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Cysteine-based Regulation of the CUL3 Adaptor Protein Keap1

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Abstract

Nrf2 (NF-E2-related factor 2) is a master transcription factor containing a powerful acidic transcriptional activation domain. Nrf2-dependent gene expression impacts cancer chemoprevention strategies, inflammatory responses, and progression of neurodegenerative diseases. Under basal conditions, association of Nrf2 with the CUL3 adaptor protein Keap1 results in the rapid Nrf2 ubiquitylation and proteasome-dependent degradation. Inhibition of Keap1 function blocks ubiquitylation of Nrf2, allowing newly synthesized Nrf2 to translocate into the nucleus, bind to ARE sites and direct target gene expression. Site-directed mutagenesis experiments coupled with proteomic analysis support a model in which Keap1 contains at least 2 distinct cysteine motifs. The first is located at Cys 151 in the BTB domain. The second is located in the intervening domain and centers around Cys 273 & 288. Adduction or oxidation at Cys151 has been shown to produce a conformational change in Keap1 that results in dissociation of Keap1 from CUL3, thereby inhibiting Nrf2 ubiquitylation. Thus, adduction captures specific chemical information and translates it into biochemical information via changes in structural conformation.

Introduction

Proteins that compose the Phase II superfamily and antioxidant enzymes provide an enzymatic line of defense against electrophiles and reactive oxygen species, two important contributors to the etiology of many human diseases. Proteins such as NAD(P)H: quinone oxidoreductase (DT-diaphorase), glutathione S-transferases, UDP-glucoronosyltransferses, MnSOD, catalase, thioredoxin, and glutamate cysteine ligase (gamma glutamylcysteine synthetase) are canonical members of the Phase II and antioxidant enzyme family and are regulated by a common cis-acting regulatory element located in the proximal promoter region. This cis-acting element was identified in mouse models and named the Electrophile-Response Element (EpRE; (Friling *et al.*, 1990), as well as identified in rat models as the Antioxidant Response Element ARE; (Favreau and Pickett, 1991). Heterodimeric binding of Nrf2 (NF-E2-related factor 2) to AREs induces target gene expression (Venugopal and Jaiswal, 1996; Venugopal and Jaiswal, 1998). Initially, Nrf2 was discovered in a screen that utilized a tandem repeat of the consensus sequences for AP1 and NF-E2 present in the locus control region in the 5' region of β-globin (Moi *et al.*, 1994). Nrf2 belongs to the basic leucine zipper (bZIP) transcription factor family and contains a powerful acidic transcriptional activation domain. Nrf2 activity impacts cancer chemoprevention strategies, inflammatory responses, and progression of neurodegenerative diseases.

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Sulfhydryl chemistry represents the basis for strategies designed to induce Nrf2-mediated Phase II gene expression (Prestera *et al.*, 1993). McMahon et al., (McMahon *et al.*, 2001) studied Phase II gene expression in the intestine of wild type and Nrf2 null animals. They found that expression of Nrf2 was required for induction of Phase II genes by synthetic sulfhydryl cancer chemopreventive agents. Sulforaphane[(−)-1-isothiocyanato-(4R0-(methylsulfinyl) butante] is a representative example. Conjugation to thiols represents a major metabolic pathway for isothiocyanates such as sulforaphane (Jiao *et al.*, 1996). This putative cancer chemoprotective agent induces the transcription of many Phase II enzymes in an Nrf2-mediated manner thereby inhibiting benzo[a]pyrene-induced tumors (Fahey *et al.*, 2002). Studies such as these demonstrate the importance of sulfhydryl chemistry and Nrf2 activation.

Nrf2 undergoes rapid ubiquitination and proteasome degradation

Under homeostatic cellular conditions Nrf2 is maintained at low levels due to CUL3-dependent E3-ubiquitin ligase mediated ubiquitination of Nrf2's amino terminus Neh2 domain at lysines 44, 50, 52, 53, 56, 64, and 68 (Itoh *et al.*, 2003; Kobayashi *et al.*, 2004; Zhang *et al.*, 2004). Ubiquitination directs proteasome-dependent degradation of Nrf2 (Sekhar *et al.*, 2002; Itoh *et al.*, 2003). Proteins targeted for Cullin-dependent ubiquitination are captured by a substrate receptor module that provides a protein recognition site and appropriate positioning within a Cullin-E3 complex. Current models of ubiquitin ligase complexes indicate that an assembled Cullin/substrate receptor module serves as a rigid scaffold to position the charged ubiquitin-conjugating E2 enzyme and its substrate. As discussed by Duda et al. (Duda *et al.*, 2008) and Saha and Deshaies (Saha and Deshaies, 2008) the distance between the E2 active site and the substrate is on the order of 50 Å, which decreases during the course of polyubiquitination. Nedd8 conjugation to the Cullin allows the E3/Ub-E2 complex to exhibit significant structural flexibility that accommodates dramatic changes in substrate geometry, thereby promoting polyubiquitination (Saifee and Zheng, 2008).

Unlike other Cullin-targeted substrates, Nrf2 ubiquitination is a constitutive process that does not require post-translational modification. Nrf2's Neh2 domain is captured by the carboxyl Kelch domain of the CUL 3 ligase substrate adaptor protein, Keap1 (Kelch-like ECH-associated protein 1) (Itoh *et al.*, 1999; Kobayashi *et al.*, 2004). Keap1 functions as a homodimer, a consequence of amino-terminal BTB (Broad complex Tramtrack, Bric-a-brac) domain interactions (Zipper and Mulcahy, 2002) that allow homodimeric binding to the amino-terminus of CUL3 (Furukawa and Xiong, 2005). Cell-based studies suggest that Nrf2 binds to Keap1 with a 2:2 stoichiometry (Lo *et al.*, 2006) whereas biophysical and biochemical studies support a 1:2 stoichiometry (Tong *et al.*, 2006a).

Induction of Nrf2-Directed Gene Expression: Identification of A Common Chemistry

Inhibition of Nrf2 ubiquitination is followed by induction of Nrf2-directed gene expression (Kobayashi *et al.*, 2004). The pioneering work of Talalay and colleagues (Talalay *et al.*, 1988; Dinkova-Kostova *et al.*, 2002; Holtzclaw *et al.*, 2004) demonstrated that induction of Nrf2-directed gene expression can be accomplished by 10 chemically diverse classes of compounds. These compounds exhibit a common chemical feature: they react with sulfhydryl groups. Thus, it was hypothesized that protein thiol modification induced Nrf2-dependent transcription (Prestera *et al.*, 1993). The knowledge that Keap1 directs the ubiquitination of Nrf2 and contains either 25 (murine) or 27 (human) cysteine residues made it the prime candidate for the hypothesized thiol sensor.

Thiol-Based Redox Sensors

In the last 10 years a significant effort has been undertaken to provide a biophysical understanding of how thiol-based redox sensors distinguish between different types of chemistries and translate chemical information into biochemical signals (Paget and Buttner,

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2003; Salmeen *et al.*, 2003; Codreanu *et al.*, 2008). Yap1 represents a well characterized model (Georgiou, 2002). Distinct cysteine residues within the transcription factor can be used to capture specific chemical information and translate it into biochemical information via changes in structural conformation, allowing redox specific modifications to induce gene transcription.

Based on the knowledge that Keap1 directs the ubiquitination of Nrf2 and that Keap1 contains a number of reactive cysteine residues, significant effort has been made to identify reactive residues and biochemically link adduction/oxidation of those residues to changes in Keap1 function. Mutation studies involving Keap1 cysteine residues have been very informative. Mutation of Cys to either Ser or Ala would not be expected to affect protein conformation significantly and consistent with this concept, the majority of the mutations did not affect Keap1 function (Zhang and Hannink, 2003). However, the absence of an effect when specific Cys residues are mutated does not demonstrate that adduction at those residues would not affect Keap1 function (Holland *et al.*, 2008). Furthermore, the observation that mutation of a specific Cys residue affects function does not demonstrate that that particular residue is reactive. But, when a mutation does affect Keap1 function, one can hypothesize that if that residue is adducted, then adduction will affect function.

The mutation studies identified 3 important Cys residues in Keap1: Cys 151, Cys 273, and Cys 288 (Zhang and Hannink, 2003). Whereas electrophiles and oxidants induce disassociation of Keap1 from CUL3, with subsequent inhibition of Nrf2 ubiquitination, mutation of Cys 151 to Ser abolished electrophile and oxidant-mediated disassociation and Keap1-directed ubiquitination (Zhang and Hannink, 2003). The role of Cys 151 in electrophile/oxidant repression of Nrf2 ubiquitination has been verified in vivo (Yamamoto *et al.*, 2008). Mutation of either Cys 273 or Cys 288 to Ala inhibits Keap1's ability to direct the constitutive ubiquitination of Nrf2. Inhibition of Nrf2 ubiquitination is followed by Nrf2 nuclear localization, with subsequent Nrf2-directed transcription (Zhang and Hannink, 2003). Ectopic expression of Keap1 containing a C273A mutation has been shown to complement (50%) a Keap1 molecule containing a C288A mutation (Wakabayashi *et al.*, 2004). This has been recapitulated in vivo (Yamamoto *et al.*, 2008).

Keap1 has been shown to be a Zn^{2+} binding protein (Dinkova-Kostova *et al.*, 2005). Mutation of Cys 273 or Cys 288 decreased the K_a for Zn^{2+} binding from 1.02 (+/− 0.19) $\times 10^{11}$ M^{-1} to approximately 1×10^{10} M^{-1}, yet the physiological significance of these observations is not understood. Mutation of Cys 273 or Cys 283 to Ser does not affect the stability of Keap1 nor its subcellular localization (Zhang and Hannink, 2003). Mutation of Cys 226, 241, 257, 273, 288, and 197 to Ala does not affect the ability of Keap1 to bind CUL3 (Kobayashi *et al.*, 2004). Mutation of Cys 273 and Cys 288 to Ala does not affect the ability of Keap1 to bind Nrf2. Using a BIAcore interaction assay, Kobayashi et al., (Kobayashi *et al.*, 2006) found that the K_d for association of wildtype Keap1 with a GFP/Nrf2-Neh2 domain was not significantly different from the K_d that was obtained when Keap1 contained C273A and C288A mutations (Kobayashi *et al.*, 2006). Currently, the mechanisms by which C273 and C288 mutations affect Keap1 function are not well understood.

A two-site recognition model has been proposed, in which oxidative modification or electophilic adduction of Keap1, particularly in the intervening domain, results in disassociation of Keap1's Kelch domain from a low affinity Nrf2/DLG binding site. The high affinity ETGE binding site in the Neh2 domain would not be affected by adduction/oxidiation of Cys residues. Loss of Kelch/DLG interaction is postulated to affect orientation of the Neh2 domain with respect to the Ub-E2 conjugating enzyme, thereby inhibiting ubiquitination (McMahon *et al.*, 2006; Tong *et al.*, 2006b). Using this model, one can hypothesize that mutation of either C273 or C288 in Keap1 results in a conformational change that impacts

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Keap1/DLG interactions. However, such a model needs to reconcile the question of Nrf2/ Keap1 stoichiometry and the plasticity outlined in current models of CUL-based ubiquitination.

Adduction of Keap1

Table 1, adapted from (Holland *et al.*, 2008) summarizes the current state of knowledge concerning cysteine adduction or oxidation of recombinant Keap1. The first column lists the domains within Keap1. The second column lists the amino acid residues in Keap1's Kelch domain shown by mutation analysis to regulate binding to Nrf2 and/or repression of Nrf2-directed gene expression (Lo *et al.*, 2006). The third column lists Cys residues that have been shown to be adducted. Cys residues list in column 3 that are in bold font have been shown by mutational studies to impact Nrf2 ubiquitination. The remaining columns (4–12) list the various reagents shown to adduct human Keap1 Cys residues. Columns (13–16) list the various reagents shown to adduct murine Keap1. Data concerning the kinetics of reactivity are not presented in this Table. Adduction results are somewhat methodology-based. Further information concerning methodology used to measure adduction of Keap1 can be obtained from the following references (Hong *et al.*, 2005a;Hong *et al.*, 2005b;Eggler *et al.*, 2007;Luo *et al.*, 2007).

For the sake of completeness it should be pointed out that 8-nitroguanosine 3',5' – cyclic monophosphate (Sawa *et al.*, 2007), carnosic acid (Satoh *et al.*, 2008), and S-nitrosocysteine (Buckley *et al.*, 2008) have all been shown to adduct Keap1 but specific residues have not been identified.

Complicating interpretation is the knowledge that some studies have used human recombinant Keap1 while others have used recombinant murine Keap1. Liebler and colleagues (Xiong, Y., Liebler, D.C., and Freeman, M., unpublished results) performed a head-to-head comparison of adduction kinetics in recombinant human and mouse Keap1 and found significant species-specific differences in reactivity. The most glaring difference relates to adduction at Cys273. As shown in Figure 1, this residue is highly reactive to IAB in murine Keap1. However, in human Keap1 reactivity of Cys273 is significantly diminished.

Inspection of the Table indicates that Cys 489 in human recombinant Keap1 is adducted by all reagents except GSSG and liqustilide (Dietz *et al.*, 2008; Holland *et al.*, 2008). Cys 489 is located in Strand C of Blade IV, between a conserved Glu (G488) and a conserved Tyr (Y490) (Li *et al.*, 2004). Less work has been done using murine Keap1 and different results have been obtained with regard to adduction at Cys 489. Whereas dexamethasone (DEX) adducts human Keap1 at Cys 489 (Liebler, 2006), it appears not to adduct Cys489 in murine Keap1 (Dinkova-Kostova *et al.*, 2002). Fifteen-deoxy-PGJ2 is a potent Nrf2 inducting agent in human and murine systems (Levonen *et al.*, 2004; Hosoya *et al.*, 2005). Yet, this reagent does not appear to adduct at mKeap1 Cys 489. Nor is there evidence that NAPQ1, another potent Nrf2 inducing agent, adducts at this residue in murine models (Copple *et al.*, 2008). Therefore, the significance of adduction at Cys 489 is not currently understood.

It is clear from Table 1 that the pattern of adduction of Keap1 Cys residues is electrophile dependent. In fact, one can classify adduction patterns into classes, analogous to the classification system developed by Prestera et al., (Prestera *et al.*, 1993). Many of the compounds shown in Table 1 adduct at Cys 151, 273, and or Cys 288. Based on the mutational analysis, it may be expected that adduction at Cys 273 or Cys 288 would inhibit Keap1-directed ubiquitination of Nrf2. Yet the molecular mechanisms that underlie inhibition are not well understood.

Cys151 is a non-conserved amino acid (Furukawa *et al.*, 2003) that resides in the BTB domain of Keap1, the domain that binds CUL3. The core BTB structure consists of 95 amino acid

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residues that fold into five alpha-helices that end with a three-stranded beta sheet (Perez-Torrado *et al.*, 2006). BTB domains can have N-terminal or C-terminal extensions to the core domain that contribute to dimerization or tetramerization. The observation that a C151S mutation inhibits electrophile and oxidant-mediated disassociation of Keap1 from CUL3 suggests that the BTB fold in Keap1 is intolerant to adduction or oxidation at this residue. Use of recombinant protein systems, as well as ectopically expressed protein in cell culture models have shown that adduction at Cys 151 induces a conformational shift in Keap1's structure that is accompanied by disassociation of Keap1 from CUL3 (Zhang and Hannink, 2003; Rachakonda *et al.*, 2008). The C151S mutation in Keap1 was shown to inhibit adduction-mediated conformational changes, as well as adduction-mediated disassociation of Keap1 from CUL3.

Relevant to this discussion is the question whether recombinant models of adduction mirror the in vivo situation. Rachakonda et al., (Rachakonda *et al.*, 2008) addressed this question using N-iodoacetyl-N-biotinylhexylenediamine (IAB) as a surrogate electrophile. Their results are presented in Table 1. IAB adducted Cys residues in recombinant human Keap1 are denoted by Xs whereas adduction in vivo is denoted by an (*). Cys residues C77, C151, C257, C288, C297, C613, and C622 were found to be adducted in vitro and in vivo. These data show that two key residues, Cys 151 and Cys 288 represent two critical targets in vivo and that their adduction can be modeled using in vitro systems. Furthermore, Rachakonda et al., (Rachakonda *et al.*, 2008) found that both Cys151 and Cys288 were rapidly adducted in vivo.

Kobayashi et al., (Kobayashi *et al.*, 2009) have investigated the role of individual Keap1 Cys residues using an elegant zebrafish model, as well as interrogating recombinant murine Keap1. Using mutation analysis and ectopically expressed protein, they compared the ability of diethyl maleate, 1,2-dithiole-3-thione, sulforaphane, tert-butylhydroquinone, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2, ebselen, 1,2-naphthoquinone, prostaglandin A_2, hydrogen peroxide, $CdCl_2$, and auranofin to stabilize Nrf2 and induce Nrf2 target gene expression in the presence of Keap1. The mutation analysis was combined with a MALDI-TOF MS analysis of murine recombinant Keap1 exposed to 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2, prostaglandin A_2, or DEM. These combined analyzes resulted in the inducers being grouped into 6 classes. Classes 1 and 2 react with Cys 151 (diethyl maleate, 1,2-dithiole-3-thione, sulforaphane, tert-butylhydroquinone, 1,2-naphthoquinone, ebselen). Classes 3 – 6 react with Cys 273 but not C151 (15-deoxy-$\Delta^{12,14}$ prostaglandin J_2, ebselen, 1,2-naphthoquinone, prostaglandin A_2, hydrogen peroxide, $CdCl_2$, auranofin). Kobayashi et al., (Kobayashi *et al.*, 2009) hypothesize that Keap1 contains at least 2 distinct cysteine motifs that capture specific chemical information and translate it into biochemical information via changes in structural conformation, similar to Yap1 (Georgiou, 2002).

Inspection of Table 1 indicates that neither sulforaphane nor GSSG adducts Cys 151, Cys 273, or Cys 288. GSSG, the oxidized form of glutathione, represents a physiological oxidant that could form either a glutathione-mixed disulfide with Keap1 or generate inter or intramolecular disulfides in Keap1. Although the Table does not present the disulfide data, Holland et al., (Holland *et al.*, 2008) found that GSSG induces disulfide bond formation between Cys residues 23 and 38, as well as residues 257 and 249 in human Keap1. GSSG induces a C319 intermolecular disulfide. Cys 23 has been shown to be mutated in breast cancer (C23Y) and inhibit Keap1-directed ubiquitination of Nrf2 (Nioi and Nguyen, 2007). Thus, Holland et al. (Holland *et al.*, 2008), postulate that disulfide formations that involve Cys 23 have the potential to affect Nrf2 stability. Further work by Holland et al., (Holland *et al.*, 2008) using molecular docking analysis supports a model in which formation of a mixed glutathione disulfide with Cys 434 and 368, which is also adducted by sulphoraphane, would occlude Nrf2 binding to the Kelch domain. This model is consistent with the multiple cysteine sensing motif model of Kobayashi et al., (Kobayashi *et al.*, 2009).

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BMCC and IAB exhibit similar adduction profiles (Table 1). BMCC adducts at Cys151, Cys273 and Cys288. Yet, BMCC does not induce Nrf2-directed gene expression in cell culture models (Hong *et al.*, 2005b). It is known that electrophile and oxidant-mediated induction of Nrf2-directed gene expression requires activation of kinase signaling (Yu *et al.*, 2000), as well as, adduction of Keap1 cysteine residues. In addition, Li and Kong (Li and Kong, 2009) have hypothesized that Nrf2 itself may be redox sensor. C183 in Nrf2 is located in a functional nuclear export signal motif. The motif is redox sensitive: oxidants and electrophiles inhibited nuclear export. A C183A mutation attenuated the redox sensitivity of Nrf2 (Li and Kong, 2009). These observations have led to the hypothesis that electrophiles and oxidants modify Nrf2 subcellular localization, in addition to modifying Keap1 function. Thus, one can envision multiple levels of redox regulation: adduction of Keap1 to inhibit Nrf2 ubiquitination, activation of signaling kinases, and modification of Nrf2 that direct its subcellular location.

Summary

Nrf2 is a master transcription factor containing a powerful acidic transcriptional activation domain. Nrf2-dependent gene expression impacts cancer chemoprevention strategies, inflammatory responses, and progression of neurodegenerative diseases. Under basal conditions, association of Nrf2 with Keap1 results in the rapid Nrf2 ubiquitylation and proteasome-dependent degradation. Inhibition of Keap1 function blocks ubiquitylation of Nrf2, allowing newly synthesized Nrf2 to translocate into the nucleus, bind to ARE sites and direct target gene expression. Site-directed mutagenesis experiments coupled with proteomic analysis support a model in which Keap1 contains at least 2 distinct cysteine motifs that capture specific chemical information and translate it into biochemical information via changes in structural conformation.

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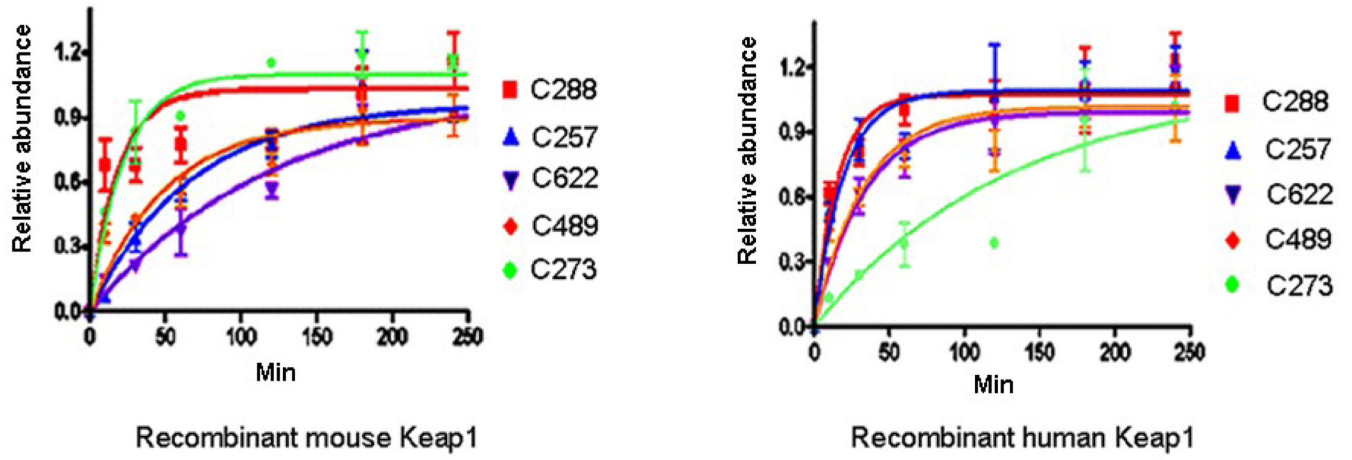

Figure 1.

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Table 1

Adduction/oxidation of recombinant Keap1, adapted from (Holland *et al.*, 2008) with permission.

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Domain		Cys	BMCC	DEX	GSSG	IAB	ISO	Liqustilide	SHO	SULF	XAN	DEM	DEX#	NAPQ1	15d-PGJ2
N-terminus		12							X	X					
		13				X*			X	X					
		23	X						X						
		38	X				X			X	X				
BTB		77	X	X	X		X			X				X	
		151	X			X*	X	X	X		X	X			
		171								X					
IVR		196	X	X		X	X			X					
		226	X	X		X	X		X	X				X	
		241				X			X						
		249		X					X	X					
		257	X			X*			X	X			X	X	
		273	X			X		X	X				X	X	X
		288	X			X*		X				X	X	X	X
		297	X		X	X*							X	X	
Kelch		319	X		X	X	X	X	X		X	X			
	Tyr 334														
		368	X		X		X		X	X		X			
	Arg 380														
	Asn 382														
		395					X								
		406													
	Arg 415														
		434	X			X	X				X	X		X	
	His 436														
	Phe 478														

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1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Arg 483														
		489	X	X	X	X	X		X	X	X	X		X	
		513			X		X			X					
		518			X		X			X					
	Tyr 525														
	Tyr 572														
C-terminus		583			X		X			X				X	
		613	X			X*	X		X		X	X	X	X	
		622				X*			X						
		624							X	X					

Human Keap1 denoted in columns identified using red numbers

BMCC: 1-biotinamido-4-(4′-[maleimidiethylcyclohexane]-carboxamido)butane: (Hong *et al.*, 2005b; Luo *et al.*, 2007)

DEX: dexamethasonemesylate adduction to human Keap1: (Liebler, 2006)

GSSG: glutathione disulfide: (Holland *et al.*, 2008)

IAB: *N*-iodoacetyl-*N*-biotinylhexylenediamine: (Hong *et al.*, 2005b; Eggler *et al.*, 2007)

ISO: Isoliquiritigenin: (Luo *et al.*, 2007)

Liqustilide: (Dietz *et al.*, 2008)

SHO: 10-Shogaol:(Luo *et al.*, 2007)

SUL: Sulforaphane: (Hong *et al.*, 2005a)

XAN: Xanthohumol: (Luo *et al.*, 2007)

X* IAB addition to Cys residue *in vivo*

Murine Keap1 denoted in columns identified using blue numbers

DEM:Diethylmaleate: (Kobayashi *et al.*, 2009)

DEX#: dexamethasonemesylate adduction to murine Keap1: (Dinkova-Kostova *et al.*, 2002; Copple *et al.*, 2008)

NAPQ1: N-acetyl-p-benzoquinoneimine: (Copple *et al.*, 2008)

15d-PGJ2: 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2:(Copple *et al.*, 2008; Kobayashi *et al.*, 2009)