleic Acids Res, 35*(Web Server issue), W460-464.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., & Yamamoto, M. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev, 13*(1), 76-86.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T., & Yamamoto, M. (2003). Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells, 8*(4), 379-391.

Kobayashi, A., Kang, M. I., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., & Yamamoto, M. (2006). Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol Cell Biol, 26*(1), 221-229.

Kuge, S., & Jones, N. (1994). YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides. *Embo j, 13*(3), 655-664.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol, 35*(6), 1547-1549.

Kumari, S., Badana, A. K., G, M. M., G, S., & Malla, R. (2018). Reactive Oxygen Species: A Key Constituent in Cancer Survival. *Biomarker insights, 13*, 1177271918755391.

Li, X., Zhang, D., Hannink, M., & Beamer, L. J. (2004). Crystal structure of the Kelch domain of human Keap1. *J Biol Chem, 279*(52), 54750-54758.

McMahon, M., Itoh, K., Yamamoto, M., & Hayes, J. D. (2003). Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem, 278*(24), 21592-21600.

McMahon, M., Lamont, D. J., Beattie, K. A., & Hayes, J. D. (2010). Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. *Proceedings of the National Academy of Sciences, 107*(44), 18838-18843.

Mészáros, B., Erdős, G., & Dosztányi, Z. (2018). IUPred2A: context-dependent prediction of protein disorder as a function of redox state and protein binding. *Nucleic Acids Research, 46*(W1), W329-W337.

Miseta, A., & Csutora, P. (2000). Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Mol Biol Evol, 17*(8), 1232-1239.

Moi, P., Chan, K., Asunis, I., Cao, A., & Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Sciences, 91*(21), 9926-9930.

Moye-Rowley, W. S., Harshman, K. D., & Parker, C. S. (1989). Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. *Genes Dev, 3*(3), 283-292.

Nam, L. B., & Keum, Y.-S. (2019). Binding partners of NRF2: Functions and regulatory mechanisms. *Archives of Biochemistry and Biophysics, 678*, 108184.

Ngo, V., Brickenden, A., Liu, H., Yeung, C., Choy, W. Y., & Duennwald, M. L. (2020). A novel yeast model detects Nrf2 and Keap1 interactions with Hsp90 (*submitted*).

Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S., & Pickett, C. B. (2003). Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem, 278*(7), 4536-4541.

Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsuji, M., Kang, M. I., Kobayashi, A., Yokoyama, S., & Yamamoto, M. (2006). Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol Cell, 21*(5), 689-700.

Petropavlovskiy, A. A., Tauro, M. G., Lajoie, P., & Duennwald, M. L. (2020). A Quantitative Imaging-Based Protocol for Yeast Growth and Survival on Agar Plates. *STAR Protocols*, 100182.

Schnell, N., Krems, B., & Entian, K. D. (1992). The PAR1 (YAP1/SNQ3) gene of Saccharomyces cerevisiae, a c-jun homologue, is involved in oxygen metabolism. *Curr Genet, 21*(4-5), 269-273.

Shibata, T., Ohta, T., Tong, K. I., Kokubu, A., Odogawa, R., Tsuta, K., Asamura, H., Yamamoto, M., & Hirohashi, S. (2008). Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci U S A, 105*(36), 13568-13573.

Simaan, H., Lev, S., & Horwitz, B. A. (2019). Oxidant-Sensing Pathways in the Responses of Fungal Pathogens to Chemical Stress Signals. *Frontiers in Microbiology, 10*, 567.

Stadtman, E. R. (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem, 62*, 797-821.

Sun, Z., Wu, T., Zhao, F., Lau, A., Birch, C. M., & Zhang, D. D. (2011). KPNA6 (Importin α7)-Mediated Nuclear Import of Keap1 Represses the Nrf2-Dependent Antioxidant Response. *Mol Cell Biol, 31*(9), 1800-1811.

Taguchi, K., Fujikawa, N., Komatsu, M., Ishii, T., Unno, M., Akaike, T., Motohashi, H., & Yamamoto, M. (2012). Keap1 degradation by autophagy for the maintenance of redox homeostasis. *Proceedings of the National Academy of Sciences, 109*(34), 13561.

Thomas, B. J., & Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. *Cell, 56*(4), 619-630.

Tong, K. I., Katoh, Y., Kusunoki, H., Itoh, K., Tanaka, T., & Yamamoto, M. (2006). Keap1 Recruits Neh2 through Binding to ETGE and DLG Motifs: Characterization of the Two-Site Molecular Recognition Model. *Mol Cell Biol, 26*(8), 2887-2900.

Tong, K. I., Padmanabhan, B., Kobayashi, A., Shang, C., Hirotsu, Y., Yokoyama, S., & Yamamoto, M. (2007). Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol Cell Biol, 27*(21), 7511-7521.

The Universal Protein Resource (UniProt). (2008). (Publication no. 10.1093/nar/gkm895). (0305-1048). https://doi.org/10.1093/nar/gkm895

Uversky, V. N. (2011). Intrinsically disordered proteins may escape unwanted interactions via functional misfolding. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1814*(5), 693-712.

Uversky, V. N. (2019). Intrinsically Disordered Proteins and Their "Mysterious" (Meta)Physics. *Frontiers in Physics, 7*(10).

Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., & Talalay, P. (2004). Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci U S A, 101*(7), 2040-2045.

Wise-Scira, O., Dunn, A., Aloglu, A. K., Sakallioglu, I. T., & Coskuner, O. (2013). Structures of the E46K mutant-type α-synuclein protein and impact of E46K mutation on the structures of the wild-type α-synuclein protein. *ACS chemical neuroscience, 4*(3), 498-508.

Xue, B., Brown, C. J., Dunker, A. K., & Uversky, V. N. (2013). Intrinsically disordered regions of p53 family are highly diversified in evolution. *Biochim Biophys Acta, 1834*(4), 725-738.

Xue, B., Dunbrack, R. L., Williams, R. W., Dunker, A. K., & Uversky, V. N. (2010). PONDR-FIT: a meta-predictor of intrinsically disordered amino acids. *Biochim Biophys Acta, 1804*(4), 996-1010.

Yamamoto, M., Kensler, T. W., & Motohashi, H. (2018). The KEAP1-NRF2 System: a Thiol-Based Sensor-Effector Apparatus for Maintaining Redox Homeostasis. *Physiol Rev, 98*(3), 1169-1203.

Zhang, D. D., & Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol, 23*(22), 8137-8151.

Chapter 4

4 Hyperactive stress response pathways in HER2+ breast cancers

Drug resistance represents one of the greatest challenges in cancer therapy. This is especially true for patients with HER2-enriched (HER2+) breast cancers, who undergo higher rates of recurrence and metastasis compared to other molecular breast cancer subtypes. While molecular chaperones such as Hsp90 guide the normal folding and proteolytic turnover of key regulators of cell growth and survival, their overexpression during oncogenesis promotes the process of tumorigenesis. Hsp90 regulates the activity of heat shock factor 1 (Hsf1) which initiates a transcriptional response to proteotoxic stress, but also facilitates a transcriptional program to sustain highly malignant cancers. Our previous studies have also identified a link between Hsp90 and Nrf2, the transcriptional master regulator of oxidative stress known to promote tumorigenesis and resistance to chemotherapeutic agents. We show here that the inducible isoform of Hsp90, *HSP90AA1*, is upregulated in HER2+ (but not HER2-) breast cancers, and this is associated with the Hsf1 and Nrf2 signalling pathways. Additionally, inhibition of Hsp90 in HER2+ breast cancer cells leads to increased mRNA expression levels for glutathione peroxidase (GPx) 2 and 3 , which may confer increased antioxidant abilities. Accordingly, *combined* inhibition of HER2, Hsp90, and GPx activity may increase response rates to targeted HER2+ breast cancer therapy.

A version of this chapter is in preparation for publication.

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4.1 Introduction

The human epidermal growth factor 2 (HER2) proto-oncogene is a membrane-bound receptor tyrosine kinase that promotes cancer growth, differentiation, and cell survival (Yarden & Sliwkowski, 2001; Gschwind et al., 2004). The *ERBB2/neu* gene, which encodes HER2, is amplified in approximately 15-30% of breast cancers and characterizes the HER2-enriched (HER2+) molecular subtype (Burstein, 2005; Mitri et al., 2012). Existing therapy for patients with HER2+ breast cancers often involves the use of a monoclonal antibody against HER2 (e.g., Trastuzumab), which provides benefit both when administered alone (Vogel et al., 2002; Baselga et al., 2005) or in combination with chemotherapy (Slamon et al., 2001; Marty et al., 2005). Despite these targeted treatment strategies, HER2+ breast cancers are still associated with low survival rates (Carey et al., 2006).

An adaptive mechanism to cellular stress is enhanced expression of antioxidant proteins and molecular chaperones and heat shock protein (Hsps) induced by the antioxidant and heat shock responses, respectively. By promoting these cytoprotective signalling pathways, cancer cells can adapt to the quickly-changing tumour microenvironment, ultimately resulting in enhanced cancer cell survival (Ciocca et al., 2013; Taguchi & Yamamoto, 2017; Rojo de la Vega et al., 2018; Yun et al., 2019; Robertson et al., 2020). Increased expression of Hsps is observed in a wide range of human cancers (Ciocca et al., 1993; Kimura et al., 1993; Kaur & Ralhan, 1995; Yano et al., 1996; Cornford et al., 2000; Whitesell & Lindquist, 2005; Hwang et al., 2009). Currently, Hsp90 is the Hsp with the most clinically relevant small-molecule inhibitors in clinical trials (ClinicalTrials.gov; Zagouri et al., 2013; Li et al., 2021). Hsp90 activity is required for the growth of several cancer types due to its capacity to chaperone the normal folding and function of a multitude of oncogenic proteins (Whitesell & Lindquist, 2005; Pick et al., 2007). Through stabilization of oncogenic client proteins, Hsp90 overexpression promotes cell survival, malignant transformation, tumour growth, and invasion (Whitesell et al., 1994; Lewis et al., 2000; Sato et al., 2000; Basso et al., 2002a; Chen et al., 2002; Teng et al., 2012; Azoitei et al., 2014), particularly through its stabilization of HER2 (Xu et al., 2002). Hsp90 inhibitors such as radicicol, display a strong, nanomolar affinity for Hsp90 (Zagouri et al., 2013; Li et al., 2021) and can suppress oncogenic transformation driven by several oncogenes, including Ras and Src (Sharma et al., 1998).

The Hsp90α1 isoform of Hsp90, encoded by the *HSP90AA1* gene, is up-regulated in response to cellular stress (Sreedhar et al., 2004). Hsp90 interacts with and regulates heat shock factor 1 (Hsf1) (Nair et al., 1996; Ali et al., 1998; Hu & Mivechi, 2003), which can mediate a transcriptional stress program that promotes resistance of cancer cells to chemotherapy and radiation (Dai et al., 2007; Mendillo et al., 2012), particularly in HER2+ breast cancers (Meng et al., 2010; Santagata et al., 2011). Similarly, cancer cells exhibit persistently high levels of reactive oxygen species (ROS) due to genetic and metabolic instability (Vander Heiden et al., 2009; Finkel, 2011) which is counteracted by Nrf2, the master transcriptional regulator of the oxidative stress response (Taguchi & Yamamoto, 2017; Rojo de la Vega et al., 2018). We have previously identified a genetic and physical interaction between Hsp90 and Nrf2 (Ngo et al., 2020, *submitted*).

Interestingly, Hsp90 regulates HER2 function by limiting HER2 heterodimerization which is required for HER2 signalling (Brennan et al., 2000; Olayioye et al., 2000). Inhibition of Hsp90 has been shown to result in the rapid degradation of the HER2 receptor (Tikhomirov & Carpenter, 2000; Solit et al., 2002) and accordingly, Hsp90 inhibitors have been used in the treatment of HER2+ breast cancer in conjunction with HER2 monoclonal antibodies (Raja et al., 2008; Canonici et al., 2018). However, HER2+ breast cancers are still associated with induced resistance (Rimawi et al., 2015; Vernieri et al., 2019) and low survival rates (Carey et al., 2006). Therefore, more work must be done to explore off-target effects and mechanisms of induced resistance.

In this study, we examined the interplay between Nrf2 and Hsf1 in breast cancer, along with the involvement of Hsp90. We explored the crosstalk between the Nrf2 and Hsf1 signalling pathways, together with their dependency on Hsp90 chaperone activity. Hsp90 inhibition was required to induce expression of stress response genes in HER2+ breast cancers, notably through increased mRNA expression of glutathione peroxidase (GPx) antioxidant genes which has been shown to reduce responsiveness to cancer therapy. This project is not yet complete, but ensuing experiments (see **Section 5.8**) will investigate the small molecule inhibition of Hsp90 (by radicicol), combined with inhibition of glutamate-cysteine ligase (GCL) (by buthionine sulfoximine, BSO (Griffith & Meister, 1979)) which diminishes the intracellular levels of the GSH cofactor required for GPx enzymatic activity. We postulate that these small-molecule inhibitors, in combination with the standard Trastuzumab and chemotherapy treatment regimen for HER2+ breast cancer, will

enhance treatment outcomes. Our work provides novel insights into the crosstalk between the heat shock and antioxidant stress responses in a translationally relevant model of breast cancer.

4.2 Results

4.2.1 *HSP90AA1* is up-regulated in HER2-enriched breast cancers

A link between Hsp90 upregulation and reduced breast cancer survival has previously been documented (Yano et al., 1996; Pick et al., 2007). Three distinct genes express cytosolic Hsp90s: *HSP90AA1* and *HSP90AA2*, encoding the two stress-inducible protein isoforms of Hsp90, Hsp90α1 and Hsp90α2, respectively, and *HSP90AB1*, encoding the constitutively-expressed Hsp90β (Sreedhar et al., 2004). Hsp90 transcript levels were examined across cancer types in the International Cancer Genome Consortium (ICGC) dataset. The stress-inducible *HSP90AA1* was most highly expressed in breast cancer relative to all other cancer subtypes (**Figure 4.1A**). Investigation of the TCGA breast cancer dataset revealed an association between elevated *HSP90AA1* transcript levels and reduced survival (**Figure 4.1B**). Alternatively, while *HSP90AB1* was also relatively highly expressed in breast cancers, elevated expression of *HSP90AB1* was not associated with patient survival (data not shown). We therefore focused our downstream analysis on mechanisms by which Hsp90α1 may promote tumorigenesis in breast cancer.

To define tumour characteristics associated with *HSP90AA1* up-regulation, we further interrogated the TCGA breast cancer dataset and report elevated *HSP90AA1* expression in the HER2-enriched molecular subtype (**Figure 4.1C**), along with HER2+ breast cancers (**Figure 4.1D**). This has previously been suggested, as Hsp90 has been shown to associate with the intracellular domain of receptor tyrosine kinases, including HER2 (Xu et al., 2002). We then assessed both protein levels (**Figure 4.1E**) and mRNA levels (**Figure 4.1F**) of Hsp90α, Hsp90α1, and HER2 across a panel of HER2+ (21PT, 21NT, 21MT-1, SKBR3) and HER2- (T47D, MCF7) breast cancer cell lines. We have included an isogenic cell line series representing distinct stages of cancer progression isolated from a single patient (21PT, 21NT, 21MT-1), which show concomitant amplification and increased expression of HER2 (Band et al., 1990).

Since the client proteins of Hsp90 have important roles in tumorigenesis and tumour survival (Lewis et al., 2000; Sato et al., 2000; Basso et al., 2002a; Chen et al., 2002), we examined which

signalling pathways are enriched in HER2+ breast cancers. Co-expression analysis was conducted for transcripts whose expression levels were positively correlated with that of *HSP90AA1* in the TCGA breast cancer cohort. The resulting list consists of 66 and 249 genes in HER2+ and HER2- breast cancers, respectively. To examine signalling pathways associated with *HSP90AA1* transcript levels in HER2+ breast cancers, we focused on 28 transcripts that positively correlate with *HSP90AA1* expression exclusively in HER2+ breast cancers (**Figure 4.1G** and **Figure S4.1A**). Pathway analysis of the input gene list revealed that the transcription factor whose target genes and transcriptional profiles match the input list most closely is Hsf1 (**Figure S4.1B**). Additionally, comparison of our gene list to transcriptional profiles with transcription factor loss-of-function datasets from the Gene Expression Omnibus (GEO) highlighted both Hsf1 and Nrf2 (**Figure S4.1C**), and there is significant overlap between the Nrf2 and Hsf1 signalling pathways, i.e., their responses as transcription factors are correlated, yet they may not be linked (Dayalan Naidu et al., 2015).

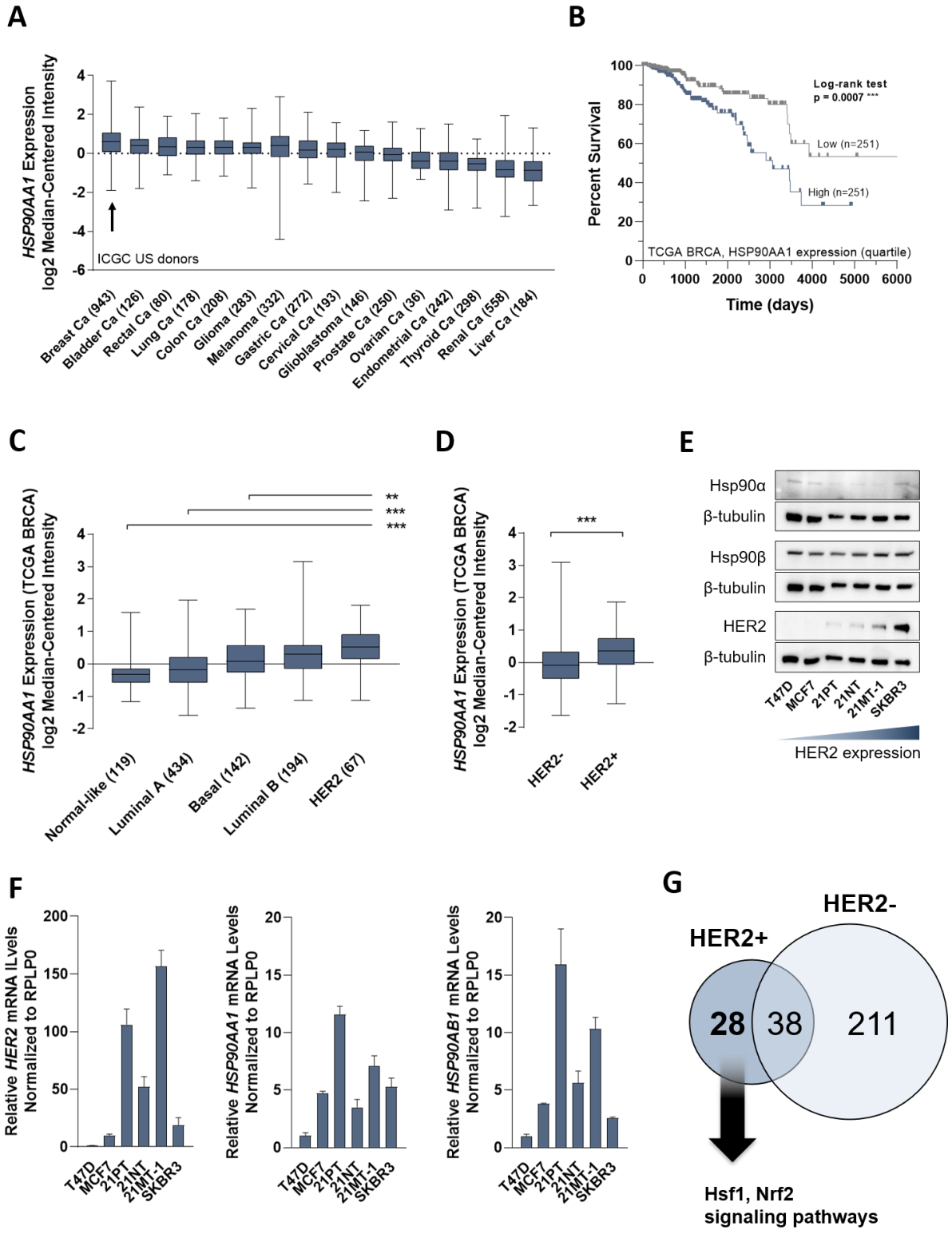

A
HSP90AA1 Expression
log2 Median-Centered Intensity
ICGC US donors
Breast Ca (943)
Bladder Ca (126)
Rectal Ca (80)
Lung Ca (178)
Colon Ca (208)
Glioma (283)
Melanoma (332)
Gastric Ca (272)
Cervical Ca (193)
Glioblastoma (146)
Prostate Ca (250)
Ovarian Ca (36)
Endometrial Ca (242)
Thyroid Ca (298)
Renal Ca (558)
Liver Ca (184)
B
Log-rank test
p = 0.0007 ***
Low (n=251)
High (n=251)
Percent Survival
TCGA BRCA, HSP90AA1 expression (quartile)
Time (days)
C
HSP90AA1 Expression (TCGA BRCA)
log2 Median-Centered Intensity
Normal-like (119)
Luminal A (434)
Basal (142)
Luminal B (194)
HER2 (67)
D
HSP90AA1 Expression (TCGA BRCA)
log2 Median-Centered Intensity
HER2-
HER2+
E
Hsp90α
β-tubulin
Hsp90β
β-tubulin
HER2
β-tubulin
T47D
MCF7
21PT
21NT
21MT-1
SKBR3
HER2 expression
F
Relative HER2 mRNA ILvels
Normalized to RPLP0
Relative HSP90AA1 mRNA Levels
Normalized to RPLP0
Relative HSP90AB1 mRNA Levels
Normalized to RPLP0
G
HER2+
HER2-
28
38
211
Hsf1, Nrf2
signaling pathways

Figure 4.1: *HSP90AA1* is up-regulated in HER2-enriched breast cancers. (A) Assessment of ICGC data (US Donor-centric) data shows *HSP90AA1* mRNA levels across tumour subtypes. *HSP90AA1* mRNA expression in breast cancer is indicated by an arrow. **(B)** Kaplan-Meier survival curve for breast cancer patients from the TCGA BRCA cohort, separated into high (upper quartile) versus low (lower quartile) *HSP90AA1* expression. **(C, D)** *HSP90AA1* mRNA expression was assessed across tumour characteristics in the TCGA BRCA cohort, including molecular subtype (C) and HER2 status (D). **(E)** Relative protein steady-state levels of HER2, Hsp90α, and Hsp90β across a panel of breast cancer cell lines, with β-tubulin serving as the internal loading control. **(F)** Relative mRNA expression of *HER2*, *HSP90AA1*, and *HSP90AB1* across a panel of breast cancer cell lines, normalized to *RPLP0* expression. **(G)** Overlap of transcripts positively associated with *HSP90AA1* mRNA levels in the TCGA breast cancer cohort by HER2 status. The 28 transcripts positively correlated with *HSP90AA1* in HER2+ breast cancers. (A, C, D, F) Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.2.2 Increased glutathione peroxidase mRNA expression levels following oxidative stress and Hsp90 inhibition in HER2+ breast cancer cells

We assessed the induction of both Nrf2 (*ABCC2, HMOX1, NQO1, GPX2*) and Hsf1 (*HSPA1A, DNAJA4*) target genes (**Figure S4.2**) across a panel of HER2+ and HER2- breast cancer cell lines, with or without the addition of an oxidative stress-inducing agent hydrogen peroxide (H_2O_2), the Hsp90 inhibitor radicicol, and combined hydrogen peroxide and radicicol by RT-qPCR (**Figure 4.2A**). Of note, the Hsp90 inhibitor geldanamycin and its derivatives have been widely used in clinical trials for the treatment of cancer (Miyata, 2005); however, recent evidence has shown that geldanamycin-derived Hsp90 inhibitors are synthetic lethal combined with Nrf2 deletion, such that activation of Nrf2 target genes by Hsp90 inhibition results in metabolism of the quinone moiety in geldanamycin that could be responsible for its synthetic lethality (Baird et al., 2020). Radicicol is an Hsp90 inhibitor that is not a geldanamycin derivative and thus lacks this toxic quinone moiety; therefore, radicicol was used in this study. Additionally, we profile mRNA expression of *NFE2L2, HSF1, HSP90AA1*, and *HSP90AB1*, as internal controls, along with several other Nrf2-

independent antioxidant genes including *GPX1*, *GPX3*, and *GPX4*. Some Nrf2 and Hsf1 target genes are not induced with the addition of even 2 mM H_2O_2 for 3 h (**Figure 4.2B**). As an internal control, we assessed the induction of the Hsf1 and Nrf2 target genes with the addition of radicicol (**Figure 4.2C**). Several of the cell lines tested showed induced expression of both *HSP90AA1* and *HSP90AB1* mRNA. With the addition of combined hydrogen peroxide and radicicol, we observed that several genes within our panel are induced by this combined treatment (**Figure 4.2D**). Of particular interest, mRNA expression levels for *GPX2* and *GPX3*, two members of the GPx family, showed large differences between combined treatment relative to treatment with hydrogen peroxide alone. Of note, this induction of *GPX2* and *GPX3* by combined treatment is most explicit in HER2+ cell lines. For mRNA expression of *NFE2L2* and *HSF1*, refer to **Figure S4.3**.

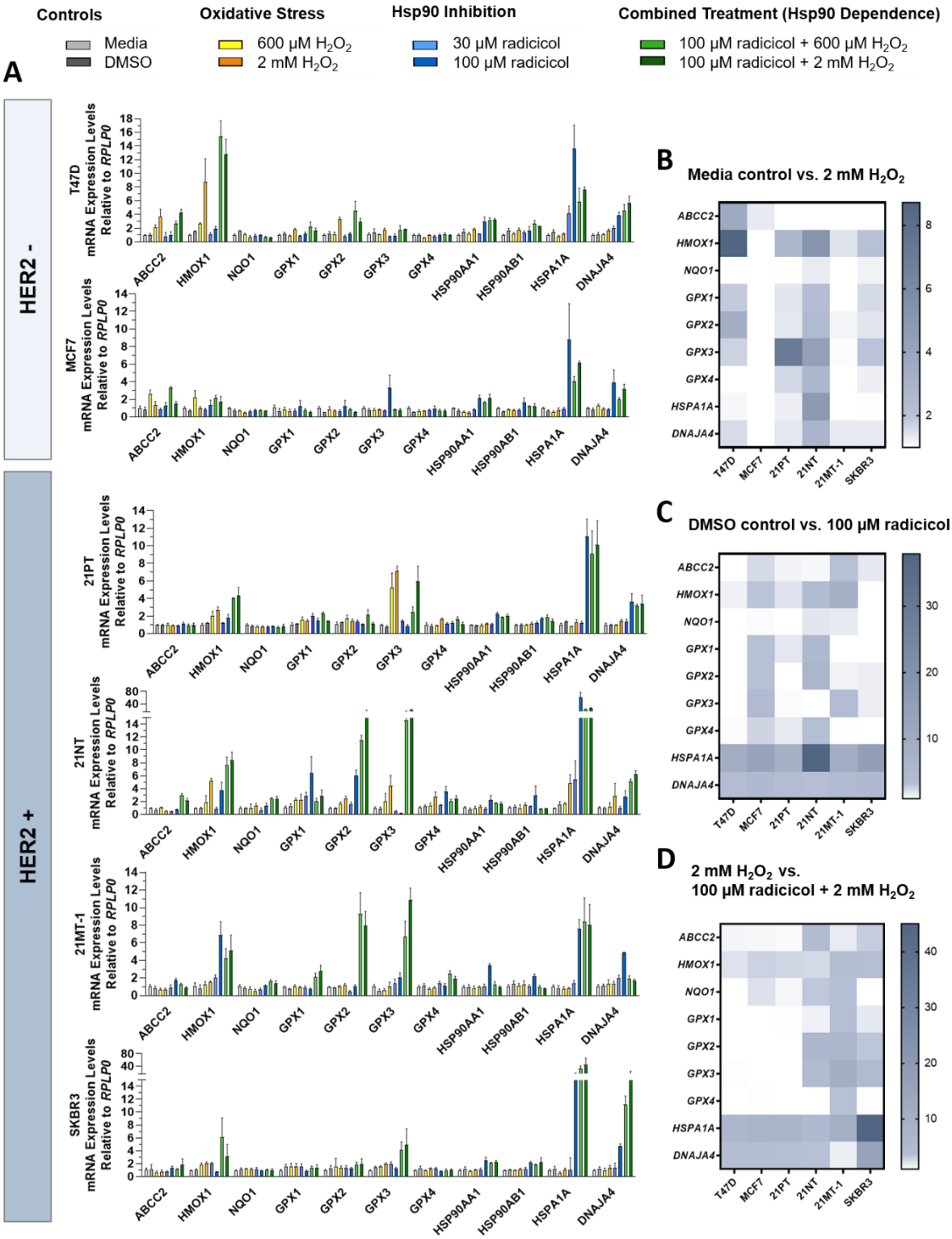
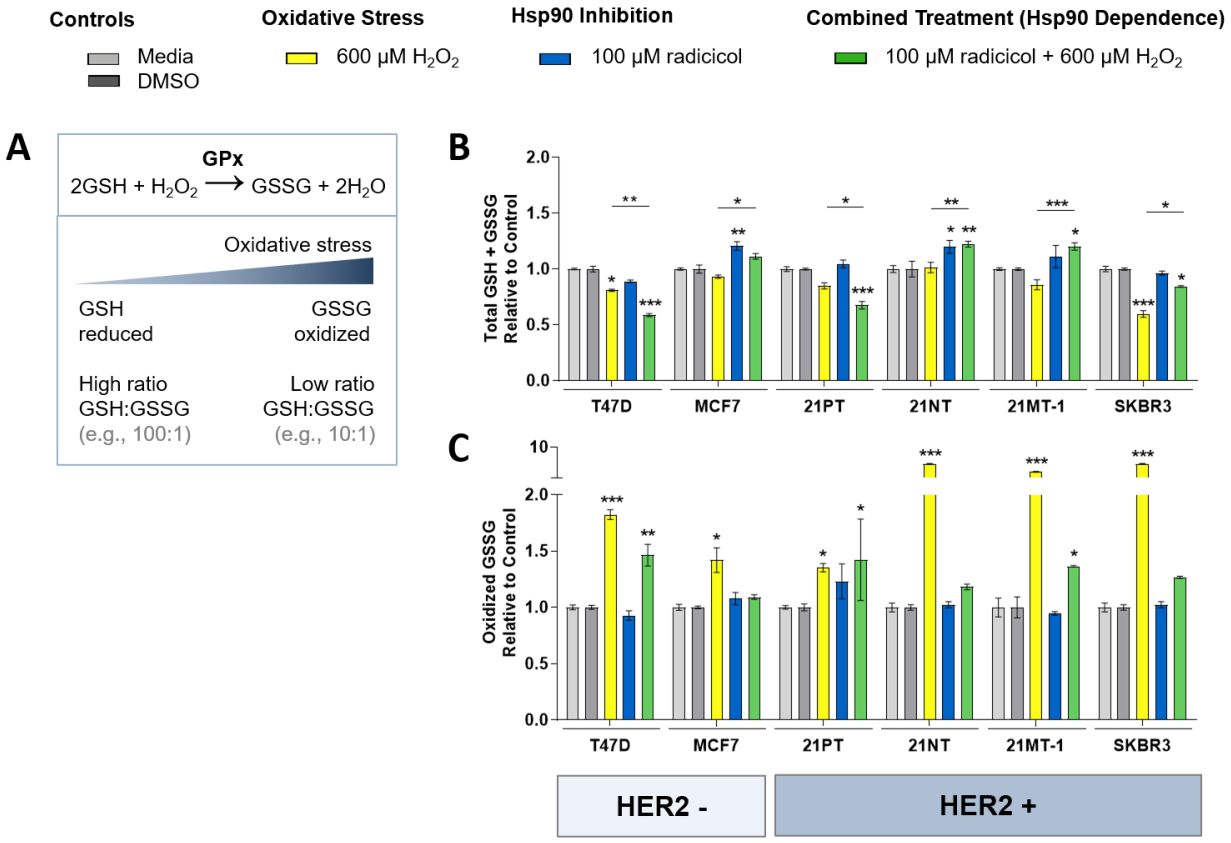

Controls
Media
DMSO
Oxidative Stress
600 μM H2O2
2 mM H2O2
Hsp90 Inhibition
30 μM radicicol
100 μM radicicol
Combined Treatment (Hsp90 Dependence)
100 μM radicicol + 600 μM H2O2
100 μM radicicol + 2 mM H2O2
A
HER2 -
HER2 +
T47D mRNA Expression Levels Relative to RPLP0
MCF7 mRNA Expression Levels Relative to RPLP0
21PT mRNA Expression Levels Relative to RPLP0
21NT mRNA Expression Levels Relative to RPLP0
21MT-1 mRNA Expression Levels Relative to RPLP0
SKBR3 mRNA Expression Levels Relative to RPLP0
ABCC2
HMOX1
NQO1
GPX1
GPX2
GPX3
GPX4
HSP90AA1
HSP90AB1
HSPA1A
DNAJA4
B
Media control vs. 2 mM H2O2
C
DMSO control vs. 100 μM radicicol
D
2 mM H2O2 vs.
100 μM radicicol + 2 mM H2O2
T47D
MCF7
21PT
21NT
21MT-1
SKBR3

Figure 4.2: Relative mRNA expression levels for *GPX2* and *GPX3* are significantly upregulated following oxidative stress with Hsp90 inhibition. (A) Relative mRNA levels of Nrf2 and Hsf1 target genes were evaluated by RT-qPCR, normalized to RPLP0 transcript levels, and depicted as fold change relative to the media control. Means derived from three biological replicates were used during analysis. **(B-D)** Heat maps summarizing the fold changes in Nrf2 and Hsf1 target gene expression for each treatment group relative to their respective controls.

4.2.3 Increased glutathione levels following oxidative stress and Hsp90 inhibition in HER2+ breast cancer cells

GPx2 and GPx3 are enzymes within the GPx family of enzymes responsible for catalyzing the detoxification of hydroperoxides (e.g., H_2O_2) through the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) (Brigelius-Flohé & Maiorino, 2013) (**Figure 4.3A**, top). GSH plays a critical role in protecting cells from oxidative damage either through direct antioxidant activity or coupled to GPx enzymatic activity (Pompella et al., 2003; Forman et al., 2009). Accordingly, decreased GSH levels, increased GSSG levels, or a decreased GSH:GSSG ratio is indicative of oxidative stress (Chai et al., 1994; Asensi et al., 1999) and implies GPx antioxidant activity through the oxidation and consumption of GSH to GSSG (**Figure 4.3A**, bottom). We sought to investigate GSH levels in the panel of HER2+ and HER2- cell lines treated with or without oxidative stress, Hsp90 inhibition, and the combined treatment of both. A luminescence-based system was used to measure total glutathione (i.e., GSH+GSSG) and oxidized GSSG, and the ratios of GSH:GSSG were calculated based on those values as a measure of oxidative stress (Asensi et al., 1999). Except for 21PT cells, all HER2+ cell lines (21NT, 21MT-1, and SKBR3, i.e., the high HER2-expressing cell lines) showed increased *total* glutathione levels with combined treatment compared to hydrogen peroxide-induced oxidative stress treatment alone (**Figure 4.3B**). For the same three HER2+ cell lines, *oxidized* GSSG levels increased significantly with oxidative stress alone (~10-fold increase relative to control), with modest increases in the remaining three cell lines (T47D, MCF7, 21PT) (~1.5-fold increase) (**Figure 4.3C**). However, when Hsp90 was inhibited in the combined treatment condition, GSSG levels dropped back to baseline even in the presence of oxidative stress (**Figure 4.3C**). Note that radicicol treatment alone does not alter total or oxidized glutathione levels. When the ratios of reduced GSH to oxidized GSSG were compared,

all cell lines show a *decreased* GSH:GSSG ratio upon oxidative stress alone (**Figure 4.3D**), indicative of antioxidant activity. This ratio is markedly low for the 21NT, 21MT-1, and SKBR3 cell lines (~0.1:1). Intriguingly, added Hsp90 inhibition in the combined treatment condition resulted in an *increased* GSH:GSSG ratio with significant differences between hydrogen peroxide treatment alone and the combined treatment condition in the 21NT, 21MT-1, and SKBR3 HER2+ cell lines that are not observed for T47D, MCF7, and 21PT cell lines (**Figure 4.3D**).


Controls
Media
DMSO
Oxidative Stress
600 μM H2O2
Hsp90 Inhibition
100 μM radicicol
Combined Treatment (Hsp90 Dependence)
100 μM radicicol + 600 μM H2O2
A
GPx
2GSH + H2O2 → GSSG + 2H2O
Oxidative stress
GSH reduced
GSSG oxidized
High ratio GSH:GSSG (e.g., 100:1)
Low ratio GSH:GSSG (e.g., 10:1)
B
Total GSH + GSSG Relative to Control
C
Oxidized GSSG Relative to Control
T47D
MCF7
21PT
21NT
21MT-1
SKBR3
HER2 -
HER2 +

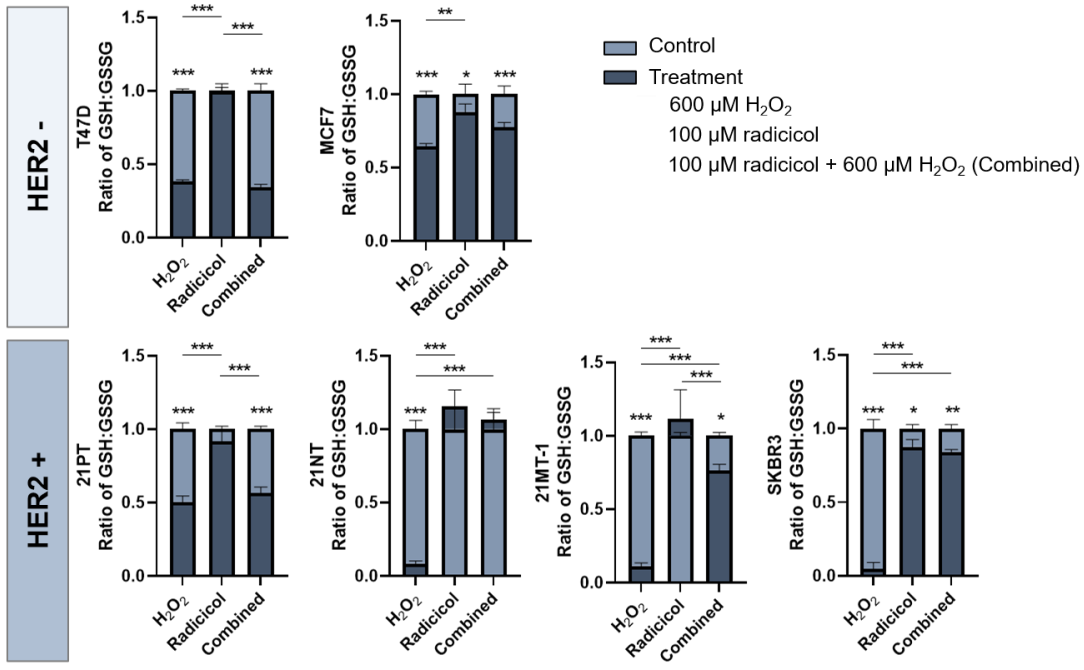

D
HER2 -
HER2 +
T47D Ratio of GSH:GSSG
MCF7 Ratio of GSH:GSSG
21PT Ratio of GSH:GSSG
21NT Ratio of GSH:GSSG
21MT-1 Ratio of GSH:GSSG
SKBR3 Ratio of GSH:GSSG
H2O2
Radicicol
Combined
Control
Treatment
600 μM H2O2
100 μM radicicol
100 μM radicicol + 600 μM H2O2 (Combined)

Figure 4.3: Glutathione levels and ratios in response to oxidative stress, Hsp90 inhibition, and combined treatment. (A) The GPx-mediated breakdown of hydrogen peroxide to water through the oxidation of GSH to GSSG in relation to levels of oxidative stress. **(B)** Total glutathione (GSH + GSSG) levels across a panel of breast cancer cell lines. **(B)** Oxidized glutathione (GSSG) levels across those same cell lines. **(D)** The ratio of reduced glutathione to oxidized glutathione (GSH:GSSG) relative to control. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.2.4 Increased response to taxane-treatment and increased ROS levels in HER2+ cells lines

Along with monoclonal antibody treatment (Trastuzumab), docetaxel and paclitaxel are two taxane-based chemotherapy drugs that have been widely used in the neoadjuvant treatment of HER2+ breast cancer (Merlin et al., 2002; Desai et al., 2008). To assess the effects of oxidative stress and Hsp90 inhibition alone and combined with chemotherapy treatment, the panel of HER2+ and HER2- cell lines were treated with hydrogen peroxide, radicicol, or combined treatment following pre-treatment with 50 nM of docetaxel or paclitaxel for a total of 24 h. Luminescence-based assays were performed to assess relative cell viability (determined by the quantification of ATP levels which indicates the presence of metabolically active cells (Crouch et al., 1993)). Pre-treatment with docetaxel or paclitaxel decreased relative cell viability compared to the untreated controls across all six cell lines for all internal treatment conditions, with pronounced efficacy in the four HER2+ cell lines compared to the HER2- cells (**Figure 4.4A**). However, the relative ratios of viability between untreated and docetaxel/paclitaxel-treated cells remained the same for each cell line. Furthermore, treatment with 600 μM H_2O_2 significantly reduced cell viability for the T47D, MCF7, and 21PT cell lines but not for the high HER2-expressing cell lines (21NT, 21MT-1, and SKBR3), which overall seem more oxidative stress-resistant than the non-/low HER2 cell lines. Although all cell lines were sensitive to very high hydrogen peroxide levels (2 mM), the high HER2-expressing cell lines still showed better overall viability (**Figure 4.4A**). Finally, except for

21NT cells and to a lesser extent for 21MT-1 cells, all other cell lines demonstrated reduced cell viability for the combined treatment conditions.

Cancer cells often show increased reactive oxygen species (ROS) levels that are generally associated with increased rates of cell proliferation and metabolism (Vander Heiden et al., 2009; Finkel, 2011). Here, ROS levels were measured at baseline and following treatment with hydrogen peroxide, radicicol, and combined treatment. As expected, all cell lines demonstrated increased ROS levels following hydrogen peroxide treatment both alone and in combination with radicicol compared to the untreated controls (**Figure 4.4B**). Interestingly, HER2+ cell lines showed significantly higher overall ROS levels upon treatment compared to HER- cell lines, whereas baseline (untreated) ROS levels were comparable across all cell lines.

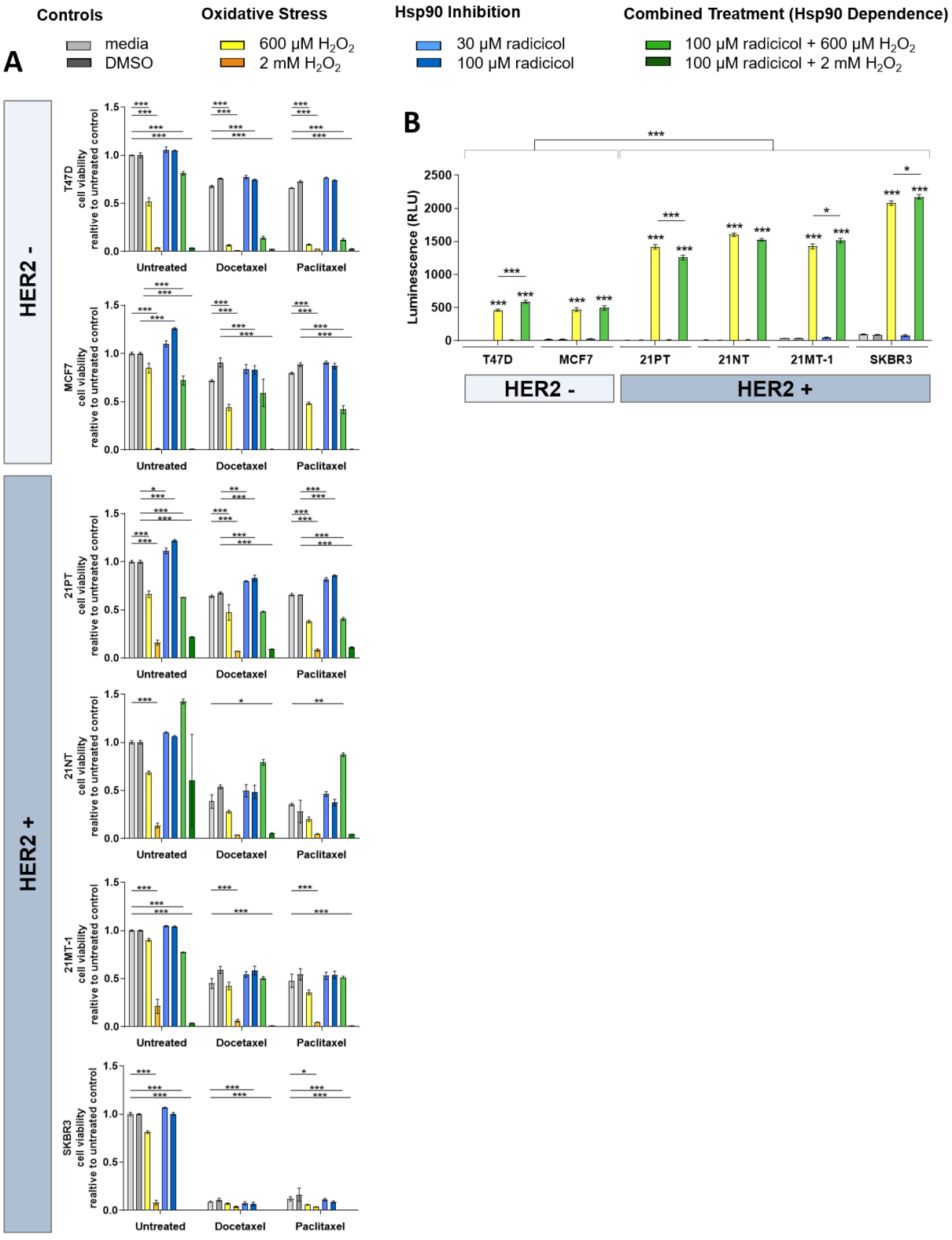

Controls
Oxidative Stress
Hsp90 Inhibition
Combined Treatment (Hsp90 Dependence)
media
DMSO
600 μM H2O2
2 mM H2O2
30 μM radicicol
100 μM radicicol
100 μM radicicol + 600 μM H2O2
100 μM radicicol + 2 mM H2O2
A
B
HER2 -
HER2 +
T47D cell viability realtive to untreated control
MCF7 cell viability realtive to untreated control
21PT cell viability realtive to untreated control
21NT cell viability realtive to untreated control
21MT-1 cell viability realtive to untreated control
SKBR3 cell viability realtive to untreated control
Untreated
Docetaxel
Paclitaxel
Luminescence (RLU)
T47D
MCF7
21PT
21NT
21MT-1
SKBR3
HER2 -
HER2 +

Figure 4.4: Relative cell viability and ROS levels in response to oxidative stress, Hsp90 inhibition, or combined treatment. (A) Cell viability across a panel of breast cancer cell lines with or without pre-treatment with chemotherapy agents, docetaxel or paclitaxel prior to treatment with hydrogen peroxide, radicicol, or both. **(B)** Relative ROS levels following treatment with hydrogen peroxide, radicicol, or both. Means derived from three biological replicates were used during analysis. Means were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are expressed as mean ± SD. $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.2.5 HER2, Hsp90α, and Hsp90β protein expression levels in response to oxidative stress and Hsp90 inhibition

(*Pending Completion*)

To assess the expression of HER2, Hsp90α, and Hsp90β protein expression levels following oxidative stress and/or Hsp90 inhibition, western blots were performed with β-Tubulin as an internal control. Initial blots were performed for three of the six breast cancer cell lines (T47D, MCF7, and 21PT) (**Figure 4.5**). However, the results cannot be interpreted due to changes in expression levels for the internal control in response to radicicol treatment. Western blots will be repeated using total protein as an internal control.

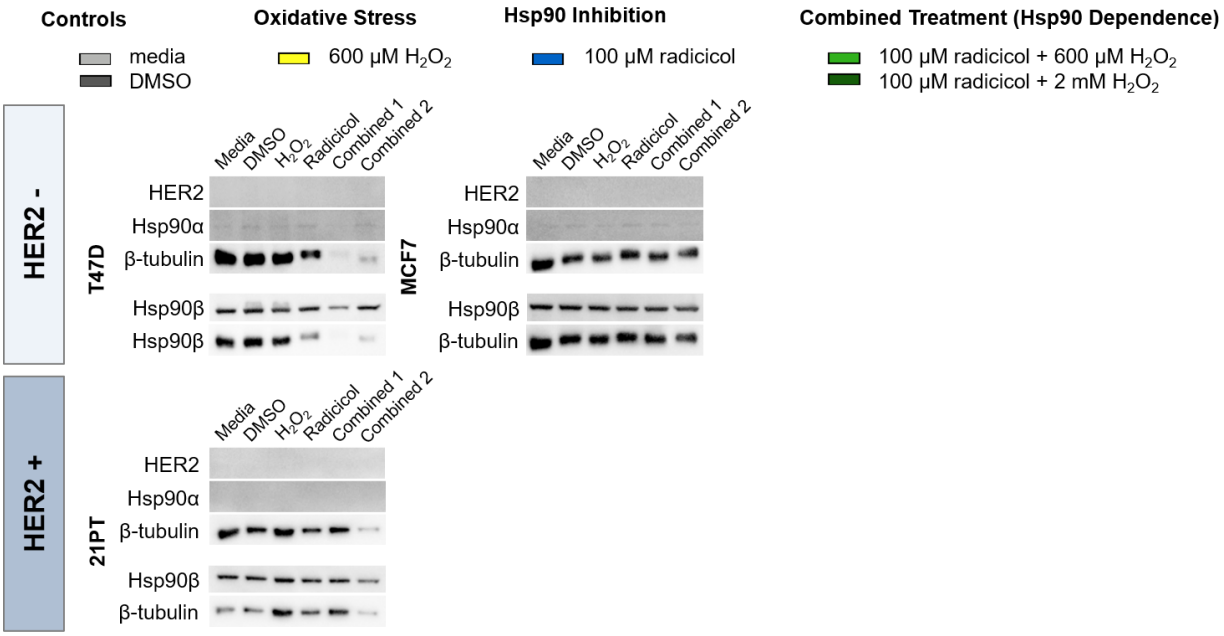


Figure 4.5: Protein expression levels in response to oxidative stress, Hsp90 inhibition, and combined treatment. Protein expression for HER2, Hsp90α, and Hsp90β across a panel of breast cancer cell lines, with β-tubulin serving as the internal loading control, for 3 of 6 cell lines.

4.2.6 Co-inhibition of Hsp90 and GPx in HER2+ breast cancer therapy

(*Pending Completion*)

We found that Hsp90 inhibition in HER2+ breast cancer cell lines induced increased expression of *GPX2* and *GPX3* which may confer enhanced cell survival through increased antioxidant capacity. To mitigate this, we propose that targeting Hsp90 and GPx in combination to enhance the efficacy of HER2+ breast cancer treatment response. HER2+ and HER2- cell lines will be treated with the standard combined HER2-targeted therapy (a Trastuzumab analogue) with chemotherapy (docetaxel or paclitaxel), in addition to the small-molecule inhibition of the Hsp90 protein (by radicicol) and GPx activity indirectly through inhibition of GSH synthesis (by buthionine sulfoximine, BSO). Cell viability and cell death will be determined. BSO is a potent inhibitor of GSH synthesis which depletes GSH levels in the cell (Griffith & Meister, 1979), resulting in reduced levels of GSH required for GPx antioxidant activity. Thus, combined radicicol

and BSO treatment are expected to render cancer cells more susceptible to cancer therapy compared to radicicol treatment alone.

4.3 Discussion

Targeting Hsp90 in cancer treatment has been a growing area of research within the past two decades (Whitesell & Lindquist, 2005; Trepel et al., 2010), although more research is required to understand which patients may receive the greatest benefit from this therapeutic intervention. The unique N-terminal ATP-binding pocket of Hsp90 is the target of several natural as well as semi-synthetic pharmacological inhibitors. The binding of this active site by pharmacological inhibitors can alter normal Hsp90 cellular functions (Roe et al., 1999; Pearl & Prodromou, 2006), leading to events such as the recruitment of E3 ubiquitin ligases and subsequent proteasome-mediated degradation of client proteins (Kobayashi et al., 2004). Hsp90 inhibitors have shown great clinical promise in the treatment of cancer due to their ability to simultaneously suppress multiple Hsp90-regulated pathways that are essential for tumour cell growth and resistance. HER2 is a direct client protein of Hsp90, and Hsp90 inhibition has been shown to result in the rapid degradation of the HER2 receptor (Basso et al., 2002b; Solit et al., 2002; Zsebik et al., 2006). Hsp90 inhibitors have therefore been used in conjunction with the HER2 monoclonal antibody treatment standard, Trastuzumab, for the treatment of HER2+ breast cancer and have shown promising results (Raja et al., 2008; Canonici et al., 2018). However, off-target effects and induced therapy resistance remain challenging in breast cancer treatment (Rimawi et al., 2015; Vernieri et al., 2019). In this work, we show that the stress-inducible isoform of Hsp90, *HSP90AA1*, is upregulated in HER2-enriched breast cancers, and Hsp90 inhibition is associated with activation of the Nrf2 and Hsf1 signalling pathways, with notable induction of Nrf2-regulated *GPX2*, and Nrf2-independent *GPX3*, both of which are genes for potent antioxidant enzymes that may contribute to cancer therapy resistance.

Previous studies have shown that HER2 receptor overexpression activates Hsf1 (Schulz et al., 2014), the master transcriptional regulator of the heat shock response against proteotoxic stress and that Hsf1 activity is negatively regulated by Hsp90 (Ali et al., 1998; Zou et al., 1998). In line with these findings, we found *HSP90AA1* transcript levels to be increased in the HER2-enriched molecular subtype, specifically in HER2+ breast cancers, and that this correlated with signalling

pathways for Hsf1 and Nrf2. The role of Nrf2 in promoting carcinogenesis and cancer therapy resistance is well established. Nrf2 hyperactivation in cancer confers protection against oxidative stress and promotes the detoxification and export of chemotherapeutic agents through the upregulation of cytoprotective antioxidant enzymes (Taguchi & Yamamoto, 2017; Rojo de la Vega et al., 2018). We therefore speculate that the observed HER2-associated increases in *GPX2* and *GPX3* upon oxidative stress with Hsp90 inhibition are protective. Recent studies have demonstrated the involvement of ROS in the development and progression of breast cancer (Zhu et al., 2016a; Ma et al., 2018; Zhong et al., 2019) and GPx plays a major role in the protection of cancer cells against ROS (Brigelius-Flohé & Kipp, 2009). We show that HER2+ cells under oxidative stress conditions exhibit higher overall ROS levels and may therefore be especially reliant on antioxidant enzymes, such as GPx. Additionally, while GPx3 is abundantly expressed in the plasma, GPx2 is preferentially expressed in the gastrointestinal tract (Brigelius-Flohé & Maiorino, 2013) but can be upregulated in malignant epithelial cells of other organs, such as the breast (Naiki-Ito et al., 2007). Increased mRNA levels for GPx2 was observed in our HER2+ breast cancer cell lines.

GPx enzymes are powerful antioxidant enzymes, and through the oxidation of reduced GSH into its oxidized GSSG, these enzymes serve to detoxify hydroperoxides, including hydrogen peroxide (Brigelius-Flohé & Maiorino, 2013). Indeed, the 21NT, 21MT-1, and SKBR3 HER2+ cell lines, all of which show the highest HER2 expression levels (confirmed by western blot analyses), showed the largest increases in GSSG levels in response to oxidative stress. This suggests that HER2+ cell lines may have increased antioxidant activity. However, upon Hsp90 inhibition by radicicol, GSSG levels dropped back to baseline which is peculiar as the HER2+ cell lines were expected to have increased GPx activity and thus, increased levels of GSSG.

As previously mentioned, a *decreased* GSH:GSSG ratio is indicative of oxidative stress (Chai et al., 1994; Asensi et al., 1999), and this was, as expected, observed for all hydrogen peroxide-treated cell lines. However, when combined with additional Hsp90 inhibition, this observed decrease in GSH:GSSG ratio was abolished for the HER2+ cell lines and the ratio was comparable to control. This is peculiar, as the expected enhancement in GPx expression should be associated with a reduced GSH:GSSG ratio. HER2+ breast cancer cells may have increased expression of glutathione reductase (GSR) for the conversion of GSSG back into GSH. This, however, seems

unlikely, as mRNA expression for *GSR* upon combined treatment shows no significant changes (**Figure S4.4**). This remains to be further explored.

Many studies have looked into the use of Nrf2 inhibitors (Robledinos-Antón et al., 2019) as adjuvants to cancer therapy to inhibit the antioxidant response (Zhu et al., 2016b). However, while GPx2 is regulated by Nrf2 (Singh et al., 2006), GPx3 is not. Thus, in this scenario, a more targeted approach is needed to mitigate the increased GPx-associated antioxidant capacity of HER2+ breast cancer cells upon Hsp90 inhibition. We thus proposed the more specific small-molecule inhibition of GPx by BSO. BSO is a potent inhibitor of the rate-limiting enzyme required for GSH synthesis, GCL (Griffith & Meister, 1979). BSO has been tested in preclinical models of cancers and has shown promise in enhancing the efficacy of anti-cancer therapeutics, including in breast cancer (Lewis-Wambi et al., 2008). We predict that inhibition of GSH biosynthesis in HER2+ breast cancer cells by BSO should reduce their GPx-associated antioxidant capacity and render cancer therapy more effective when combined with Hsp90 inhibition. This will be determined in our ensuing studies.

Hsp90 inhibition has been widely studied in the context of breast cancer treatment; however, mechanisms of induced resistance and off-target effects remained underexplored. This work begins to decipher the functional interplay between HER2 and Hsp90 and identifies the specific role of GPx enzymes in HER2+ breast cancer. By assessing the efficacy of HER2+ breast cancer therapies, such as Trastuzumab, in conjunction with small-molecule inhibitors of Hsp90 and GPx activity, we provide new insight into the combined use of these inhibitors in the improvement of treatment outcomes for HER2+ breast cancer and open new avenues for breast cancer treatment in general.

4.4 Materials and Methods

4.4.1 Cell lines and culture conditions

T47D cells were maintained in Roswell Park Memorial Institute (RPMI)1640 media supplemented with 10% FBS. MCF7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. The 21PT, 21NT, and 21MT-1 cell lines were maintained in α Minimum Essential Medium (αMEM) supplemented with 2 mM L-glutamine, 1 μg/mL insulin,

12.5 ng/mL EGF, 2.8 µM hydrocortisone, 10 mM HEPES, 1mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 µg/mL gentamycin sulfate, and 10% FBS. SKBR3 cells were maintained in McCoy's 5A media supplemented with 10% FBS. All reagents for the culture of breast cancer cell lines were obtained from Gibco Inc. (Manassas, VA, USA).

4.4.2 Inhibitor treatment

Cells (T47D, MCF7, 21PT, 21NT, 21MT-1, and SKBR3) were seeded in a 6-well plate at $0.5x10^6$ cells per well. The following day, cells were treated (in triplicate) with one of the following six treatments: 600 µM H_2O_2 (3 h), 2 mM H_2O_2 (3 h), 30 µM radicicol (Cayman Chemical, 12772-57-5; 6h), 100 µM radicicol (6 h), and combined treatments with 100 µM radicicol (6 h) with 600 µM H_2O_2 (3 h) and 100 µM radicicol (6 h) with 2 mM H_2O_2 (3 h). Controls included media only and DMSO treatment (final concentration of 0.1% DMSO for 6 h for all DMSO and radicicol-treated samples).

4.4.3 RNA isolation and quantitative reverse transcription PCR (RT-qPCR)

RNA was isolated using the RNA Extraction Kit (Pure Reagents, KIT-RNA-ISO-MAM-75), and 500 ng of RNA was converted into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1632). The PowerUp SYBR Green Master Mix (Applied Biosystems, A25742) was utilized for quantitative PCR with the primer sequences listed in **Table 4.1**. The output values were normalized to *RPLP0* expression.

Table 4.1: Primer sequences utilized for RT-qPCR in mRNA studies.

mRNA Probe	Primer Sequences (Forward and Reverse, 5' to 3')
ABCC2	F: AGTCTTAGCAGGTGTTGGGG R: GACTAAAGGCCAGCAGGTTCT
DNAJA4	F: AGGTGATAAAGCACGGGGAC R: GCCAGTTCTGCTCATTGGGA
GPX1	F: GGAGAACGCCAAGAACGAAG R: AGCATGAAGTTGGGCTCGAA
GPX2	F: GGATACCAGCCCACCTTCAC R: GGTAGGCGAAGACAGGATGC
GPX3	F: GAGCTTGCACCATTCGGTCT R: TTAGGGACAAAGCCTCCACC
GPX4	F: GCCTTCCCGTGTAACCAGT R: GCGAACTCTTTGATCTCTTCGT
HMOX1	F: CCCCAACGAAAAGCACATCC R: AGACAGCTGCCACATTAGGG
HSF1	F: CAGCTTCCACGTGTTCGAC R: GGCCATGTTGTTGTGCTTGA
HSPA1A	F: TAACCCCATCATCAGCGGAC R: AGCTCCAAAACAAAAACAGCAA
HSP90AA1	F: GCTGGACAGCAAACATGGAG R: AGACAGGAGCGCAGTTTCAT
HSP90AB1	F: ATTGTGACCAGCACCTACGG R: CATGGTGGAGTTGTCCCGAA
NQO1	F: TGGAAGAAACGCCTGGAGAAT R: CTGGTTGTCAGTTGGGATGG
RPLP0	F: CCTCATATCCGGGGGAATGTG R: GCAGCAGCTGGCACCTTATTG

4.4.4 Preparation of protein lysates

Cells (T47D, MCF7, 21PT, 21NT, 21MT-1, and SKBR3) were seeded in a 6-well plate at $1x10^6$ cells per well. The following day, 500 µl of RIPA buffer (150 mM NaCl, 1% Triton-X, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-base; pH 8.0) with Pierce Protease Inhibitor Tablets (Thermo Fisher Scientific, A32965) was added to confluent 6-well dishes. Cells were scraped, collected in a microcentrifuge tube, incubated on ice for 10 min, and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected, and the protein concentration per sample was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) by comparison to BSA standards.

4.4.5 Electrophoresis and western blot analysis

Protein (20 µg) was resolved on a 10% SDS-PAGE gel. The membrane was blocked with 5% milk in PBST and incubated with one of the following primary antibodies overnight at 4°C: rabbit anti-HER2 (Invitrogen, MA5-15050), mouse anti-Hsp90α (Abcam, ab79849), mouse anti-Hsp90β (Abcam, ab53497), or rat anti-β-Tubulin (Abcam, ab6160). The membrane was incubated with an HRP-conjugated secondary antibody, either anti-rabbit (Abcam, ab6721), anti-mouse (Abcam, ab6728), or anti-rat (Abcam, ab97057) as required, for 1 h at room temperature. Western blots were visualized using the Clarity Western ECL Substrate kit (Bio-Rad, 1705061) and images were taken using the ChemiDoc Imaging System (Bio-Rad). In ensuing experiments, densitometric quantification will be performed using ImageLab (Bio-Rad) and quantities will be normalized to total protein expression.

4.4.6 Cell viability, ROS, and glutathione assays

Cells (T47D, MCF7, 21PT, 21NT, 21MT-1, and SKBR3) were seeded in a white 96-well plate at 20 000 cells per well. The following day, cells were treated (in triplicate) with hydrogen peroxide, radicicol, and combined treatments as outlined in Section 4.4.2. Cell viability was measured using the CellTiter-Glo 2.0 Cell Viability Assay (Promega, G9242) and ROS levels were measured using the ROS-Glo H_2O_2 Assay (Promega, G8820). Glutathione levels were measured using the GSH/GSSG-Glo Assay (Promega, V6611). All assays were performed according to the

manufacturer's protocol. Fluorescence and luminescence were measured using the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek).

4.4.7 Bioinformatics analyses

The International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas breast cancer (TCGA BRCA) datasets were interrogated, and data were exported using XenaBrowser (Goldman et al., 2020). *HSP90AA1* expression was compared across cancer type (ICGC), breast cancer molecular subtype (TCGA BRCA), and HER2 status (TCGA BRCA). The aforementioned datasets are listed in **Table 4.2**.

To examine transcripts that were co-expressed with *HSP90AA1* in HER2- and HER2+ breast cancers within the TCGA dataset, gene lists were constructed using Venny 2.1 (Oliveros, 2007). Enrichr pathway analysis (Chen et al., 2013) was conducted with the input list consisting of 28 genes positively correlated with *HSP90AA1* only in HER2+ breast cancers. Results assessing Transcription Factor (TF) Interactions and TF-loss of function (LOF) (GEO) expression data are reported. The combined score takes into account the p-value and z-score, with the calculation CombinedScore = ln(p)*z, with p representing the p-value and z representing the z-score (Kuleshov et al., 2016). Pharmacological inhibitor data was obtained from CancerRxGene (Yang et al., 2013) and searching for Hsp90 inhibitors. Cell line characterization with respect to HER2 status was obtained from previously published studies (Suzuki et al., 2009; Valabrega et al., 2011; Wang et al., 2011; Kalous et al., 2012; Di et al., 2014; Dai et al., 2017).

Table 4.2: Publicly available datasets utilized for analysis.

Dataset	Reference (or Link)
International Cancer Genome Consortium (ICGC)	https://dcc.icgc.org
TCGA Breast Cancer (BRCA)	https://portal.gdc.cancer.gov/projects/TCGA-BRCA
CancerRxGene	https://www.cancerrxgene.org

4.4.8 Statistical analysis

Statistical analyses were conducted using Prism 8 (GraphPad Software). Statistical significance was obtained by performing a one-way ANOVA with Tukey post hoc for comparison between groups, or the Student's t-test for comparison between two groups (with a minimum of 3 biological replicates). Error bars represent standard deviation. P-values less than 0.05 were considered statistically significant. Significance levels are indicated using asterisks, where * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$. Shapiro-Wilk tests were performed for all data sets to ensure normality.

4.5 Supplementary Figures

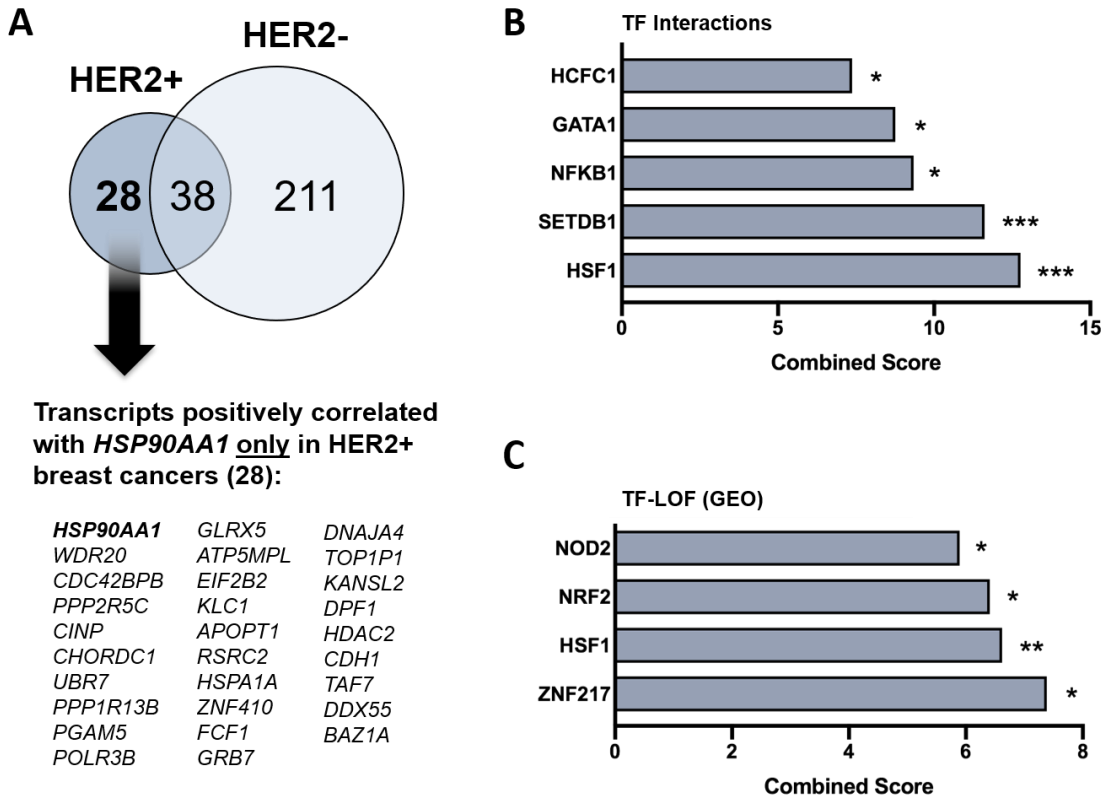


Figure S4.1: *HSP90AA1* expression is associated with Nrf2 and Hsf1 signalling pathways in HER2+ breast cancers. Overlap of transcripts positively associated with *HSP90AA1* mRNA levels in the TCGA breast cancer cohort by HER2 status. The 28 transcripts positively correlated with *HSP90AA1* in HER2 positive breast cancers. Pathway analysis was conducted on this gene set, with results from **(B)** Transcription Factor (TF) Interactions and **(C)** TF loss of function (LOF) (GEO) expression data reported.

	Gene	Protein Encoded	Function
Nrf2	*NFE2L2*	Nuclear factor erythroid 2-related factor 2 (Nrf2)	Transcription factor regulates the oxidative stress response
	ABCC1	Multidrug resistance-associated protein 2 (Mrp2)	ATPase activity coupled to transmembrane movement for detoxification
	NQO1	NAD(P)H dehydrogenase:quinone 1 (NQO1)	Reduces quinone to hydroquinone
	HMOX1	Heme oxygenase 1 (HO-1)	Degrades heme to biliverdin
	GPX2	Glutathione peroxidase 2 (GPx2)	Reduces H_2O_2 and hydroperoxides
	GSS	Glutathione synthetase (GSS)	Condensation of γ-glutamylcysteine and glycine to glutathione (GSH) for glutathione biosynthesis
	GSR	Glutathione-disulfide reductase (GSR)	Reduces GSSG to GSH for GSH regeneration
	Gene	**Protein Encoded**	**Function**
Hsf1	*HSF1*	Heat shock factor 1 (Hsf1)	Transcription factor that regulates the heat shock response
	HSP90AA1	Heat shock protein 90kDa alpha (Hsp90α)	Stress inducible isoform of the molecular chaperone Hsp90
	HSP90AB1	Heat shock protein 90kDa beta (Hsp90β)	Constitutively active isoform of the molecular chaperone Hsp90
	HSPA1A	Heat shock 70 kDa protein 1 (Hsp70)	Molecular chaperone
	DNAJA1	DnaJ homolog subfamily A member 1 (Hsp40)	Co-chaperone for HspA8/Hsc70

Figure S4.2: List of Nrf2 and Hsf1 target genes evaluated by RT-qPCR. Target genes and their associated protein and protein function are listed.

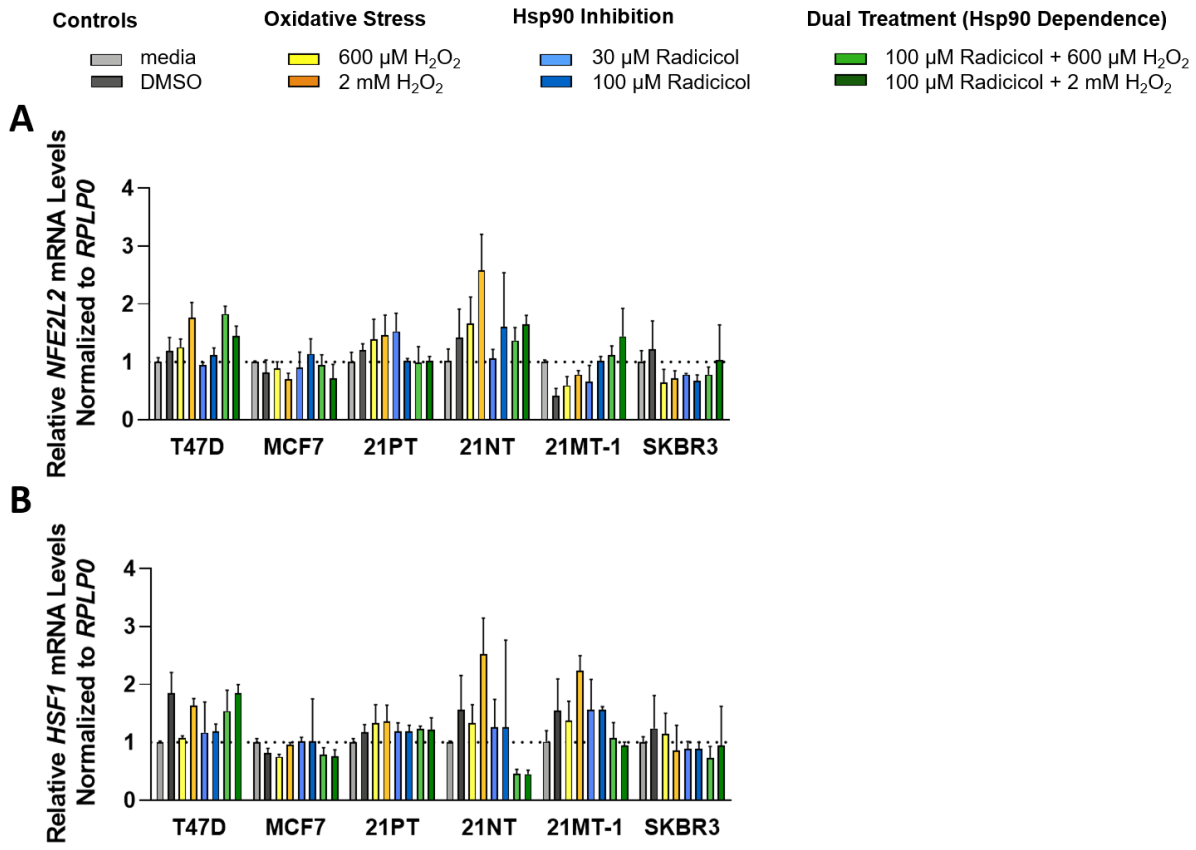


Figure S4.3: Relative mRNA levels for *NFE2L2* and *HSF1*. Genes were evaluated by RT-qPCR, normalized to *RPLP0* transcript levels, and depicted as fold change relative to the media control. Means derived from three biological replicates were used during analysis. Data are expressed as mean ± SD.

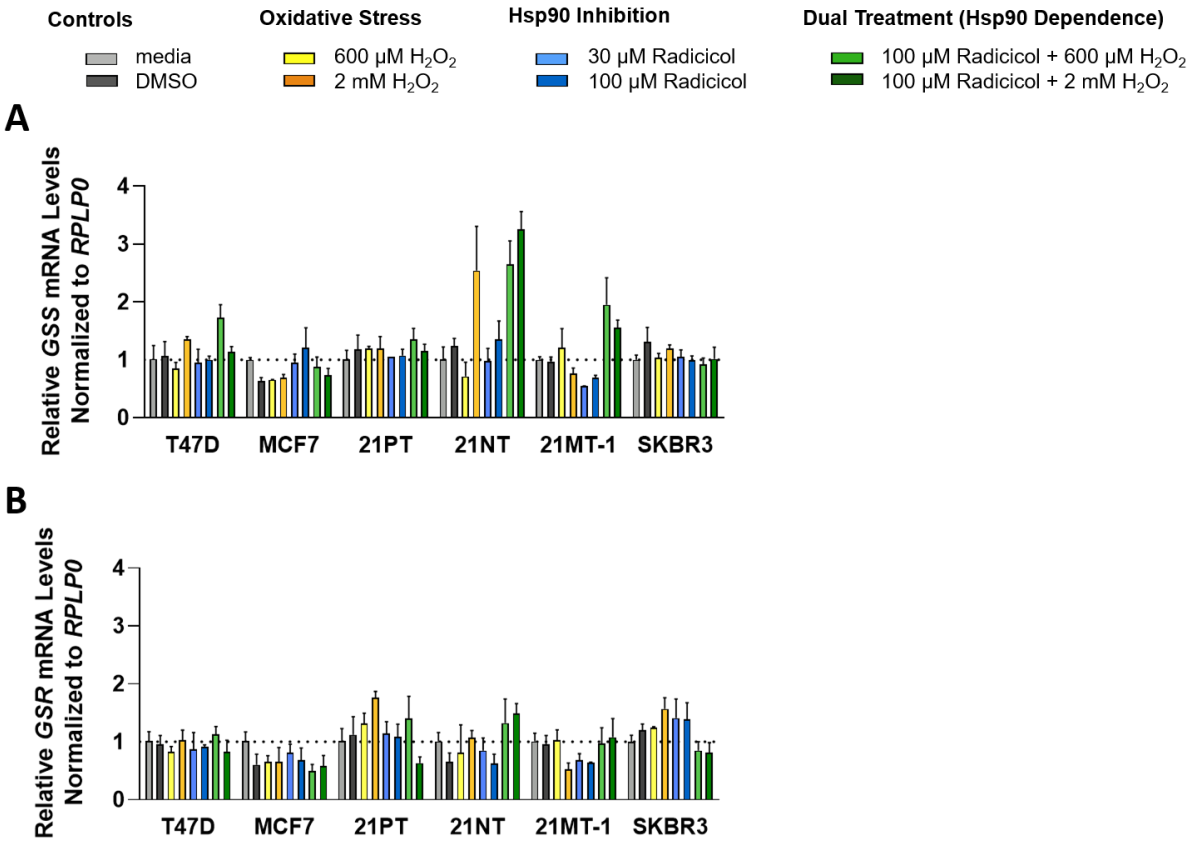


Figure S4.4: Relative mRNA levels for *GSS* and *GSR*. Genes were evaluated by RT-qPCR, normalized to *RPLP0* transcript levels, and depicted as fold change relative to the media control. Means derived from three biological replicates were used during analysis. Data are expressed as mean ± SD.

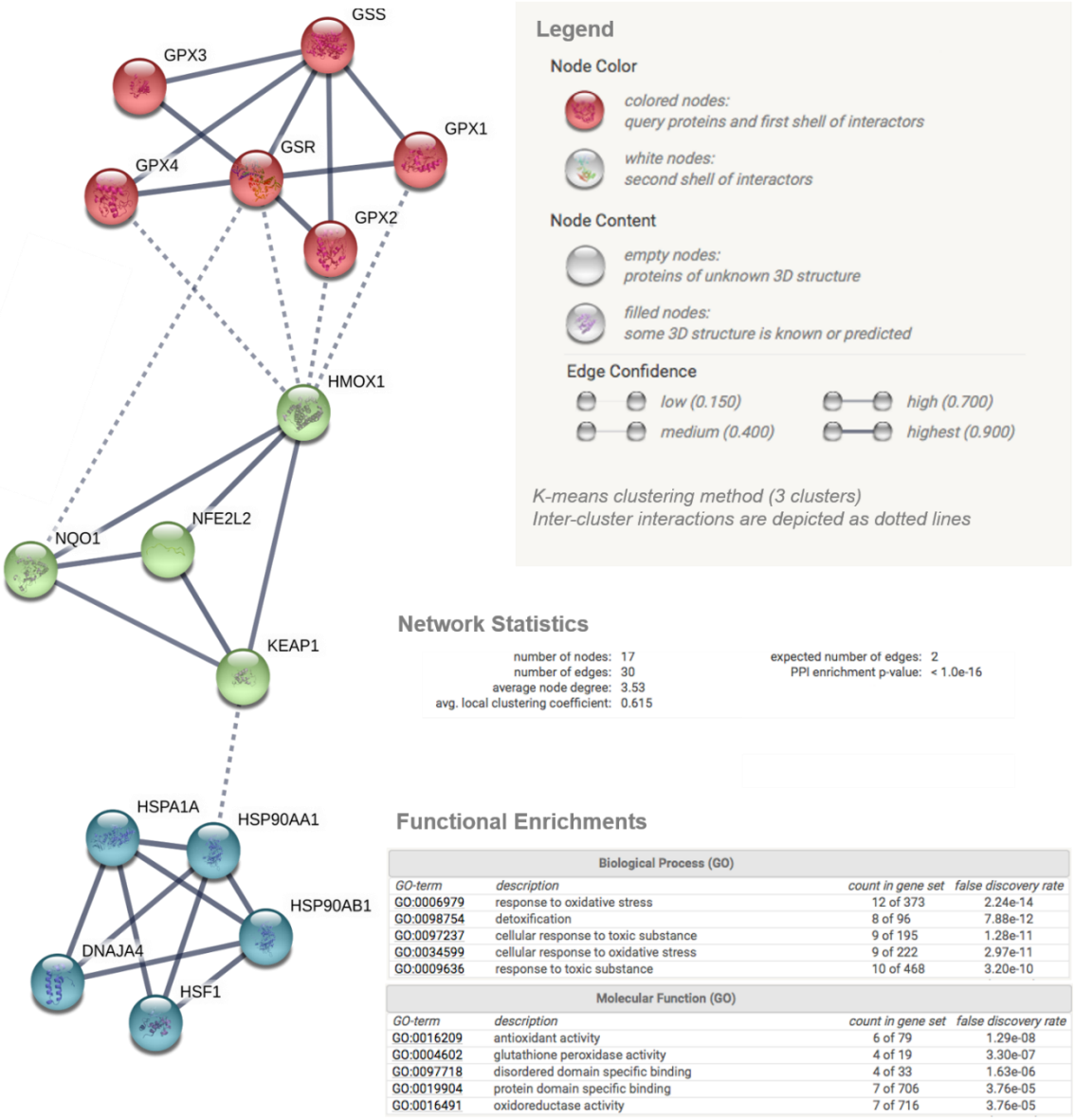


Figure S4.5: STRING interaction of Nrf2 and Hsf1 target genes. Nrf2 target genes are shown in blue and Hsf1 target genes are shown in green. Glutathione peroxidase (GPx)-associated genes are illustrated in red. Network statistics and functional enrichments are shown. Data are derived from the STRING Consortium.

4.6 References

Ali, A., Bharadwaj, S., O'Carroll, R., & Ovsenek, N. (1998). HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. *Mol Cell Biol, 18*(9), 4949-4960.

Asensi, M., Sastre, J., Pallardo, F. V., Lloret, A., Lehner, M., Garcia-de-la Asuncion, J., & Viña, J. (1999). [23] Ratio of reduced to oxidized glutathione as indicator of oxidative stress status and DNA damage *Methods in Enzymology* (Vol. 299, pp. 267-276): Academic Press.

Azoitei, N., Diepold, K., Brunner, C., Rouhi, A., Genze, F., Becher, A., Kestler, H., van Lint, J., Chiosis, G., Koren, J., Fröhling, S., Scholl, C., & Seufferlein, T. (2014). HSP90 Supports Tumor Growth and Angiogenesis through PRKD2 Protein Stabilization. *Cancer Res, 74*(23), 7125.

Baird, L., Suzuki, T., Takahashi, Y., Hishinuma, E., Saigusa, D., & Yamamoto, M. (2020). Geldanamycin-Derived HSP90 Inhibitors Are Synthetic Lethal with NRF2. *Mol Cell Biol, 40*(22), e00377-00320.

Band, V., Zajchowski, D., Swisshelm, K., Trask, D., Kulesa, V., Cohen, C., Connolly, J., & Sager, R. (1990). Tumor progression in four mammary epithelial cell lines derived from the same patient. *Cancer Res, 50*(22), 7351-7357.

Baselga, J., Carbonell, X., Castañeda-Soto, N. J., Clemens, M., Green, M., Harvey, V., Morales, S., Barton, C., & Ghahramani, P. (2005). Phase II study of efficacy, safety, and pharmacokinetics of trastuzumab monotherapy administered on a 3-weekly schedule. *J Clin Oncol, 23*(10), 2162-2171.

Basso, A. D., Solit, D. B., Chiosis, G., Giri, B., Tsichlis, P., & Rosen, N. (2002a). Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J Biol Chem, 277*(42), 39858-39866.

Basso, A. D., Solit, D. B., Munster, P. N., & Rosen, N. (2002b). Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. *Oncogene, 21*(8), 1159-1166.

Brennan, P. J., Kumagai, T., Berezov, A., Murali, R., & Greene, M. I. (2000). HER2/neu: mechanisms of dimerization/oligomerization. *Oncogene, 19*(53), 6093-6101.

Brigelius-Flohé, R., & Kipp, A. (2009). Glutathione peroxidases in different stages of carcinogenesis. *Biochim Biophys Acta, 1790*(11), 1555-1568.

Brigelius-Flohé, R., & Maiorino, M. (2013). Glutathione peroxidases. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1830*(5), 3289-3303.

Burstein, H. J. (2005). *The distinctive nature of HER2-positive breast cancers* (0028-4793). Retrieved from

Canonici, A., Qadir, Z., Conlon, N. T., Collins, D. M., O'Brien, N. A., Walsh, N., Eustace, A. J., O'Donovan, N., & Crown, J. (2018). The HSP90 inhibitor NVP-AUY922 inhibits growth of HER2 positive and trastuzumab-resistant breast cancer cells. *Invest New Drugs, 36*(4), 581-589.

Carey, L. A., Perou, C. M., Livasy, C. A., Dressler, L. G., Cowan, D., Conway, K., Karaca, G., Troester, M. A., Tse, C. K., Edmiston, S., Deming, S. L., Geradts, J., Cheang, M. C., Nielsen, T. O., Moorman, P. G., Earp, H. S., & Millikan, R. C. (2006). Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA, 295*(21), 2492-2502.

Chai, Y. C., Ashraf, S. S., Rokutan, K., Johnston, R. B., Jr., & Thomas, J. A. (1994). S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide. *Arch Biochem Biophys, 310*(1), 273-281.

Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., Clark, N. R., & Ma'ayan, A. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics, 14*(1), 128.

Chen, G., Cao, P., & Goeddel, D. V. (2002). TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell, 9*(2), 401-410.

Ciocca, D. R., Arrigo, A. P., & Calderwood, S. K. (2013). Heat shock proteins and heat shock factor 1 in carcinogenesis and tumor development: an update. *Arch Toxicol, 87*(1), 19-48.

Ciocca, D. R., Clark, G. M., Tandon, A. K., Fuqua, S. A., Welch, W. J., & McGuire, W. L. (1993). Heat shock protein hsp70 in patients with axillary lymph node-negative breast cancer: prognostic implications. *J Natl Cancer Inst, 85*(7), 570-574.

ClinicalTrials.gov. ClinicalTrials.gov Available from U.S. National Library of Medicine ClinicalTrials.gov Retrieved December 16, 2020, from U.S. National Library of Medicine https://www.clinicaltrials.gov/

Cornford, P. A., Dodson, A. R., Parsons, K. F., Desmond, A. D., Woolfenden, A., Fordham, M., Neoptolemos, J. P., Ke, Y., & Foster, C. S. (2000). Heat shock protein expression independently predicts clinical outcome in prostate cancer. *Cancer Res, 60*(24), 7099-7105.

Crouch, S. P., Kozlowski, R., Slater, K. J., & Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J Immunol Methods, 160*(1), 81-88.

Dai, C., Whitesell, L., Rogers, A. B., & Lindquist, S. (2007). Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell, 130*(6), 1005-1018.

Dai, X., Cheng, H., Bai, Z., & Li, J. (2017). Breast Cancer Cell Line Classification and Its Relevance with Breast Tumor Subtyping. *J Cancer, 8*(16), 3131-3141.

Dayalan Naidu, S., Kostov, R. V., & Dinkova-Kostova, A. T. (2015). Transcription factors Hsf1 and Nrf2 engage in crosstalk for cytoprotection. *Trends Pharmacol Sci, 36*(1), 6-14.

Desai, N. P., Trieu, V., Hwang, L. Y., Wu, R., Soon-Shiong, P., & Gradishar, W. J. (2008). Improved effectiveness of nanoparticle albumin-bound (nab) paclitaxel versus polysorbate-based docetaxel in multiple xenografts as a function of HER2 and SPARC status. *Anti-cancer drugs, 19*(9), 899-909.

Di, Z., Klop, M. J., Rogkoti, V. M., Le Dévédec, S. E., van de Water, B., Verbeek, F. J., Price, L. S., & Meerman, J. H. (2014). Ultra high content image analysis and phenotype profiling of 3D cultured micro-tissues. *PLoS ONE, 9*(10), e109688.

Finkel, T. (2011). Signal transduction by reactive oxygen species. *Journal of Cell Biology, 194*(1), 7-15.

Forman, H. J., Zhang, H., & Rinna, A. (2009). Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med, 30*(1-2), 1-12.

Goldman, M. J., Craft, B., Hastie, M., Repečka, K., McDade, F., Kamath, A., Banerjee, A., Luo, Y., Rogers, D., Brooks, A. N., Zhu, J., & Haussler, D. (2020). Visualizing and interpreting cancer genomics data via the Xena platform. *Nature Biotechnology, 38*(6), 675-678.

Griffith, O. W., & Meister, A. (1979). Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *J Biol Chem, 254*(16), 7558-7560.

Gschwind, A., Fischer, O. M., & Ullrich, A. (2004). The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer, 4*(5), 361-370.

Hu, Y., & Mivechi, N. F. (2003). HSF-1 interacts with Ral-binding protein 1 in a stress-responsive, multiprotein complex with HSP90 in vivo. *J Biol Chem, 278*(19), 17299-17306.

Hwang, Y. J., Lee, S. P., Kim, S. Y., Choi, Y. H., Kim, M. J., Lee, C. H., Lee, J. Y., & Kim, D. Y. (2009). Expression of heat shock protein 60 kDa is upregulated in cervical cancer. *Yonsei medical journal, 50*(3), 399-406.

Kalous, O., Conklin, D., Desai, A. J., O'Brien, N. A., Ginther, C., Anderson, L., Cohen, D. J., Britten, C. D., Taylor, I., Christensen, J. G., Slamon, D. J., & Finn, R. S. (2012). Dacomitinib (PF-00299804), an irreversible Pan-HER inhibitor, inhibits proliferation of HER2-amplified breast cancer cell lines resistant to trastuzumab and lapatinib. *Mol Cancer Ther, 11*(9), 1978-1987.

Kaur, J., & Ralhan, R. (1995). Differential expression of 70-kDa heat shock-protein in human oral tumorigenesis. *Int J Cancer, 63*(6), 774-779.

Kimura, E., Enns, R. E., Thiebaut, F., & Howell, S. B. (1993). Regulation of HSP60 mRNA expression in a human ovarian carcinoma cell line. *Cancer Chemotherapy and Pharmacology, 32*(4), 279-285.

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., & Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol, 24*(16), 7130-7139.

Kuleshov, M. V., Jones, M. R., Rouillard, A. D., Fernandez, N. F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S. L., Jagodnik, K. M., Lachmann, A., McDermott, M. G., Monteiro, C. D., Gundersen, G. W., & Ma'ayan, A. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res, 44*(W1), W90-97.

Lewis-Wambi, J. S., Kim, H. R., Wambi, C., Patel, R., Pyle, J. R., Klein-Szanto, A. J., & Jordan, V. C. (2008). Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res, 10*(6), R104.

Lewis, J., Devin, A., Miller, A., Lin, Y., Rodriguez, Y., Neckers, L., & Liu, Z. G. (2000). Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation. *J Biol Chem, 275*(14), 10519-10526.

Li, L., Chen, N.-N., You, Q.-D., & Xu, X.-L. (2021). An updated patent review of anticancer Hsp90 inhibitors (2013-present). *Expert Opinion on Therapeutic Patents, 31*(1), 67-80.

Ma, L., Fu, Q., Xu, B., Zhou, H., Gao, J., Shao, X., Xiong, J., Gu, Q., Wen, S., Li, F., Shen, L., Chen, G., Fang, H., & Lyu, J. (2018). Breast cancer-associated mitochondrial DNA haplogroup promotes neoplastic growth via ROS-mediated AKT activation. *Int J Cancer, 142*(9), 1786-1796.

Marty, M., Cognetti, F., Maraninchi, D., Snyder, R., Mauriac, L., Tubiana-Hulin, M., Chan, S., Grimes, D., Antón, A., Lluch, A., Kennedy, J., O'Byrne, K., Conte, P., Green, M., Ward, C., Mayne, K., & Extra, J. M. (2005). Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. *J Clin Oncol, 23*(19), 4265-4274.

Mendillo, M. L., Santagata, S., Koeva, M., Bell, G. W., Hu, R., Tamimi, R. M., Fraenkel, E., Ince, T. A., Whitesell, L., & Lindquist, S. (2012). HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. *Cell, 150*(3), 549-562.

Meng, L., Gabai, V. L., & Sherman, M. Y. (2010). Heat-shock transcription factor HSF1 has a critical role in human epidermal growth factor receptor-2-induced cellular transformation and tumorigenesis. *Oncogene, 29*(37), 5204-5213.

Merlin, J. L., Barberi-Heyob, M., & Bachmann, N. (2002). In vitro comparative evaluation of trastuzumab (Herceptin®) combined with paclitaxel (Taxol®) or docetaxel (Taxotere®) in HER2-expressing human breast cancer cell lines. *Annals of Oncology, 13*(11), 1743-1748.

Mitri, Z., Constantine, T., & O'Regan, R. (2012). The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy. *Chemotherapy research and practice, 2012*, 743193-743193.

Miyata, Y. (2005). Hsp90 inhibitor geldanamycin and its derivatives as novel cancer chemotherapeutic agents. *Curr Pharm Des, 11*(9), 1131-1138.

Naiki-Ito, A., Asamoto, M., Hokaiwado, N., Takahashi, S., Yamashita, H., Tsuda, H., Ogawa, K., & Shirai, T. (2007). Gpx2 is an overexpressed gene in rat breast cancers induced by three different chemical carcinogens. *Cancer Res, 67*(23), 11353-11358.

Nair, S. C., Toran, E. J., Rimerman, R. A., Hjermstad, S., Smithgall, T. E., & Smith, D. F. (1996). A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1, and the aryl hydrocarbon receptor. *Cell Stress Chaperones, 1*(4), 237-250.

Ngo, V., Brickenden, A., Liu, H., Yeung, C., Choy, W. Y., & Duennwald, M. L. (2020). A novel yeast model detects Nrf2 and Keap1 interactions with Hsp90 (*submitted*).

Olayioye, M. A., Neve, R. M., Lane, H. A., & Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *Embo j, 19*(13), 3159-3167.

Oliveros, J. C. (2007). VENNY. An interactive tool for comparing lists with Venn Diagrams. Retrieved from https://bioinfogp.cnb.csic.es/tools/venny/index.html

Pearl, L. H., & Prodromou, C. (2006). Structure and Mechanism of the Hsp90 Molecular Chaperone Machinery. *Annual Review of Biochemistry, 75*(1), 271-294.

Pick, E., Kluger, Y., Giltnane, J. M., Moeder, C., Camp, R. L., Rimm, D. L., & Kluger, H. M. (2007). High HSP90 Expression Is Associated with Decreased Survival in Breast Cancer. *Cancer Res, 67*(7), 2932.

Pompella, A., Visvikis, A., Paolicchi, A., Tata, V. D., & Casini, A. F. (2003). The changing faces of glutathione, a cellular protagonist. *Biochemical Pharmacology, 66*(8), 1499-1503.

Raja, S. M., Clubb, R. J., Bhattacharyya, M., Dimri, M., Cheng, H., Pan, W., Ortega-Cava, C., Lakku-Reddi, A., Naramura, M., Band, V., & Band, H. (2008). A combination of Trastuzumab and 17-AAG induces enhanced ubiquitinylation and lysosomal pathway-dependent ErbB2 degradation and cytotoxicity in ErbB2-overexpressing breast cancer cells. *Cancer biology & therapy, 7*(10), 1630-1640.

Rimawi, M. F., De Angelis, C., & Schiff, R. (2015). Resistance to Anti-HER2 Therapies in Breast Cancer. *American Society of Clinical Oncology Educational Book*(35), e157-e164.

Robertson, H., Dinkova-Kostova, A. T., & Hayes, J. D. (2020). NRF2 and the Ambiguous Consequences of Its Activation during Initiation and the Subsequent Stages of Tumourigenesis. *Cancers (Basel), 12*(12).

Robledinos-Antón, N., Fernández-Ginés, R., Manda, G., & Cuadrado, A. (2019). Activators and Inhibitors of NRF2: A Review of Their Potential for Clinical Development. *Oxid Med Cell Longev, 2019*, 9372182-9372182.

Roe, S. M., Prodromou, C., O'Brien, R., Ladbury, J. E., Piper, P. W., & Pearl, L. H. (1999). Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem, 42*(2), 260-266.

Rojo de la Vega, M., Chapman, E., & Zhang, D. D. (2018). NRF2 and the Hallmarks of Cancer. *Cancer Cell, 34*(1), 21-43.

Santagata, S., Hu, R., Lin, N. U., Mendillo, M. L., Collins, L. C., Hankinson, S. E., Schnitt, S. J., Whitesell, L., Tamimi, R. M., Lindquist, S., & Ince, T. A. (2011). High levels of nuclear heat-shock factor 1 (HSF1) are associated with poor prognosis in breast cancer. *Proc Natl Acad Sci U S A, 108*(45), 18378-18383.

Sato, S., Fujita, N., & Tsuruo, T. (2000). Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A, 97*(20), 10832-10837.

Schulz, R., Streller, F., Scheel, A. H., Rüschoff, J., Reinert, M. C., Dobbelstein, M., Marchenko, N. D., & Moll, U. M. (2014). HER2/ErbB2 activates HSF1 and thereby controls HSP90 clients including MIF in HER2-overexpressing breast cancer. *Cell Death & Disease, 5*(1), e980-e980.

Sharma, S. V., Agatsuma, T., & Nakano, H. (1998). Targeting of the protein chaperone, HSP90, by the transformation suppressing agent, radicicol. *Oncogene, 16*(20), 2639-2645.

Singh, A., Rangasamy, T., Thimmulappa, R. K., Lee, H., Osburn, W. O., Brigelius-Flohé, R., Kensler, T. W., Yamamoto, M., & Biswal, S. (2006). Glutathione peroxidase 2, the major cigarette smoke-inducible isoform of GPX in lungs, is regulated by Nrf2. *Am J Respir Cell Mol Biol, 35*(6), 639-650.

Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., & Norton, L. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med, 344*(11), 783-792.

Solit, D. B., Zheng, F. F., Drobnjak, M., Münster, P. N., Higgins, B., Verbel, D., Heller, G., Tong, W., Cordon-Cardo, C., Agus, D. B., Scher, H. I., & Rosen, N. (2002). 17-Allylamino-17-demethoxygeldanamycin Induces the Degradation of Androgen Receptor and HER-2/neu and Inhibits the Growth of Prostate Cancer Xenografts. *Clinical Cancer Research, 8*(5), 986.

Sreedhar, A. S., Kalmar, E., Csermely, P., & Shen, Y. F. (2004). Hsp90 isoforms: functions, expression and clinical importance. *FEBS Lett, 562*(1-3), 11-15.

Suzuki, S., Nakasato, M., Shibue, T., Koshima, I., & Taniguchi, T. (2009). Therapeutic potential of proapoptotic molecule Noxa in the selective elimination of tumor cells. *Cancer Sci, 100*(4), 759-769.

Taguchi, K., & Yamamoto, M. (2017). The KEAP1–NRF2 System in Cancer. *Frontiers in Oncology, 7*, 85.

Teng, Y., Ngoka, L., Mei, Y., Lesoon, L., & Cowell, J. K. (2012). HSP90 and HSP70 proteins are essential for stabilization and activation of WASF3 metastasis-promoting protein. *J Biol Chem, 287*(13), 10051-10059.

Tikhomirov, O., & Carpenter, G. (2000). Geldanamycin induces ErbB-2 degradation by proteolytic fragmentation. *J Biol Chem, 275*(34), 26625-26631.

Trepel, J., Mollapour, M., Giaccone, G., & Neckers, L. (2010). Targeting the dynamic HSP90 complex in cancer. *Nature Reviews Cancer, 10*, 537.

Valabrega, G., Capellero, S., Cavalloni, G., Zaccarello, G., Petrelli, A., Migliardi, G., Milani, A., Peraldo-Neia, C., Gammaitoni, L., Sapino, A., Pecchioni, C., Moggio, A., Giordano, S., Aglietta, M., & Montemurro, F. (2011). HER2-positive breast cancer cells resistant to trastuzumab and lapatinib lose reliance upon HER2 and are sensitive to the multitargeted kinase inhibitor sorafenib. *Breast Cancer Res Treat, 130*(1), 29-40.

Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science, 324*(5930), 1029-1033.

Vernieri, C., Milano, M., Brambilla, M., Mennitto, A., Maggi, C., Cona, M. S., Prisciandaro, M., Fabbroni, C., Celio, L., Mariani, G., Bianchi, G. V., Capri, G., & de Braud, F. (2019). Resistance mechanisms to anti-HER2 therapies in HER2-positive breast cancer: Current knowledge, new research directions and therapeutic perspectives. *Critical Reviews in Oncology/Hematology, 139*, 53-66.

Vogel, C. L., Cobleigh, M. A., Tripathy, D., Gutheil, J. C., Harris, L. N., Fehrenbacher, L., Slamon, D. J., Murphy, M., Novotny, W. F., Burchmore, M., Shak, S., Stewart, S. J., & Press, M. (2002). Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol, 20*(3), 719-726.

Wang, Y. C., Morrison, G., Gillihan, R., Guo, J., Ward, R. M., Fu, X., Botero, M. F., Healy, N. A., Hilsenbeck, S. G., Phillips, G. L., Chamness, G. C., Rimawi, M. F., Osborne, C. K., & Schiff, R. (2011). Different mechanisms for resistance to trastuzumab versus lapatinib in HER2-positive breast cancers--role of estrogen receptor and HER2 reactivation. *Breast Cancer Res, 13*(6), R121.

Whitesell, L., & Lindquist, S. L. (2005). HSP90 and the chaperoning of cancer. *Nat Rev Cancer, 5*(10), 761-772.

Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E., & Neckers, L. M. (1994). Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone

ansamycins: essential role for stress proteins in oncogenic transformation. *Proceedings of the National Academy of Sciences, 91*(18), 8324.

Xu, W., Marcu, M., Yuan, X., Mimnaugh, E., Patterson, C., & Neckers, L. (2002). Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc Natl Acad Sci U S A, 99*(20), 12847-12852.

Yang, W., Soares, J., Greninger, P., Edelman, E. J., Lightfoot, H., Forbes, S., Bindal, N., Beare, D., Smith, J. A., Thompson, I. R., Ramaswamy, S., Futreal, P. A., Haber, D. A., Stratton, M. R., Benes, C., McDermott, U., & Garnett, M. J. (2013). Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. *Nucleic Acids Res, 41*(Database issue), D955-961.

Yano, M., Naito, Z., Tanaka, S., & Asano, G. (1996). Expression and roles of heat shock proteins in human breast cancer. *Jpn J Cancer Res, 87*(9), 908-915.

Yarden, Y., & Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol, 2*(2), 127-137.

Yun, C. W., Kim, H. J., Lim, J. H., & Lee, S. H. (2019). Heat Shock Proteins: Agents of Cancer Development and Therapeutic Targets in Anti-Cancer Therapy. *Cells, 9*(1).

Zagouri, F., Sergentanis, T. N., Chrysikos, D., Papadimitriou, C. A., Dimopoulos, M. A., & Psaltopoulou, T. (2013). Hsp90 inhibitors in breast cancer: a systematic review. *Breast, 22*(5), 569-578.

Zhong, G., Qin, S., Townsend, D., Schulte, B. A., Tew, K. D., & Wang, G. Y. (2019). Oxidative stress induces senescence in breast cancer stem cells. *Biochem Biophys Res Commun, 514*(4), 1204-1209.

Zhu, D., Shen, Z., Liu, J., Chen, J., Liu, Y., Hu, C., Li, Z., & Li, Y. (2016a). The ROS-mediated activation of STAT-3/VEGF signaling is involved in the 27-hydroxycholesterol-induced angiogenesis in human breast cancer cells. *Toxicology letters, 264*, 79-86.

Zhu, J., Wang, H., Chen, F., Fu, J., Xu, Y., Hou, Y., Kou, H. H., Zhai, C., Nelson, M. B., Zhang, Q., Andersen, M. E., & Pi, J. (2016b). An overview of chemical inhibitors of the Nrf2-ARE signaling pathway and their potential applications in cancer therapy. *Free Radical Biology and Medicine, 99*, 544-556.

Zou, J., Guo, Y., Guettouche, T., Smith, D. F., & Voellmy, R. (1998). Repression of Heat Shock Transcription Factor HSF1 Activation by HSP90 (HSP90 Complex) that Forms a Stress-Sensitive Complex with HSF1. *Cell, 94*(4), 471-480.

Zsebik, B., Citri, A., Isola, J., Yarden, Y., Szöllősi, J., & Vereb, G. (2006). Hsp90 inhibitor 17-AAG reduces ErbB2 levels and inhibits proliferation of the trastuzumab resistant breast tumor cell line JIMT-1. *Immunology Letters, 104*(1), 146-155.

Chapter 5

5 Discussion

5.1 Summary of Chapters

The chapters in this work investigate key cellular and molecular aspects of the Keap1-Nrf2 antioxidant pathway, including genetic and physical protein-protein interactions of Nrf2, and the folding and misfolding of Nrf2 and Keap1 under conditions of oxidative stress. From there, a convergence between the antioxidant and heat shock response pathways was observed and subsequent work examined this crosstalk in a clinically relevant model of breast cancer.

5.1.1 A novel yeast model for Nrf2 and Keap1 detects their interaction with Hsp90

In **Chapter 2**, we established and characterized a yeast model of human Nrf2, Keap1, and other proteins that interact with and regulate Nrf2. This chapter introduces yeast as a novel tool to study Nrf2 in a living test tube scenario, as yeast do not express Nrf2 or any close homolog; thus, existing and new interactions can be studied independently of external regulatory mechanisms. Our Nrf2 yeast model recapitulates previously described Nrf2 interactions in mammalian cells, including the key interaction between Nrf2 and Keap1 that regulates Nrf2 activity in the cell. Using the complementary experimental tools available for yeast, a previously unexplored interaction between Nrf2 and the molecular chaperone Hsp90 was detected, in addition to that of Keap1 and Hsp90. Through this Nrf2-Hsp90 interaction, this work starts to establish an important nexus between the antioxidant and heat shock responses.

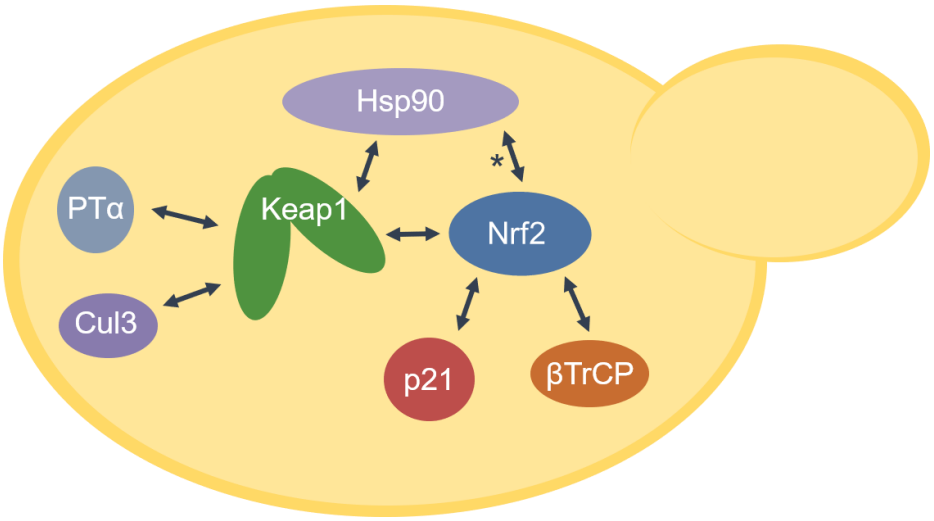


Figure 5.1: Summary of the observed Nrf2 interactions in yeast. Using yeast growth assays and the split-ubiquitin system to study genetic interactions and protein-protein interactions, respectively, known and previously unexplored (*) interactions within the Nrf2 protein network were detected.

5.1.2 Oxidative stress-induced inclusion formation of Nrf2 and Keap1

Chapter 3 examined aspects of protein oxidative damage, misfolding, and inclusion formation for Nrf2 and Keap1 using various experimental models. We found that both in yeast and mammalian cells, Nrf2 and Keap1 form intracellular protein inclusions upon exposure to oxidative stress, which is mediated, at least in part, by aberrant disulfide bond formation, shown through biochemical analyses using purified proteins. The intrinsically disordered nature of Nrf2 facilitates its tendency for misfolding (which can also expose oxidation-prone cysteine residues), while the high cysteine content of Keap1 promotes intra- and intermolecular disulfide bond formation by thiol oxidation, leading to protein misfolding under oxidative stress conditions. This protein inclusion formation was found to be oxidative stress-specific and dose-dependent. Although the exact functional consequences of this phenomenon remain to be explored, these findings propose a possible role of protein misfolding as a regulatory mechanism during oxidative stress conditions and indicate the importance of protein folding—mediated by heat shock proteins and molecular chaperones such as Hsp90—in regulating protein function.

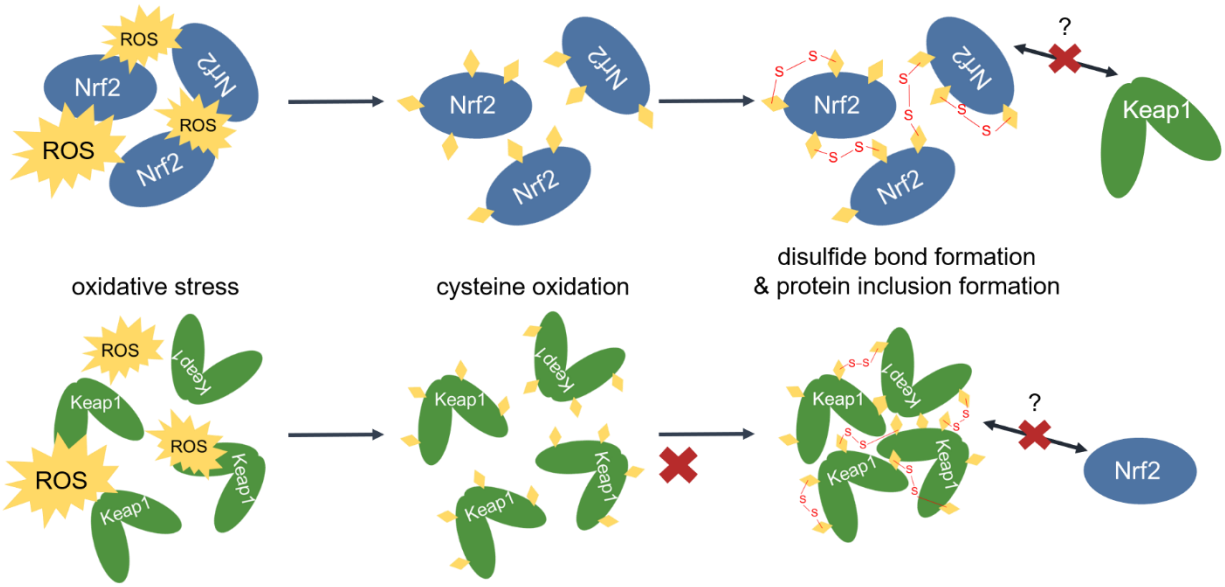


Figure 5.2: Nrf2 and Keap1 misfold and form protein inclusions upon oxidative stress. Treatment with hydrogen peroxide to elicit oxidative stress resulted in the formation of Nrf2 and Keap1 inclusions in yeast, cultured mammalian cells, and purified proteins.

5.1.3 Hyperactive stress response pathways in HER2+ breast cancers

Chapter 4 investigated the interplay between the antioxidant and heat shock responses and their dependence on Hsp90 chaperone activity in cancer cells. Nrf2 is highly overexpressed in cancer, which contributes to cancer cell growth, proliferation, and resistance to therapy (Takahashi et al., 2010; Li et al., 2011; Hartikainen et al., 2012). The Nrf2 and Hsf1 signalling pathways were examined in the context of breast cancer to explore the clinically relevant problem of cancer therapy resistance. Initial bioinformatic analyses revealed that HER-enriched breast cancers are among the highest expressers of *HSP90AA1*. We therefore investigated HER2+ and HER2- breast cancer cells under conditions of stress using Hsp90 inhibitors, which have been commonly used in the treatment of breast cancer (Zhong et al., 2019). Intriguingly, inhibition of Hsp90 leads to the off-target upregulation of two glutathione peroxidase antioxidant genes specifically in HER2-enriched cell lines during oxidative stress conditions. In alignment with the findings in Chapter 2, this suggests that there is crosstalk between the heat shock and antioxidant responses within these highly malignant cell lines. Ensuing experiments will explore a treatment regimen that addresses

this off-target effect by introducing an indirect inhibitor of GPx enzymatic activity to eliminate the antioxidant advantage that HER2-enriched breast cancer cells acquire upon Hsp90 inhibition.

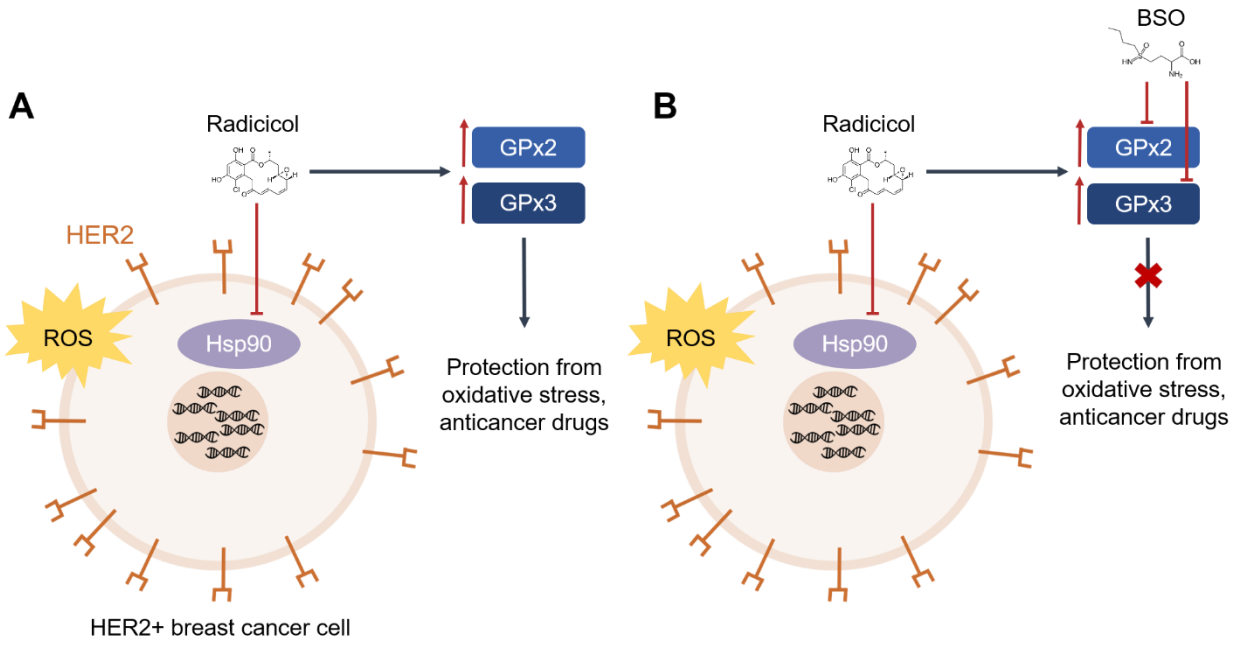


Figure 5.3: Hsp90 inhibition in HER2+ breast cancer leads to GPx overexpression during oxidative stress. (A) Inhibition of Hsp90 by radicicol under oxidative stress leads to the upregulation of *GPX2* and *GPX3* in HER2-enriched breast cancer cells. **(B)** Indirect impairment of GPx enzymatic activity by BSO is predicted to hinder this antioxidant advantage and render cancer cells more susceptible to cancer therapy.

5.2 Exploring Nrf2 Interactions

Nrf2 activity is strictly regulated by its interactions with other proteins, namely its key interaction with Keap1 through the binding of its DLG and ETGE motifs to the Kelch domain of Keap1 (Tong et al., 2006; Tong et al., 2007). Indeed, mutations within either motif impair the Keap1-Nrf2 interaction, as observed for our yeast DLG and ETGE mutant variants, L30F and T80R (Shibata et al., 2008). These mutations impair the ability of Keap1 to rescue the toxicity of Nrf2 expressed in yeast, implying these mutations abolish the genetic Keap1-Nrf2 interaction. Numerous human

cancers have demonstrated gain-of-function mutations in *NFE2L2* and loss-of-function mutations in *KEAP1* (Padmanabhan et al., 2006; Singh et al., 2006; Nioi & Nguyen, 2007; Shibata et al., 2008; Ooi et al., 2013). While it is unlikely that Nrf2 mutations *cause* cancer, Nrf2 mutations may enhance the growth and development of existing cancer cells by conferring enhanced antioxidant abilities to mitigate the stress of a hostile tumour environment (Taguchi & Yamamoto, 2017; Rojo de la Vega et al., 2018).

When Keap1-dependent Nrf2 regulation fails, the question remains whether other mechanisms of Keap1-independent regulation can adequately compensate. As previously discussed, there are many forms of non-canonical Nrf2 regulation via the direct interaction and competitive inhibition of proteins that bind to either Nrf2 or Keap1. Some major ones include Nrf2-binding βTrCP, p21, RXRα, and BRCA1 (Chen et al., 2009; Rada et al., 2011; Chowdhry et al., 2013; Gorrini et al., 2013; Wang et al., 2013; Xu et al., 2018), and Keap1-binding p62 and ProTα (Karapetian et al., 2005; Copple et al., 2010; Fan et al., 2010; Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010). Some of these proteins (βTrCP, p21, and ProTα) were included in our yeast studies and their respective interactions were confirmed using yeast growth assays and the split-ubiquitin system. Unfortunately, our studies did not include the many other interacting proteins, nor were these select interactions that were chosen investigated in detail. Future work may investigate Nrf2-associated genetic and physical protein-protein interactions in greater detail by taking advantage of the many tools available for yeast studies. Of note, to establish the Nrf2 yeast model, the split-ubiquitin system was used to detect known interactions; however, the high throughput capacity of the split-ubiquitin system was not exploited, e.g., it can be used to screen whole human cDNA fusion libraries to identify previously undiscovered interactions. Seeing as Nrf2 is highly intrinsically disordered and can thus bind to a wide range of targets, exploiting this system will likely result in the discovery of numerous unexplored Nrf2-binding partners. This system may also be used for the detection of novel small molecule enhancers and inhibitors that modulate these interactions in high throughput screens. Nevertheless, our yeast model of Nrf2 allowed us to discover the interaction between heat shock protein Hsp90 and Nrf2, the two key players in the heat shock and antioxidant responses, which inspired subsequent studies that sought to explore this intriguing nexus.

5.3 Oxidative Damage and Nrf2 Regulation

Although Nrf2 is primarily regulated through its protein interactions, other factors may influence Nrf2 activity, such as competitive binding to the ARE by transcriptional repressors (e.g., Bach1 (Dhakshinamoorthy et al., 2005)), phosphorylation of Nrf2 (e.g., GSK3, PKC (Huang et al., 2002; Rada et al., 2011; Chowdhry et al., 2013)), and the intracellular re-localization of Nrf2 (e.g., PGAM5 (Lo & Hannink, 2008)), among others. Given that Nrf2 is the key regulator of oxidative stress, the extent of oxidation within the Nrf2 protein itself may also influence its activity in the cell. Nrf2 contains six cysteine residues and Keap1 contains 27; cysteines are unique in that they contain highly reactive sulfur-based thiol groups that are prone to oxidation by ROS and disulfide bond formation with other cysteines (Stadtman, 1993). Indeed, we found that in yeast and cultured mammalian cells, Nrf2 and Keap1 are oxidized and form protein inclusions upon oxidative stress. Results using purified proteins showed that this was, at least in part, disulfide bond-dependent. However, the presence of cysteine residues alone is not sufficient for misfolding, as the Kelch domain of Keap1, which contains eight of the 27 cysteine residues in Keap1, did not misfold or form inclusions upon oxidative stress. Keap1 misfolding could be a highly regulated event, or the outcome of protein damage, but this remains to be explored. We speculate that perhaps under conditions of high oxidative stress, Keap1 and/or Nrf2 may misfold and form aberrant protein inclusions that impair the Keap1-Nrf2 interaction. Since Keap1 activity is dependent on its interaction with Nrf2, its activity may be completely inactivated when Keap1 misfolds, which allows for Nrf2 hyperactivation. On the other hand, Nrf2's transcription factor activity is not dependent on the Keap1-Nrf2 interaction and it may therefore still be functional, but this must also be further explored.

5.4 Reversibility of Cysteine Oxidation Inclusions

Apart from the ER, where temporary disulfide bonds help to stabilize proteins during the folding process (Jansens et al., 2002), most cysteines in the cell are kept reduced. Thioredoxin is a ubiquitous antioxidant enzyme chiefly responsible for thiol-redox control to reduce disulfide bonds and protect proteins from oxidative inclusion formation and inactivation (Collet & Messens, 2010). The question remains then, if oxidized Nrf2 and Keap1 inclusions are reversible by mechanisms such as disulfide bond reduction by thioredoxin and possible refolding by molecular chaperones,

and to what extent is this oxidative damage tolerated before it becomes irreversible and toxic. This question is particularly important in the context of neurodegenerative diseases where oxidative damage and protein misfolding and aggregation/inclusion formation are common hallmarks across many different neurodegenerative diseases (Soto, 2003). Pre-treatment of HeLa cells with the antioxidant NAC (but not vitamin C) reduced the formation of Nrf2 inclusions upon oxidative stress, suggesting that ROS scavenging may alleviate or prevent, to a certain extent, oxidative cysteine modifications; however, more evidence is required to confirm this. The use of live-cell imaging would be very informative and allow us to observe, using time-lapse microscopy, the formation of these stress-induced inclusions, and to determine if these inclusions can be reversed over time either by endogenous cellular mechanisms or through exogenous chemical treatments.

The other arising question is whether molecular chaperones, which assist in the refolding of misfolded proteins can refold cytosolic, disulfide bond-mediated, misfolded protein inclusions such as those observed for Nrf2 and Keap1. Within yeast, Hsp104 cooperates with Hsp70 and Hsp40 to function as a disaggregase that mediates the dissolution of protein aggregates and inclusions to restore their function or facilitate their clearance from the cell (Glover & Lindquist, 1998; Goloubinoff et al., 1999). However, metazoans, including humans, do not possess a known Hsp104 homologue so it remains unclear how some protein aggregates and inclusions are handled in these cells and which molecular chaperones are important for this (Shorter, 2008). It is also unlikely that molecular chaperones alone are capable of reducing disulfide bonds without the help of thioredoxins as they do not possess the mechanisms for disulfide bond reduction.

5.5 Cysteine Oxidation in Protein Regulation

Cysteines are one of the most highly conserved amino acid residues (Marino & Gladyshev, 2010), as observed in our cysteine alignments studies for Nrf2 and Keap1 in Chapter 3. Accordingly, cysteines are crucial to many cellular processes and often occur in the functional site of proteins (regulatory, catalytic, cofactor-binding, etc.) (Marino & Gladyshev, 2010). Within the Keap1-Nrf2 antioxidant pathway, specific cysteines within Keap1 are required for sensing oxidative stress and are thus critical for regulation of the antioxidant response (Dinkova-Kostova et al., 2002; Zhang & Hannink, 2003; Wakabayashi et al., 2004; McMahon et al., 2010). Similarly, the Nrf2-interacting and Parkinson's disease-associated protein, DJ-1, relies on the oxidation of Cys106 to

perform its cytoprotective function (Kim et al., 2009), while the E3 ubiquitin ligase activity of another Parkinson's-associated protein, parkin, requires the S-nitrosylation (addition of a nitric oxide group) to critical cysteine residues (Chung et al., 2004). Evidently, oxidative modifications such as cysteine oxidation are important for protein function. Aberrant cystine oxidation of Keap1 and Nrf2 and inclusion formation may thus function as an "on/off switch" for Nrf2 regulation under higher oxidative stress conditions.

Future studies must investigate the functional consequences of stress-induced inclusion formation of Nrf2 and Keap1. Perhaps under high levels of oxidative stress, Keap1, upon forming aberrant intra- and inter-molecular disulfide bonds, misfolds, is inactivated, and cannot bind to Nrf2 to target it for degradation, thus permanently allowing free Nrf2 to activate the antioxidant response. Conversely, oxidized Nrf2 may be unable to bind Keap1, therefore escaping Keap1-mediated degradation. This could be an adaptive or maladaptive mechanism of Nrf2 regulation under high oxidative stress conditions. In this regard, determining the reversibility of this cysteine oxidation and misfolding event is important, as irreversible oxidative damage and inactivation may lead to constitutive Nrf2 activation, as observed in many human cancers (Praslicka et al., 2016). Accordingly, in rapidly dividing cancer cells where ROS production is high (Reuter et al., 2010), or in the process of ageing where there are marked decreases in antioxidant capacity (Liguori et al., 2018), Nrf2, Keap1, and other proteins may be over-oxidized, resulting in the loss or dysregulation of their activity. This is in line with the "redox stress hypothesis" (Sohal & Orr, 2012) which proposes an age-associated, pro-oxidizing shift in the redox state of cells that results in the over-oxidation of protein thiols, thereby impairing signalling pathways. This may also be applicable for human diseases beyond ageing, such as cancer progression and cancer therapy resistance.

5.6 Off-Target Effects and Crosstalk Between Cell Stress Pathways

Hsp90 inhibitors have been the focus of many anti-cancer treatment regimens, particularly for HER2+ breast cancer since inhibition of Hsp90 results in degradation of the HER2 receptor (Tikhomirov & Carpenter, 2000; Solit et al., 2002). Although some clinical trials have shown promising results, others have been halted due to adverse side effects or cancer recurrence following treatment (Schopf et al., 2017). Off-target effects may explain the latter and must be

investigated to optimize treatment outcomes. In Chapter 4, we observe the off-target up-regulation of the antioxidant pathways through *GPX2* and *GPX3* during oxidative stress by Hsp90 inhibition. Thus, in highly malignant and advanced cancer cell lines such as HER2+ cells, inhibition of one stress response pathway seems to call upon a different stress pathway to alleviate the insult encountered by the cancer cell. Indeed, the Nrf2-regulated antioxidant enzyme GPx2, which is normally exclusively expressed in the intestinal epithelium (Brigelius-Flohé & Maiorino, 2013), was recruited in response to oxidative stress and Hsp90 inhibition, thereby suggesting crosstalk between the two stress response pathways. The heat shock response and antioxidant response pathways tend to be studied as separate cell stress response pathways and therapeutics targeting chaperones and antioxidant enzymes are often used independently without considering the possible influence of one pathway on the other. This may hinder the efficacy of the therapeutic, thus, more research should be conducted to investigate these types of off-target effects and the connection between different cellular stress responses before entering the clinical trial stage.

5.7 Limitations

5.7.1 Chapter 2

Yeast is a powerful model organism to study protein interactions and the basic mechanisms of cellular pathways. Yeast and human cells share fundamental commonalities for many conserved cellular processes, including those regulating protein quality control (Mohammadi et al., 2015), making the results directly relevant to humans. However, the yeast model also has limitations. Yeast are single-cell organisms that are much simpler than humans and some conserved pathways are more simplified in yeast (e.g., the unfolded protein response in yeast contains one pathway while humans have three (Wu et al., 2014)); therefore, results are not always directly translatable. Moreover, yeast does not express any close Nrf2 homologue. While this allows for the advantage of minimizing interference with endogenous Nrf2 regulation as it occurs in mammalian cells (i.e., yeast as a "living test tube"), this also has its limitations in that endogenous factors that may have otherwise influenced the interaction or mechanism are not present, which may ultimately lead to imprecise results. For instance, in our yeast model of Nrf2, Cul3 is not present to mediate the Keap1-dependent ubiquitination of Nrf2 because the Keap1-Nrf2 pathway does not exist in yeast. Additionally, the plasmids used in our genetic interaction studies were high-copy expression

plasmids that result in protein overexpression in yeast cells. It is therefore possible that this artificially high copy number introduces unforeseen variables regarding genetic and protein-protein interactions. Nonetheless, many important discoveries that are relevant to this work have been discovered using yeast. Quite notably, in 1999, a yeast two-hybrid screen using the Neh2 domain of Nrf2 as 'bait' led to the discovery of Keap1 a the canonical Nrf2 repressor [42]. This highlights the strength of yeast as a tool for detecting protein interactions.

5.7.2 Chapter 3

To assess Nrf2 and Keap1 oxidative stress-induced protein inclusion formation, yeast and cultured mammalian cells were used. Experiments were primarily conducted in HeLa cells, with minor additional experiments in the 21MT-1 and SKBR3 breast cancer cell lines to confirm some results observed in HeLa. It is important to note that all three cell lines are cancer cell lines and may thus carry mutations or other aberrations, which could undermine experimental outcomes. Although this inclusion formation was observed in yeast, future experiments must still be conducted in non-cancerous cell lines and eventually animal models to verify that our findings are not cancer cell-specific. Additionally, due to the rapid growth of cells in artificial culture (especially cancer cells), there is a high chance of mutation and genetic variation within the cell population (Hastings & Franks, 1983). This may lead to heterogeneity within the cell population that cannot be easily be distinguished, leading to inaccurate results. This is a limitation for all traditional cell culture experiments in general, including those in Chapter 4.

As mentioned before, observations regarding the temporal formation and possible solubilization of these protein inclusions were not yet determined. In our experiments, cells were treated with hydrogen peroxide for a total of 3 h, but visualization after 30 mins and 1, 2 and 3 h of hydrogen peroxide treatment (data not shown) but did not yield any significant differences, implying that this oxidation event may be very rapid and more precise imaging methods are required. Finally, although this study documented the formation of stress-induced protein inclusions that was verified using three different models (yeast, mammalian cells, and purified protein) and different forms of cell stress, functional analyses will need to be addressed in future studies.

5.7.3 Chapter 4

The main limitation of Chapter 4 is that it is currently incomplete due to the restrictions imposed by the COVID-19 pandemic starting in March 2020 that prevented laboratory access and hindered some of my work. For this reason, the key experiment involving the proposed co-inhibition of Hsp90 and GPx could not be performed. Additionally, the western blots using a total protein internal loading control could not be completed. These experiments will be completed following my Ph.D. defence for publication purposes.

With regards to experimental limitations, because this study involves the use of artificially cultured cells, it is also subject to the limitations discussed in Section 5.7.2, namely the high likelihood of mutations and resultant population heterogeneity. Additionally, because these cells are cultured in a monolayer, cell-cell interactions and cell-cell signalling that may have existed between these cells within a tumour cannot be accurately be recapitulated, making the results less transferable to a clinically relevant scenario. This limitation may be addressed with the use of 3D cell culture systems which have been gaining traction over the past decade for the advantages they provide over traditional 2D cell culture (Ravi et al., 2015). Importantly for this study, this method allows for the formation of scaffolds and matrices and more closely mimics the tissue architecture and microenvironment of a tumour, thereby providing more accurate drug response studies (Ravi et al., 2015). However, 3D cell cultures are time-consuming, labour-intensive, and expensive (Lee et al., 2019) and therefore not a feasible option for all laboratories. An additional limitation is that our RT-qPCR studies were limited to a select number of Nrf2 and Hsf1 genes rather than assessing transcriptome-wide changes (e.g., by RNAseq (Wang et al., 2009)) due to the high associated costs. Nrf2 and Hsp90 are involved in many cellular processes and regulate a vast array of proteins, thus, additional off-target effects associated with Hsp90 inhibition could exist that go beyond what is observed in this study. We are therefore dependent on future work and other research groups to fill in these gaps.

5.8 Future Directions

Many aspects of this work provide exciting trajectories to be explored in future studies. Regarding the interaction between Nrf2 and Keap1 with Hsp90, although previous work has revealed that Hsp90 prefers Kelch domains (Taipale et al., 2012) like those found in Keap1, the exact binding between Nrf2 and Hsp90 and the underlying molecular mechanisms and functional outcomes are still unknown. Future work could use the yeast model of Nrf2 to perform domain analyses, in addition to biophysical techniques such as nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC) to determine the exact interaction sites for these two proteins. Furthermore, the Nrf2 yeast model could be used to screen for small-molecule inhibitors or compounds that modulate Nrf2 interactions with other proteins or to screen for additional genetic and protein-protein interactions that have not yet been described. As Nrf2 is an intrinsically disordered protein, it has the potential to bind to an unusually vast array of proteins, some of which may still be unknown.

For our protein oxidation studies, live-cell imaging would be very informative, and expansion into non-cancer cell lines and animal models, as well as the use of different oxidative stress compounds, is important, as discussed above. Other key experiments would be to determine the functional outcome of Nrf2 and Keap1 inclusion formation, particularly to determine if the Nrf2 antioxidant response can still be activated if Nrf2 or Keap1 are included, and to determine the degree of reversibility (i.e., by thioredoxin and/or molecular chaperones). Domain analyses may also reveal if certain domains are more prone to misfolding than others, or if the full-length protein is necessary for inclusion formation. We found that the Kelch domain of Keap1 alone is not sufficient for inclusion formation, but the other domains remain to be studied. Finally, given the harsh microenvironment of a rapidly dividing cancer cell where ROS is prevalent, it would be interesting to determine if stress-induced Nrf2 and Keap1 inclusions are present in cancer patient samples using immunohistochemistry (IHC). These results encourage us to start such pathological studies in breast cancer.

For our Hsp90 inhibition studies in HER2+ breast cancer, short-term directions include completing the remaining experiments to assess the co-inhibition of Hsp90 and GPx activity. Cell viability and cell death assays will be used to determine the efficacy of Trastuzumab and chemotherapy treatment with this co-inhibition. Western blots will also be completed. For long-term experiments,

considering that cancer cells can develop resistance to therapy, it would be important to determine if HER2+ breast cancer cells will become resistant to our proposed combined treatment regimen. Resistance to Trastuzumab (Luque-Cabal et al., 2016; Li et al., 2018) and chemotherapy (Ji et al., 2019; Lainetti et al., 2020) have been well documented; however, the question remains if resistance will be delayed or inhibited with the co-inhibition of Hsp90 and GPx, or if cancer cells can develop resistance to Hsp90 inhibitors in general. A single mutation in the N-terminal ATP-binding site of Hsp90 has been found to result in resistance to Hsp90 inhibitors (Duerfeldt & Blagg, 2009) but the mechanism remains unknown and it is unclear if this happens in a prolonged cancer treatment scenario. Lastly, HER2 plays an important role in cancer cell proliferation and tumour metastasis in other cancers apart from breast cancer, with HER2 overexpression being detected in pancreatic cancer (Stoecklein et al., 2004) and HER2 as a predictor of poor prognosis in ovarian cancer (Luo et al., 2018). If the results in HER2+ breast cancer turn out to be promising, they could be extended into these other HER2+ cancer subtypes.

5.9 Overall Significance

The antioxidant and heat shock responses are often discussed independently as two separate pathways regulating two different types of cellular stress. In this work, we show that there is convergence and crosstalk between these pathways using a yeast model for Nrf2 and a human breast cancer model for HER2-enriched breast cancer. We also examined aspects of protein folding by investigating the misfolding of Nrf2 and Keap1 under oxidative stress conditions. Our findings suggest that oxidative damage to Nrf2 and Keap1 and their interactions with Hsp90 alter their regulation and function within the cellular antioxidant stress response. Taken together, we explored aspects of two key cellular stress response pathways and considered their implications in protein regulation, folding, and function, and suggest that investigating these key pathways in parallel may provide an additional layer of knowledge that is relevant to both basic science and clinical research.

5.10 Graphical Summary

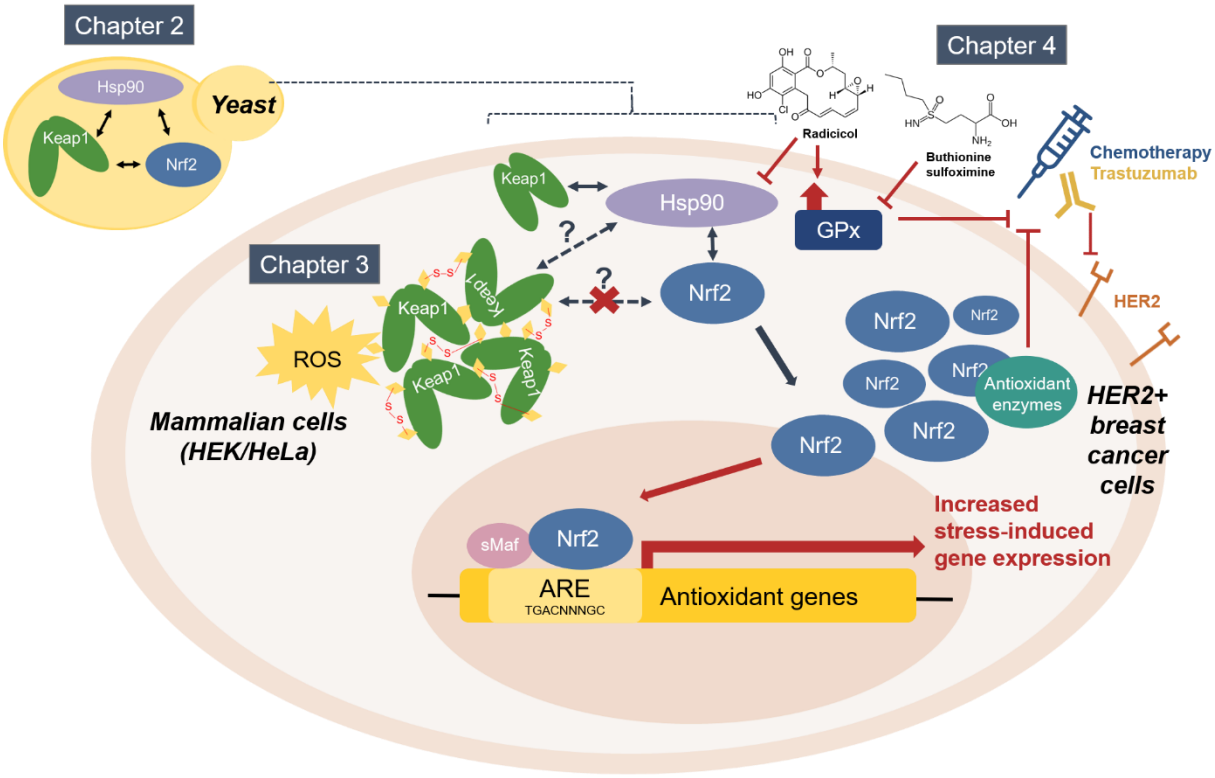


Figure 5.4: **Graphical summary of Chapters 2, 3, and 4.** A novel yeast model of Nrf2 detects the interaction between Nrf2 and Keap1 with Hsp90. Furthermore, oxidative damage to Nrf2 and Keap1 and their interactions with Hsp90 alter their regulation and function within the cellular antioxidant stress response. This nexus between the antioxidant and heat shock responses is thus an important area of research.

5.11 References

Brigelius-Flohé, R., & Maiorino, M. (2013). Glutathione peroxidases. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1830*(5), 3289-3303.

Chen, W., Sun, Z., Wang, X. J., Jiang, T., Huang, Z., Fang, D., & Zhang, D. D. (2009). Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol Cell, 34*(6), 663-673.

Chowdhry, S., Zhang, Y., McMahon, M., Sutherland, C., Cuadrado, A., & Hayes, J. D. (2013). Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene, 32*(32), 3765-3781.

Chung, K. K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J. C., Marsh, L., Dawson, V. L., & Dawson, T. M. (2004). S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science, 304*(5675), 1328-1331.

Collet, J.-F., & Messens, J. (2010). Structure, Function, and Mechanism of Thioredoxin Proteins. *Antioxidants & Redox Signaling, 13*(8), 1205-1216.

Copple, I. M., Lister, A., Obeng, A. D., Kitteringham, N. R., Jenkins, R. E., Layfield, R., Foster, B. J., Goldring, C. E., & Park, B. K. (2010). Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway. *J Biol Chem, 285*(22), 16782-16788.

Dhakshinamoorthy, S., Jain, A. K., Bloom, D. A., & Jaiswal, A. K. (2005). Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. *J Biol Chem, 280*(17), 16891-16900.

Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., & Talalay, P. (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences, 99*(18), 11908.

Duerfeldt, A. S., & Blagg, B. S. J. (2009). Hydrating for Resistance to Radicicol. *ACS Chem Biol, 4*(4), 245-247.

Fan, W., Tang, Z., Chen, D., Moughon, D., Ding, X., Chen, S., Zhu, M., & Zhong, Q. (2010). Keap1 facilitates p62-mediated ubiquitin aggregate clearance via autophagy. *Autophagy, 6*(5), 614-621.

Glover, J. R., & Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell, 94*(1), 73-82.

Goloubinoff, P., Mogk, A., Zvi, A. P., Tomoyasu, T., & Bukau, B. (1999). Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proc Natl Acad Sci U S A, 96*(24), 13732-13737.

Gorrini, C., Baniasadi, P. S., Harris, I. S., Silvester, J., Inoue, S., Snow, B., Joshi, P. A., Wakeham, A., Molyneux, S. D., Martin, B., Bouwman, P., Cescon, D. W., Elia, A. J., Winterton-Perks, Z., Cruickshank, J., Brenner, D., Tseng, A., Musgrave, M., Berman, H. K., Khokha, R., Jonkers, J., Mak, T. W., & Gauthier, M. L. (2013). BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival. *Journal of Experimental Medicine, 210*(8), 1529-1544.

Hartikainen, J. M., Tengström, M., Kosma, V.-M., Kinnula, V. L., Mannermaa, A., & Soini, Y. (2012). Genetic Polymorphisms and Protein Expression of NRF2 and Sulfiredoxin Predict Survival Outcomes in Breast Cancer. *Cancer Res, 72*(21), 5537.

Hastings, R. J., & Franks, L. M. (1983). Cellular heterogeneity in a tissue culture cell line derived from a human bladder carcinoma. *British Journal Of Cancer, 47*(2), 233-244.

Huang, H. C., Nguyen, T., & Pickett, C. B. (2002). Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J Biol Chem, 277*(45), 42769-42774.

Jain, A., Lamark, T., Sjøttem, E., Larsen, K. B., Awuh, J. A., Øvervatn, A., McMahon, M., Hayes, J. D., & Johansen, T. (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J Biol Chem, 285*(29), 22576-22591.

Jansens, A., van Duijn, E., & Braakman, I. (2002). Coordinated nonvectorial folding in a newly synthesized multidomain protein. *Science, 298*(5602), 2401-2403.

Ji, X., Lu, Y., Tian, H., Meng, X., Wei, M., & Cho, W. C. (2019). Chemoresistance mechanisms of breast cancer and their countermeasures. *Biomedicine & Pharmacotherapy, 114*, 108800.

Karapetian, R. N., Evstafieva, A. G., Abaeva, I. S., Chichkova, N. V., Filonov, G. S., Rubtsov, Y. P., Sukhacheva, E. A., Melnikov, S. V., Schneider, U., Wanker, E. E., & Vartapetian, A. B. (2005). Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Mol Cell Biol, 25*(3), 1089-1099.

Kim, Y. C., Kitaura, H., Taira, T., Iguchi-Ariga, S. M., & Ariga, H. (2009). Oxidation of DJ-1-dependent cell transformation through direct binding of DJ-1 to PTEN. *Int J Oncol, 35*(6), 1331-1341.

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y.-S., Ueno, I., Sakamoto, A., Tong, K. I., Kim, M., Nishito, Y., Iemura, S.-i., Natsume, T., Ueno, T., Kominami, E., Motohashi, H., Tanaka, K., & Yamamoto, M. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nature Cell Biology, 12*, 213.

Lainetti, P. F., Leis-Filho, A. F., Laufer-Amorim, R., Battazza, A., & Fonseca-Alves, C. E. (2020). Mechanisms of Resistance to Chemotherapy in Breast Cancer and Possible Targets in Drug Delivery Systems. *Pharmaceutics, 12*(12).

Lau, A., Wang, X. J., Zhao, F., Villeneuve, N. F., Wu, T., Jiang, T., Sun, Z., White, E., & Zhang, D. D. (2010). A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol Cell Biol, 30*(13), 3275-3285.

Lee, D., Pathak, S., & Jeong, J.-H. (2019). Design and manufacture of 3D cell culture plate for mass production of cell-spheroids. *Scientific Reports, 9*(1), 13976.

Li, G., Guo, J., Shen, B.-Q., Yadav, D. B., Sliwkowski, M. X., Crocker, L. M., Lacap, J. A., & Phillips, G. D. L. (2018). Mechanisms of Acquired Resistance to Trastuzumab Emtansine in Breast Cancer Cells. *Molecular Cancer Therapeutics, 17*(7), 1441.

Li, Q. K., Singh, A., Biswal, S., Askin, F., & Gabrielson, E. (2011). KEAP1 gene mutations and NRF2 activation are common in pulmonary papillary adenocarcinoma. *J Hum Genet, 56*(3), 230-234.

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Gargiulo, G., Testa, G., Cacciatore, F., Bonaduce, D., & Abete, P. (2018). Oxidative stress, aging, and diseases. *Clinical interventions in aging, 13*, 757-772.

Lo, S.-C., & Hannink, M. (2008). PGAM5 tethers a ternary complex containing Keap1 and Nrf2 to mitochondria. *Experimental Cell Research, 314*(8), 1789-1803.

Luo, H., Xu, X., Ye, M., Sheng, B., & Zhu, X. (2018). The prognostic value of HER2 in ovarian cancer: A meta-analysis of observational studies. *PLoS ONE, 13*(1), e0191972-e0191972.

Luque-Cabal, M., García-Teijido, P., Fernández-Pérez, Y., Sánchez-Lorenzo, L., & Palacio-Vázquez, I. (2016). Mechanisms Behind the Resistance to Trastuzumab in HER2-Amplified Breast Cancer and Strategies to Overcome It. *Clinical Medicine Insights. Oncology, 10*(Suppl 1), 21-30.

Marino, S. M., & Gladyshev, V. N. (2010). Cysteine function governs its conservation and degeneration and restricts its utilization on protein surfaces. *Journal of Molecular Biology, 404*(5), 902-916.

McMahon, M., Lamont, D. J., Beattie, K. A., & Hayes, J. D. (2010). Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. *Proceedings of the National Academy of Sciences, 107*(44), 18838-18843.

Mohammadi, S., Saberidokht, B., Subramaniam, S., & Grama, A. (2015). Scope and limitations of yeast as a model organism for studying human tissue-specific pathways. *BMC Systems Biology, 9*(1), 96.

Nioi, P., & Nguyen, T. (2007). A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity. *Biochem Biophys Res Commun, 362*(4), 816-821.

Ooi, A., Dykema, K., Ansari, A., Petillo, D., Snider, J., Kahnoski, R., Anema, J., Craig, D., Carpten, J., Teh, B. T., & Furge, K. A. (2013). CUL3 and NRF2 mutations confer an NRF2 activation phenotype in a sporadic form of papillary renal cell carcinoma. *Cancer Res, 73*(7), 2044-2051.

Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsuji, M., Kang, M. I., Kobayashi, A., Yokoyama, S., & Yamamoto, M. (2006). Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol Cell, 21*(5), 689-700.

Praslicka, B. J., Kerins, M. J., & Ooi, A. (2016). The complex role of NRF2 in cancer: A genomic view. *Current opinion in toxicology, 1*, 37-45.

Rada, P., Rojo, A. I., Chowdhry, S., McMahon, M., Hayes, J. D., & Cuadrado, A. (2011). SCF/β-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Mol Cell Biol, 31*(6), 1121-1133.

Ravi, M., Paramesh, V., Kaviya, S. R., Anuradha, E., & Solomon, F. D. P. (2015). 3D Cell Culture Systems: Advantages and Applications. *Journal of Cellular Physiology, 230*(1), 16-26.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med, 49*(11), 1603-1616.

Rojo de la Vega, M., Chapman, E., & Zhang, D. D. (2018). NRF2 and the Hallmarks of Cancer. *Cancer Cell, 34*(1), 21-43.

Schopf, F. H., Biebl, M. M., & Buchner, J. (2017). The HSP90 chaperone machinery. *Nature Reviews Molecular Cell Biology, 18*(6), 345-360.

Shibata, T., Ohta, T., Tong, K. I., Kokubu, A., Odogawa, R., Tsuta, K., Asamura, H., Yamamoto, M., & Hirohashi, S. (2008). Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci U S A, 105*(36), 13568-13573.

Shorter, J. (2008). Hsp104: a weapon to combat diverse neurodegenerative disorders. *Neurosignals, 16*(1), 63-74.

Singh, A., Misra, V., Thimmulappa, R. K., Lee, H., Ames, S., Hoque, M. O., Herman, J. G., Baylin, S. B., Sidransky, D., Gabrielson, E., Brock, M. V., & Biswal, S. (2006). Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. *PLoS Med, 3*(10), e420.

Sohal, R. S., & Orr, W. C. (2012). The redox stress hypothesis of aging. *Free Radical Biology and Medicine, 52*(3), 539-555.

Solit, D. B., Zheng, F. F., Drobnjak, M., Münster, P. N., Higgins, B., Verbel, D., Heller, G., Tong, W., Cordon-Cardo, C., Agus, D. B., Scher, H. I., & Rosen, N. (2002). 17-Allylamino-17-demethoxygeldanamycin Induces the Degradation of Androgen Receptor and HER-2/neu and Inhibits the Growth of Prostate Cancer Xenografts. *Clinical Cancer Research, 8*(5), 986.

Soto, C. (2003). Unfolding the role of protein misfolding in neurodegenerative diseases. *Nature Reviews Neuroscience, 4*(1), 49-60.

Stadtman, E. R. (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem, 62*, 797-821.

Stoecklein, N. H., Luebke, A. M., Erbersdobler, A., Knoefel, W. T., Schraut, W., Verde, P. E., Stern, F., Scheunemann, P., Peiper, M., Eisenberger, C. F., Izbicki, J. R., Klein, C. A., & Hosch, S. B. (2004). Copy Number of Chromosome 17 but Not HER2 Amplification Predicts Clinical Outcome of Patients With Pancreatic Ductal Adenocarcinoma. *Journal of Clinical Oncology, 22*(23), 4737-4745.

Taguchi, K., & Yamamoto, M. (2017). The KEAP1–NRF2 System in Cancer. *Frontiers in Oncology, 7*, 85.

Taipale, M., Krykbaeva, I., Koeva, M., Kayatekin, C., Westover, K. D., Karras, G. I., & Lindquist, S. (2012). Quantitative Analysis of Hsp90-Client Interactions Reveals Principles of Substrate Recognition. *Cell, 150*(5), 987-1001.

Takahashi, T., Sonobe, M., Menju, T., Nakayama, E., Mino, N., Iwakiri, S., Nagai, S., Sato, K., Miyahara, R., Okubo, K., Hirata, T., Date, H., & Wada, H. (2010). Mutations in Keap1 are a potential prognostic factor in resected non-small cell lung cancer. *J Surg Oncol, 101*(6), 500-506.

Tikhomirov, O., & Carpenter, G. (2000). Geldanamycin induces ErbB-2 degradation by proteolytic fragmentation. *J Biol Chem, 275*(34), 26625-26631.

Tong, K. I., Katoh, Y., Kusunoki, H., Itoh, K., Tanaka, T., & Yamamoto, M. (2006). Keap1 Recruits Neh2 through Binding to ETGE and DLG Motifs: Characterization of the Two-Site Molecular Recognition Model. *Mol Cell Biol, 26*(8), 2887-2900.

Tong, K. I., Padmanabhan, B., Kobayashi, A., Shang, C., Hirotsu, Y., Yokoyama, S., & Yamamoto, M. (2007). Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol Cell Biol, 27*(21), 7511-7521.

Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., & Talalay, P. (2004). Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci U S A, 101*(7), 2040-2045.

Wang, H., Liu, K., Geng, M., Gao, P., Wu, X., Hai, Y., Li, Y., Li, Y., Luo, L., Hayes, J. D., Wang, X. J., & Tang, X. (2013). RXRalpha inhibits the NRF2-ARE signaling pathway through a direct interaction with the Neh7 domain of NRF2. *Cancer Res, 73*(10), 3097-3108.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews. Genetics, 10*(1), 57-63.

Wu, H., Ng, B. S., & Thibault, G. (2014). Endoplasmic reticulum stress response in yeast and humans. *Biosci Rep, 34*(4).

Xu, P., Liu, Q., Xie, Y., Shi, X., Li, Y., Peng, M., Guo, H., Sun, R., Li, J., Hong, Y., Liu, X., & Xu, G. (2018). Breast cancer susceptibility protein 1 (BRCA1) rescues neurons from cerebral ischemia/reperfusion injury through NRF2-mediated antioxidant pathway. *Redox Biology, 18*, 158-172.

Zhang, D. D., & Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol, 23*(22), 8137-8151.

Zhong, G., Qin, S., Townsend, D., Schulte, B. A., Tew, K. D., & Wang, G. Y. (2019). Oxidative stress induces senescence in breast cancer stem cells. *Biochem Biophys Res Commun, 514*(4), 1204-1209.

Curriculum Vitae
Vy Ngo

Education

2015 Sep-2021 Mar **University of Western Ontario** (Western University)
Department of Pathology and Laboratory Medicine, London, ON, Canada
Ph.D. in Pathology and Laboratory Medicine
Ph.D. advisor: Martin Duennwald, Ph.D.
Dissertation: Nrf2 regulation by Hsp90, oxidation, and in breast cancer

2010 Sep-2014 Apr **McMaster University**
Department of Psychology, Neuroscience & Behaviour, Hamilton, ON, Canada
Honours B.Sc. in Biology and Psychology
Thesis advisor: Daniel Goldreich, Ph.D.
Honours thesis: Testing a Bayesian observer model of the cutaneous rabbit illusion (2013-2014)

Research Positions

2015 Sep-2021 Mar **Graduate Research Assistant** (for Dr. Martin Duennwald)
University of Western Ontario, Department of Pathology and Laboratory Medicine, London, ON.

2014 May-2015 Jul **Research Assistant** (for Dr. Daniel Goldreich)
McMaster University, Department of Psychology, Neuroscience & Behaviour, Hamilton, ON.

2012 Sep-2014 Apr **Undergraduate Research Student** (for Dr. Daniel Goldreich)
McMaster University, Department of Psychology, Neuroscience & Behaviour, Hamilton, ON.

Teaching Positions

2019
2018, 2017 Sep-Dec **Graduate Teaching Assistant** (for Dr. Candace Gibson)
Pathology 2420A: "*Pathology for Nursing Students*", University of Western Ontario, Department of Pathology and Laboratory Medicine, London, ON.
****Nominated twice*** *for a Graduate Student Teaching Assistant Award*

2017 Jan-Apr **Graduate Teaching Assistant** (for Dr. Martin Duennwald)
Pathology 3700G: "*Modern Approaches in Biomedical and Pathology Research*", University of Western Ontario, Department of Pathology and Laboratory Medicine, London, ON.

Teaching Certifications

2020 Jul **Western Certificate in University Teaching and Learning**
University of Western Ontario, London, ON.

A 50-hour program designed to enhance the quality of teaching by graduate students; components include microteaching sessions, 10 workshops, 2 short courses, and the completion of a teaching dossier and written project (mock course syllabus).

2020 Apr **Advanced Teaching Program (ATP) Certificate**
University of Western Ontario, London, ON.

A 20-hour course designed for advanced graduate students to develop practical teaching skills for current and future teaching roles.

2020 Jan **Teaching Assistant Training Program (TATP) Certificate**
University of Western Ontario, London, ON.

A hands-on program for Teaching Assistants (TAs) to learn about fair grading practices, diversity in the classroom, lesson design, and giving students feedback on written work.

Honours and Awards

2021 Apr **Dr. Cameron A. Wallace Graduate Student Award**
Department of Pathology and Laboratory Medicine, University of Western Ontario, London, ON.

Recognizes a student's accomplishments in Pathology research and course work undertaken during their graduate program.

2020 Dec **Young Investigator Award**
Society for Redox Biology and Medicine (SfRBM).

Presented to students and postdoctoral fellow members based on a submitted research abstract and the presentation of the work at the SfRBM's Annual Conference, in poster or oral symposia (top 20%).

2020 Sep-Dec, 2019 Sep-Aug, 2018 Sep-Aug **Translational Breast Cancer Research (TBCRU) Studentship**
London Regional Cancer Program funded in part by the Breast Cancer Society of Canada, University of Western Ontario, London, ON.

Awarded by the Translational Breast Cancer Research Unit to encourage and support trainees who are carrying out translational breast cancer research.

Applied for annually; years and amounts awarded:
2020 (1 term), $3000
2019 (3 terms), $3000 accepted of $18 000 award*
2018 (3 terms), $18 000.

*As the recipient of another external funding source (i.e., QEII-GSST), only $3000 of $18 000 could be awarded to top up the maximum award levels.

2020 Apr **Dr. Frederick Winnett Luney Graduate Research Award**
Department of Pathology and Laboratory Medicine, University of Western Ontario, London, ON.

Awarded to a graduate student in a research-based M.Sc./M.Cl.Sc. and Ph.D. Pathology and Laboratory Medicine Program, based on academic achievement and research merit.

2019 Jun **Graduate Student Teaching Assistant Award – *Nominated***
Society of Graduate Students, University of Western Ontario, London, ON.

Recognizes a Graduate Teaching Assistant for their extraordinary efforts and dedication to teaching at the University of Western Ontario.

2019 Jun **CSMB Travel Award**
Canadian Society of Molecular Biosciences (CSMB).

2019 May **Queen Elizabeth II Graduate Scholarship in Science & Technology (QEII-GSST)**
University of Western Ontario, London, ON; $15,000.

A merit-based scholarship available to domestic students in graduate study undertaking research in science and technology fields.

2019 Mar **Dr. M. Daria Haust Award for the Best Basic Science Presentation**
Department of Pathology and Laboratory Medicine, University of Western Ontario, London, ON.

Recognizes a graduate student's excellence in research design and performance as well as the presentation of the research topic.

2018 Jun **Graduate Student Teaching Assistant Award – *Nominated***
Society of Graduate Students, University of Western Ontario, London, ON.

Recognizes a Graduate Teaching Assistant for their extraordinary efforts and dedication to teaching at the University of Western Ontario.

2018 May **SOGS Travel Award**
Society of Graduate Students, University of Western Ontario, London, ON.

2017 Sep-2018 Aug **Dr. Frederick Winnett Luney Graduate Scholarship**
Department of Pathology and Laboratory Medicine, University of Western Ontario, London, ON; $10,000.

Awarded to a graduate student in a research-based MSc/MClSc and Ph.D. Pathology and Laboratory Medicine Program, based on academic achievement and research merit.

2010 Oct **Governor General's Academic Medal**, Governor-General of Canada.

2010 Oct **Queen Elizabeth II Aiming for the Top Scholarship**; $3000.

2010 Sep **The McMaster President's Award**, McMaster University; $3500.

Publications

a) Publication Summary

	Published/ (In press)	Submitted/ (In preparation)	Career Totals
Peer-Reviewed Articles	3	3(1)	6 (1)
Abstracts (National/International)	5	-	5
Abstracts (Regional/Institutional)	10	-	10
			21 (1)

b) Peer-Reviewed Articles

1. **Ngo, V.**, Krstic, M., Goodale, D., Allan, A. L., & Duennwald, M. L. (2021). Hyperactive stress response pathways in HER2+ breast cancers. (*In preparation*)

2. **Ngo, V.**, Karunatilleke, N., Song, Z., Brickenden, A., Choy, W. Y., & Duennwald, M. L. (2020). Oxidative stress-induced inclusion formation of Nrf2 and Keap1. (*Submitted)*

3. **Ngo, V.**, Brickenden, A., Liu, H., Yeung, C., Choy, W. Y., & Duennwald, M. L. (2020). A novel yeast model detects Nrf2 and Keap1 interactions with Hsp90. (*Submitted*)

4. Karunatilleke, N., Fast, C., **Ngo, V.**, Brickenden, A., Duennwald., M. L, & Choy, W. Y. (2020). Nrf2, a key regulator of the cellular oxidative stress response, is an intrinsically disordered protein. (*Submitted*)

5. Biswas, S., Feng, B., Chen, S., Liu, J., Aref-Eshghi, E., Gonder, J., **Ngo, V.**, Sadikovic, B., & Chakrabarti, S. (2021). The long non-coding RNA HOTAIR is a critical epigenetic mediator of angiogenesis in diabetic retinopathy. *Investig Ophthalmol Vis Sci, 62(3)*: 20-20.

6. Spratt, D., Barber, K., Marlatt, N., **Ngo, V.**, Macklin, J., Xiao, Y., Konermann, L., Duennwald, M. L, & Shaw, G. (2019). A Subset of Calcium-binding S100 Proteins Show Preferential Heterodimerization. *The FEBS Journal, 286(10)*: 1859-1876.

7. Tong, J., **Ngo, V.**, & Goldreich, D. (2016). Tactile length contraction as Bayesian inference. *J Neurophysiol, 116(2)*: 369-379.

Abstracts and Presentations

a) National/International Meetings

2020 Nov **Ngo, V.**, Karunatilleke, N., Song, Z., Brickenden, A., Krstic, M., Choy, W. Y., & Duennwald, M. L. Oxidative stress-induced aggregation of Keap1 impairs Nrf2 regulation. Poster presentation at the 27th Annual Conference of the Society for Redox Biology and Medicine (SfRBM). Virtual platform.

****Awarded*** *a Young Investigator Award*

2019 Jun **Ngo, V.**, Krstic, M., Brickenden, A., Karunatilleke, N., Choy, W. Y., & Duennwald, M. L. Novel crosstalk between the antioxidant and heat shock responses in cancer. ***Selected*** for an oral presentation at the 62nd Annual Conference of the Canadian Society for Molecular Biosciences (CSMB) Model Systems in Cancer Research. Montreal, QC, Canada.

2019 Oct Karunatilleke, N., Fast, C., **Ngo, V.**, Brickenden, A., Duennwald, M. L., Konermann, L., & Choy, W. Y. Nrf2, the key transcription factor that regulates the cellular response to oxidative stress, is intrinsically disordered. Co-author for oral presentation by N. K. at the 18th International Conference on Structural Biology. London, England, UK.

2018 Jul **Ngo, V.** & Duennwald, M. L. Oxidative stress-induced aggregation of Keap1 impairs Keap1-dependent Nrf2 regulation. ***Selected*** for a lightning talk and poster presentation at the FASEB Science Research Conferences: Protein Folding in the Cell. Olean, NY, USA.

2018 Apr **Ngo, V.** & Duennwald, M. L. Nrf2 regulation in the antioxidant response in cancer: Stress-induced aggregation of Nrf2 and Keap1. ***Selected*** for a lightning talk and poster presentation at the 11th Conference on Signaling in Normal and Cancer Cells. Banff, AB, Canada.

b) Regional/Institutional Meetings

2019 Mar **Ngo, V.**, Krstic, M., Karunatilleke, N., Song, Z., Brickenden, A., Allan, A. L., Choy, W. Y., & Duennwald, M. L. Oxidative stress-induced aggregation of Keap1 impairs Keap1-dependent Nrf2 regulation. ***Selected*** for a platform presentation at Pathology and Laboratory Medicine Research Day. London, ON.

****Awarded*** *the Dr. M. Daria Haust Award for the Best Basic Science Presentation*

2018 May **Ngo, V.** & Duennwald, M. L. Nrf2 regulation in the antioxidant response in cancer. Poster presentation at London Health Research Day. London, ON.

2018 Apr **Ngo, V.** & Duennwald, M. L. Nrf2 regulation in the antioxidant response in cancer. Poster presentation at Pathology and Laboratory Medicine Research Day. London, ON.

2017 Mar **Ngo, V.** & Duennwald, M. L. The interaction between p21 and Nrf2 regulates oxidative stress in cancer. ***Selected*** for a platform presentation at Pathology and Laboratory Medicine Research Day. London, ON.

2017 Mar **Ngo, V.** & Duennwald, M. L. The interaction between p21 and Nrf2 regulates oxidative stress in cancer. Poster presentation at Pathology and Laboratory Medicine Research Day. London, ON.

2016 Apr **Ngo, V.** & Duennwald, M. L. Nrf2 interactions with p21, prothymosin alpha, and Keap1 in cancer. Poster presentation at Pathology and Laboratory Medicine Research Day. London, ON.

2016 Mar **Ngo, V.** & Duennwald, M. L. Nrf2 interactions with p21, prothymosin alpha, and Keap1 in cancer. Poster presentation at London Health Research Day. London, ON.

2015 May Tong, J., **Ngo, V.**, & Goldreich, D. Testing a Bayesian model of perceptual length contraction illusions. Poster presentation at the Southern Ontario Neuroscience Association Conference. Niagara Falls, ON.

2015 Feb **Ngo, V.**, Tong, J., & Goldreich, D. Testing a Bayesian model of the tactile rabbit and tau illusions. Poster presentation at the Lake Ontario Visionary Establishment Conference. Hamilton, ON.

2014 Apr **Ngo, V.**, Tong, J., & Goldreich, D. Testing a Bayesian observer model of the cutaneous rabbit illusion. Poster presentation at the NeuroXchange Conference. Hamilton, ON.

Professional Memberships

2019 Jan- **Society for Redox Biology and Medicine (SfRBM)**
Member

2017 May- **Canadian Society for Molecular Biosciences (CSMB)**
Member

2019 Sep-2020 Oct **Graduate Education Committee (GEC) (Research)**
Department of Pathology and Laboratory Medicine, University of Western Ontario, London, ON.
Graduate Student Representative

2017 Sep-2020 Aug **Western Pathology Association (WPA)**
Department of Pathology and Laboratory Medicine, University of Western Ontario, London, ON.
GEC Representative (2019-2020)
Co-President (2018-2019)
Vice President of Finance and Communications (2017-2018)

2016 Sep-2020 Jun **Pathology and Laboratory Medicine Research Day Committee**
Department of Pathology and Laboratory Medicine, University of Western Ontario, London, ON.
Graduate Student Representative

2015 Sep-2018 Aug **Schulich Graduate Students Council (SGSC)**
Schulich School of Medicine & Dentistry, University of Western Ontario, London, ON.
Student Representative