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p62 links autophagy and Nrf2 signaling

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ABSTRACT

The Nrf2-Keap1-ARE pathway is a redox and xenobiotic sensitive signaling axis that functions to protect cells against oxidative stress, environmental toxicants, and harmful chemicals through the induction of cytoprotective genes. To enforce strict regulation, cells invest a great deal of energy into the maintenance of the Nrf2 pathway to ensure rapid induction upon cellular insult and rapid return to basal levels once the insult is mitigated. Because of the protective role of Nrf2 transcriptional programs, controlled activation of the pathway has been recognized as a means for chemoprevention. On the other hand, constitutive activation of Nrf2, due to somatic mutations of genes that control Nrf2 degradation, promotes carcinogenesis and imparts chemoresistance to cancer cells. Autophagy, a bulk protein degradation process, is another tightly regulated complex cellular process that functions as a cellular quality control system to remove damaged proteins or organelles. Low cellular nutrient levels can also activate autophagy, which acts to restore metabolic homeostasis through the degradation of macromolecules to provide nutrients. Recently, these two cellular pathways were shown to intersect through the direct interaction between p62 (an autophagy adaptor protein) and Keap1 (the Nrf2 substrate adaptor for the Cul3 E3 ubiquitin ligase). Dysregulation of autophagy was shown to result in prolonged Nrf2 activation in a p62-dependent manner. In this review, we will discuss the progress that has been made in dissecting the intersection of these two pathways and the potential tumor-promoting role of prolonged Nrf2 activation.

Contents

1. Introduction

The nuclear factor erytheroid-derived-2-like 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1)-antioxidant response element (ARE) field has expanded at an extraordinary rate since the cloning of Nrf2 [1,2]. Over two decades of research, it has been firmly established that the Nrf2-Keap1-ARE pathway is an adaptive cellular response conferring protection against oxidative and xenobiotic stress. Modification of Keap1 cysteine residues leads to inhibition of Nrf2 ubiquitylation and stabilization of Nrf2, allowing Nrf2 to accumulate in the cytosol and then to translocate into the nucleus where it binds to a small Maf protein and activates transcription of genes containing antioxidant response elements (AREs) in their regulatory regions [3–5] (Fig. 1, canonical pathway).

In addition to Keap1-mediated regulation, recently, two other

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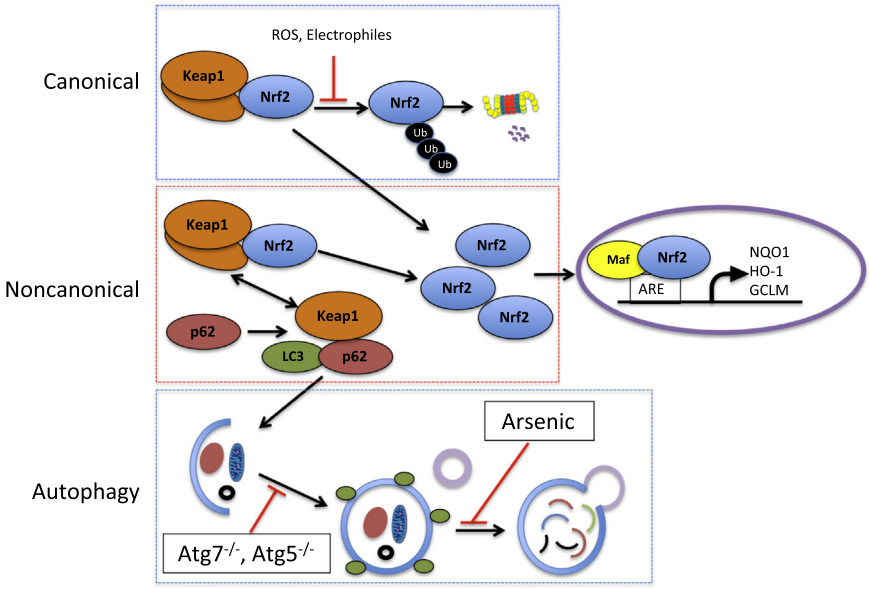

Fig. 1. The canonical and noncanonical regulatory pathways of Nrf2 signaling. (1) Canonical pathway: under normal conditions, Nrf2 is bound to the E3 ubiquitin ligase adaptor protein Keap1, which leads to its ubiquitylation and proteasomal degradation. When Keap1 is challenged with ROS or electrophiles, critical cysteines are modified, blocking Nrf2 ubiquitylation, increasing the level of Nrf2 and activating the ARE-mediated transcription. (2) Noncanonical pathway: when autophagic flux is compromised and p62 accumulates, Keap1 is sequestered by p62 and can no longer bind Nrf2, leading to increased Nrf2 signaling. (3) Autophagy pathway: the pathway can be dysregulated by the blockage of autophagosome maturation (e.g. deletion of *Atg7* or *Atg5*) or the fusion of autophagosome-lysosome (e.g. arsenic treatment).

E3 ubiquitin ligases have been found to regulate the protein level of Nrf2. In 2010, the Neh6 domain of Nrf2 was shown to be phosphorylated by GSK-3β, creating a phosphodegron, which is recognized by the Skp1-Cul1-Rbx1-βTrCP E3 ubiquitin ligase complex [6,7]. Furthermore, our group has found that Hrd1, an integral endoplasmic reticulum (ER) membrane E3 ligase, negatively regulates Nrf2 in patients with liver cirrhosis [8]. These Keap1-independent mechanisms of Nrf2 regulation highlight the functional importance of Nrf2 beyond redox sensing.

Macroautophagy (henceforth "autophagy") is an important catabolic process that degrades cytoplasmic components in bulk (Fig. 1, autophagy) [9–11]. Autophagy is responsible for the clearance of damaged organelles, long-lived proteins, or misfolded proteins. This degradation process acts to preserve cellular homeostasis and to defend against oxidative or proteotoxic stress. Both intracellular and extracellular signals can induce autophagy. These signals include oxidative stress, ER stress, or nutrient deprivation [12]. Autophagy also plays an important role in eliminating intracellular pathogens, in antigen presentation, and in maintaining cellular longevity [13,14]. Given its many critical roles, it is not surprising that impaired autophagic function contributes to a diverse collection of diseases such as cancer, neurodegeneration, cardiomyopathy, Crohn's disease, diabetes, and fatty liver disease [10,12]. On the other hand, increased autophagic activity allows cancer cells to cope with high metabolic and proteotoxic stress, which is essential to their survival [15]. Thus, similar to Nrf2, autophagy plays seemingly contradictory roles in cancer [9,16,17], and the roles of Nrf2 and autophagy in cancer are context-dependent.

The crosstalk between the Nrf2-Keap1-ARE axis and autophagy was revealed in 2010 when five separate groups independently confirmed the association between p62/sequestosome 1(SQSTM1), an autophagy adaptor, and Keap1 [18–22]. Following this finding, great progress has been made to understand the mechanistic underpinnings linking the Nrf2 and autophagy pathways, the functional consequence of autophagy dysregulation, and prolonged Nrf2 signaling.

1.1. *p62-dependent Nrf2 activation – the noncanonical pathway*

p62 is an autophagy adaptor protein, initially identified as a tyrosine kinase p56lck binding protein, that gained more attention when mutations in p62 were found to be associated with Paget's disease of bone [23–25]. However, the functional importance of p62 did not emerge until Komatsu et al. reported an association between p62 and LC3, indicating a role for p62 in autophagy [26]. In this study, p62 was found to mediate the formation of protein aggregates destined for autophagic turnover, which suggested that p62 facilitated selective degradation of protein cargo via autophagy [26]. It is now understood that p62 works as an adaptor, binding ubiquitylated protein aggregates and delivering them to the autophagosomes.

Although the p62-mediated induction of Nrf2 with subsequent nuclear translocation and activation of ARE-driven genes was first reported in 2007 [27], the molecular mechanism was not fully elucidated until the discovery of the association between p62 and Keap1 [18–22]. Three of the five groups confirmed that the 349-DPSTGE-354 motif in the Keap1-interacting region (KIR) domain of p62, which resembles the Keap1-interating ETGE motif in the Neh2 domain of Nrf2, accounts for the direct interaction between p62 and Keap1 [18–20]. This interaction allows p62 to sequester Keap1 into the autophagosomes, which impairs the ubiquitylation of Nrf2, leading to activation of the Nrf2 signaling pathway (Fig. 1, noncanonical pathway). However, the details of the association between p62, Keap1, and LC3 remain controversial. For example, the LC3-interacting region (LIR) domain in p62 is close to the KIR domain in p62. This adjacency was shown to cause competitive binding between LC3 and Keap1 to p62 [20]. In contrast, another study showed that Keap1-p62-LC3 complex formation was required for the clearance of ubiquitin aggregates in response to oxidative stress [21]. This controversy remains to be solved.

In addition to the sequestration of Keap1 by p62 into aggregates or autophagosomes, Keap1 was found to be a p62-regulated substrate for autophagy-mediated degradation. Therefore, p62 plays a key role in controlling Keap1 turnover, as indicated by the results showing that overexpression of p62 significantly decreased the half-life of Keap1 whereas knockdown of p62

increased it [22]. It was shown that the protein level of Keap1 in the liver was much lower in wild type mice compared to liver-specific *Atg7*-deficient or *p62*-deficient mice [28]. These results indicate that p62-mediated Keap1 turnover, in addition to physical sequestration of Keap1, also contributes to the noncanonical activation of Nrf2 (Fig. 1, canonical pathway).

Recent structural and functional studies have further illuminated the details of the noncanonical mechanism of Nrf2 activation. Previous structural studies demonstrated that Keap1 interacts through its Kelch domain with either the DLG or the ETGE motif of Nrf2 in a 2:1 ratio. According to the hinge and latch model, the ETGE motif has a higher affinity for Keap1 than the DLG motif, which causes the latter to dynamically associate and dissociate from Keap1 to generate oscillating "closed" (associated) and "open" (dissociated) conformations [29–33]. Interestingly, p62 contains an STGE motif that binds to the Kelch domain of Keap1 and it has been speculated that this binding occurs during the open conformation of the Keap1-Nrf2 complex [33]. Another study demonstrated that phosphorylation of p62 S351 within this motif significantly increased the affinity between p62 and Keap1, which resulted in prolonged accumulation of Nrf2 and transcriptional upregulation of its target genes [34]. To date, however, the kinase responsible for p62 phosphorylation at S351 has not been identified [34]. Recently, Sestrins, especially Sestrin2 was found to be in complex with p62, Keap1, and Rbx1, and the association of these proteins facilitated p62-dependent autophagic degradation of Keap1 and subsequent activation of Nrf2 [35]. Another study suggests that Sestrin2 interacted with Unc-51-like kinase 1 (ULK1) and p62 to promote p62 phosphorylation by ULK1 at S403, which facilitated p62-mediated degradation of cargo proteins including Keap1 [36].

2. Prolonged Nrf2 activation results from the noncanonical mechanism – a novel mechanism of arsenic action

It is reasonable to envision that this noncanonical mechanism of Nrf2 induction results in prolonged Nrf2 signaling, relative to the canonical pathway, because protein aggregates have to be resolved and new Keap1 protein has to be made in order to restore the functional, canonical Nrf2-Keap1-ARE axis. This was experimentally confirmed during mechanistic investigations of toxic and carcinogenic effects of arsenic. Inorganic arsenic, an environmentally ubiquitous toxic metalloid that pollutes drinking water, soil, and air all over the world, has been implicated in many human diseases including cancer, cardiovascular diseases, neurological diseases, respiratory diseases, and potentially diabetes [37–41]. It seems reasonable that there would be different cellular signaling events or outcomes elicited by acute high doses *vs.* chronic low doses of arsenic exposure. Some detrimental outcomes of arsenic toxicity resulting from acute exposure, typically higher than environmentally relevant doses, include ROS production, metabolic stress due to an alteration of enzyme function, DNA damage, inhibition of DNA repair mechanisms, and activation of cell death pathways such as necrosis and apoptosis [42–46]. In contrast, chronic exposure to environmentally relevant low doses of arsenic results in tumorigenesis without provoking a measurable amount of the aforementioned cellular responses. Recently, our group determined that autophagic flux was blocked after exposure to low doses of arsenic, which may represent one of the underlying mechanisms of arsenic carcinogenicity [47,48]. In this study, we used low doses of arsenic to understand a long standing dilemma: why arsenic, a carcinogenic metal, is also able to induce the chemopreventive Nrf2 signaling pathway and whether there is a distinction in the way Nrf2 is activated by arsenic *vs.* chemopreventive compounds such as sulforaphane. Through detailed investigations, we found that sodium arsenite employs a unique mechanism to activate Nrf2, without modification of Keap1 cysteines, but with diminished Nrf2 ubiquitylation [49]. Subsequently, we determined that arsenic activated Nrf2 in a p62-dependent (noncanonical) manner, through inhibition of autophagic flux [47,48]. Arsenic was shown to increase the accumulation of autophagosomes and the colocalization of p62, Keap1, and LC3, which in turn led to chronic and sustained activation of Nrf2 [47,48]. Generally, autophagy activation coincides with diminished p62 levels, so the accumulation of p62 would suggest blockage of autophagy rather than induction. It remains to be determined how arsenic blocks autophagic flux.

In addition to arsenic, another metal was found to work by a similar mechanism to activate the Nrf2 signaling pathway. In cadmium-transformed human lung bronchial epithelial BEAS-2B cells, an acquired autophagy deficiency was observed, which led to constitutive accumulation of p62 and Nrf2, giving rise to apoptosis-resistant cells [50]. More interestingly, cadmium behaves differently in BEAS-2B parental cells *vs.* cadmium-transformed BEAS-2B cells. In non cadmium-transformed BEAS-2B cells, cadmium served as an autophagy inducer, but it lost the ability to induce autophagy in cadmium-transformed cells, as indicated by a lack of GFP-LC3 puncta in response to short term cadmium treatment [50]. Therefore, long-term exposure to cadmium might affect the initiation or elongation steps of autophagy in these transformed cells.

In addition to metals, the central nervous system stimulant methamphetamine and the skin sensitizer 1-fluoro-2,4-dinitrobenzene were reported to induce the Nrf2 signaling pathway through impaired autophagic flux and p62 accumulation in a mouse atrial cardiac cell line and in a human monocytic cell line, respectively [51,52]. These studies provide a critical link between autophagy dysregulation and prolonged Nrf2 signaling, which provides important environmental health implications.

3. The pathological consequences of prolonged Nrf2 activation through the p62-mediated noncanonical mechanism

Starting in 2006, the "dark side" of Nrf2 has gradually emerged. Somatic mutations in Nrf2 or its negative regulators (Keap1 and Cul3) have been shown to be responsible for the high levels of Nrf2 in certain tumors, such as lung cancer, melanoma, renal clear cell carcinoma, and hepatocellular carcinoma [53–57]. High levels of Nrf2 in these cancer cells provide a cellular environment conducive to growth and survival under detrimental conditions. Furthermore, Nrf2 has proven to contribute to chemoresistance [17,58]. More importantly, adjuvant therapy using the Nrf2 inhibitor brusatol imparted drug sensitivity to chemoresistant cancer cells, and enhanced the survival of mice bearing KRas-induced lung tumors [59,60]. Therefore, persistent and constitutive Nrf2 activation due to genetic mutations has been recognized to be pathogenic, especially in cancer. This "dark side" role of Nrf2 in cancer due to persistent Nrf2 activation led us to postulate that prolonged Nrf2 activation through the p62-mediated noncanonical mechanism during low dose, chronic arsenic exposure may promote cell transformation.

Recently, studies using autophagy-defective mouse models provided strong evidence that prolonged Nrf2 activation, due to autophagy dysregulation, leads to tissue injuries and cancer. For instane, when autophagy is ablated due to deletion of *Atg5*, *Atg7*, or *Beclin-1*, p62-Keap1 aggregates accumulate in the cytosol, resulting in prolonged Nrf2 activation [61–64]. Atg7 serves as an E1 ubiquitin-activating enzyme in the initiation of autophagosome synthesis. It plays an important role in the elongation step of the phagophore by activating Atg12 and LC3-I mediated conjugation

systems [65]. Mice deficient in the *Atg7* gene have impaired autophagosome formation [66]. In these mice, phagophore elongation is blocked and accumulation of protein aggregates rich in p62 and Keap1 is observed [18]. Therefore, *Atg7*-deficient mice provided a valuable *in vivo* model for mechanistic studies aimed at dissecting the crosstalk between the autophagy and Nrf2 pathways.

A positive correlation between p62 and Nrf2 was initially observed in a liver-specific *Atg7* knockout mouse [67]. Subsequently, several reports confirmed the accumulation of Nrf2 in the liver as being due to p62-mediated Keap1 inactivation, which in turn triggered liver damage, inflammation, fibrosis, and tumorigenesis [18,28,35,61,63,68]. For example, hepatocellular adenoma was detected in liver-specific *Atg7*-deficient mice at the age of 7 months. In these mice, p62- and Keap1-containing protein aggregates accumulated and Nrf2 was persistently activated [61]. Interestingly, similar aggregates were detected in human hepatocellular carcinomas (HCC), with activation of the Nrf2 pathway. In these HCC cell lines, deletion of p62 using a zinc finger nuclease system blocked aggregate formation and Nrf2 activation, and suppressed anchorage-independent growth [61]. Another study using the same liver specific Atg7-deficient mice confirmed aberrant p62 and Keap1 accumulation in aggregates and prolonged Nrf2 activation. Liver damage was observed in these mice, as displayed by swelling of hepatocytes, infiltration of inflammatory cells, and collapse of the hepatic lobule [28]. In support of Nrf2 activation as a crucial factor for liver injury, concurrent *p62* or *Nrf2* ablation in *Atg7*-deficient mice corrected the pathological effects observed in *Atg7*-deficient mice [18,26,28].

In addition to liver injury and tumorigenesis, similar noncanonical activation of Nrf2 was observed in lung, epidermal keratinocytes and melanocytes in cell type-specific *Atg7*-deficient mice. In these studies, autophagy deficiency was shown to disturb redox homeostasis through persistent accumulation of Nrf2, which contributed to airway hyper-responsiveness, impaired removal of oxidized phospholipids and protein aggregates and hypopigmentation of the skin [69–71]. In another study, latent Kaposi's sarcoma-associated herpesvirus infection was reported to induce aberrant Nrf2 accumulation due to p62-mediated Keap1 inactivation, which was associated with the initiation and development of Kaposi's sarcoma [72].

The impaired autophagic pathway due to *Atg7* deficiency caused not only p62 accumulation and prolonged Nrf2 activation, but also increased the level of polyubiquitylated protein aggregates and inclusion bodies. Interestingly, increases in protein aggregates or inclusion bodies in liver and brain from *Atg7*-deficient mice were completely suppressed by simultaneous loss of either *p62* or *Nrf2*, thus the accumulation of poly-ubiquitylated proteins is thought to be a consequence of prolonged Nrf2 activation [68]. Further studies are warranted to understand the requirement of poly-ubiquitylated protein aggregates in the pathogenesis resulting from prolonged Nrf2 activation and the molecular mechanism driving protein aggregate formation.

Intriguingly, in $BRaf^{V600E}$-driven lung tumors, although *Atg7* deficiency led to robust tumor induction at an early stage (40% more at 5 weeks post-BRaf activation), tumor growth was eventually retarded and the morphology of the tumors switched from adenoma or adenocarcinoma to oncocytoma, a benign tumor with accumulation of defective mitochondria, due to limited glutamine supply. Therefore, the mice bearing *Atg7*-deficient tumors had better survival rates [73,74]. Furthermore, in $BRaf^{V600E}$-driven tumors, *Nrf2* deficiency and *Atg7* deficiency share similar phenotypes in that deletion of either promotes early tumorigenesis but extended survival of mice by promoting oncocytomas [73,74]. In accordance with this finding that basal levels of autophagy are necessary for cancer cell growth, a basal amount of autophagy is also required for normal functions of melanocytes since *Atg7* deficiency caused premature senescence and impaired pigment production in these cells [71]. Therefore, it may be concluded that the roles of autophagy and Nrf2 in cancer are context-dependent. A comprehensive understanding of the context-dependent mechanistic details is crucial before optimal therapeutic modulation of Nrf2 and autophagy can be achieved.

The understanding of the prolonged Nrf2 activation through this noncanonical mechanism explains why high levels of Nrf2 were observed in certain cancer cells that do not bear mutations in genes controlling the expression of Nrf2. For example, persistent activation of Nrf2 in human hepatocellular carcinoma cells was due to Keap1-p62 aggregate formation, implicating the involvement of the noncanonical mechanism of Nrf2 activation, instead of somatic mutations in *Nrf2*, *Keap1*, or *Cul3*[61]. In hepatocellular carcinoma patients, the co-localization of p62 and Keap1 in aggregates is as high as 25.5% [61], compared to the 8% mutation rate of *Keap1*[55]. Therefore, the impaired production of autophagosomes (e.g. *Atg7*-deficient mice) or blocked autophagic flux (e.g. in response to low dose arsenic exposure) may be a contributing factor to achieve high Nrf2 levels in the absence of somatic mutations in genes controlling Nrf2 expression levels, leading to tumor development and progression.

4. Conclusion

Both the autophagy and Nrf2-Keap1 pathways antagonize cellular stress by upregulating a battery of antioxidant and cellular defense genes. Typically, intermittent activation of Nrf2 through the canonical pathway confers cellular protection and functional integrity. Conversely, constitutive activation of Nrf2, due to somatic mutations in genes regulating Nrf2, leads to tumor promotion and contributes to chemoresistance [75]. Similarly, prolonged activation of Nrf2 through the noncanonical mechanism seems to be detrimental, resulting in tissue injuries, inflammation, and tumorigenesis, the “dark side” of Nrf2. Autophagy, another cellular stress response, is crucial to proteostasis and organelle health in cells. Recent findings indicate that these two pathways are intimately linked by the autophagy adaptor protein, p62. The observation of this interconnectedness has revealed the mechanistic underpinnings of prolonged Nrf2 signaling. When autophagy is compromised, p62 sequesters Keap1 into aggregates, Nrf2 is then stabilized and ARE-regulated genes are upregulated. Because this system lacks the normal, facile means of deactivating Nrf2 signaling, this leads to sustained activation of the Nrf2-ARE system. Although some details remain to be illuminated, these studies have provided new paradigms for the treatment of disease. Conceivably, Nrf2 inhibitors can be used to suppress the prolonged Nrf2 activation resulting from autophagy defects. The detailed mechanistic understanding of the interaction between the Nrf2-Keap1-ARE axis and autophagy is a clear demonstration of the ongoing need for detailed mechanistic pictures of biological and pathological phenomena to facilitate the discovery of new therapies.

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