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Regulation of the Keap1–Nrf2 pathway by p62/SQSTM1

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Abstract

Nrf2 is a transcription factor responsible for gene expression of a series of anti-oxidant proteins and detoxifying enzymes. Keap1, an adaptor protein of Cullin-3 ubiquitin ligase, senses electrophilic or oxidative stresses and then arrests ubiquitination of Nrf2, leading to Nrf2 activation. In addition to this canonical pathway, one Nrf2 target (p62/SQSTM1) competitively binds to Keap1 to activate Nrf2. The p62/SQSTM1-Keap1-Nrf2 axis is linked to selective autophagy and regulated by post-translational modifications such as sequential phosphorylation and ubiquitination of p62/SQSTM1. Importantly, this non-canonical pathway is hyper-activated in autophagy-deficient mouse livers and tissues of hepatocellular carcinoma. In this review, we delineate a molecular mechanism of Nrf2-activation by p62/SQSTM1, and describe its physiological role as well as the pathophysiological significance in autophagy-knockout livers and human hepatocellular carcinoma.

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Keywords

p62/Sqstm1, Keap1–Nrf2 system, Autophagy, Selective autophagy, Hepatocellular carcinoma.

1. Introduction

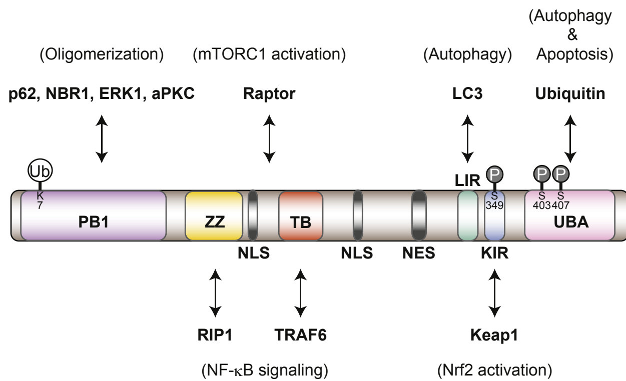
p62/SQSTM1 (hereafter referred to as p62) is a stress-inducible and multifunctional protein [1,2] with multiple domains, including a Phox1 and Bem1p (PB1) domain, a zinc finger (ZZ), two nuclear localization signals (NLSs), a TRAF6 binding (TB) domain, a nuclear export signal (NES), an LC3-interacting region (LIR), a Keap1-interacting region (KIR), and a ubiquitin-associated (UBA) domain (Figure 1). While p62 has been extensively studied as a scaffold protein for atypical PKC, ERK1, NF-κB, caspase-8, and mTORC1 [3], a growing body of evidence has revealed that p62 serves as an adaptor between selective autophagy and ubiquitin signaling [4,5]. p62 forms helical filaments based on a PB1 domain scaffold [6], which easily aggregate into large structures with ubiquitinated proteins under stress conditions [7–9]. Such p62-aggregate structures are degraded in an autophagy-dependent manner [8–10]. p62 is usually accumulated in autophagy-deficient mouse tissues, and p62-positive aggregate structures [11*] are a typical feature of impaired autophagy [12]. Likewise, p62-positive aggregate structures have been identified in tissues of human patients, including in association with neurodegenerative diseases, liver disorders, and cancers [13,14].

The Keap1–Nrf2 system is a major oxidative stress response pathway [15,16]. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a basic leucine zipper (bZIP) transcription factor, and its heterodimer with small Maf proteins controls the expression of anti-oxidant proteins that protect against oxidative damage triggered by injury and inflammation. Kelch-like ECH-associated protein 1 (Keap1) is an adaptor protein of Cullin-3-based ubiquitin ligase. The Keap1 forms a homodimer that recognizes ETGE and DLGex motifs of one Nrf2 molecule through the same binding pocket located at the bottom surface of Keap1 [17–20]. This two-site binding is indispensable for ubiquitination of Nrf2. While ETGE tightly binds to Keap1, the binding affinity of DLGex is much weaker than that of ETGE, and the binding is quickly dissociated, fine-tuning the ubiquitination of Nrf2 [19,20]. Upon modification of specific cysteine residues of Keap1 by oxidants, Nrf2 escapes from the Keap1 interaction and translocates to the nucleus to induce Nrf2-target gene expression [21–24]. This Keap1–Nrf2 pathway enables rapid and transient activation of Nrf2 in response to stressors (Figure 2).

2. Molecular mechanism of Nrf2-activation by p62

Loss of autophagy in mouse livers causes robust Nrf2-activation, accompanied by severe accumulation of p62 [11*]. Increased oxidative stress due to impaired autophagy was initially assumed to be a primary cause for the Nrf2-activation, but mass spectrometry analyses by several independent groups identified the direct interaction between p62 and Keap1, which inhibits the ability of Keap1 to trap Nrf2, followed by Nrf2-stabilization and activation [25**, 26**, 27]. One Nrf2

Figure 1



Schematic domain structures of p62. p62 interacts with p62, NBR1, ERK1, and atypical PKC (aPKC) through Phox and Bem1 (PB1)-mediated homooligomerization or heterooligomerization. K7 ubiquitination of PB1, mediated by TRIM21, inhibits the oligomerization. The zinc finger (ZZ) and TRAF6-binding domain (TB) interact with the RIP and TRAF6, respectively, to regulate NF-κB signaling. Raptor interacts with the linker region between ZZ and TB for mTORC1 activation. LC3-interacting region (LIR) and the C-terminal ubiquitin-associated domain (UBA) engage the sequestration of ubiquitinated substrates into the autophagosome. Keap1-interacting region (KIR) binds to Keap1. Phosphorylation of Ser403 and Ser407 residues on UBA domain and Ser349 residue on KIR occur in response to selective autophagy. The UBA domain also interacts with ubiquitinated caspase8 to facilitate the apoptosis signal. NLS and NES indicate nuclear localization signal and nuclear export signal, respectively.

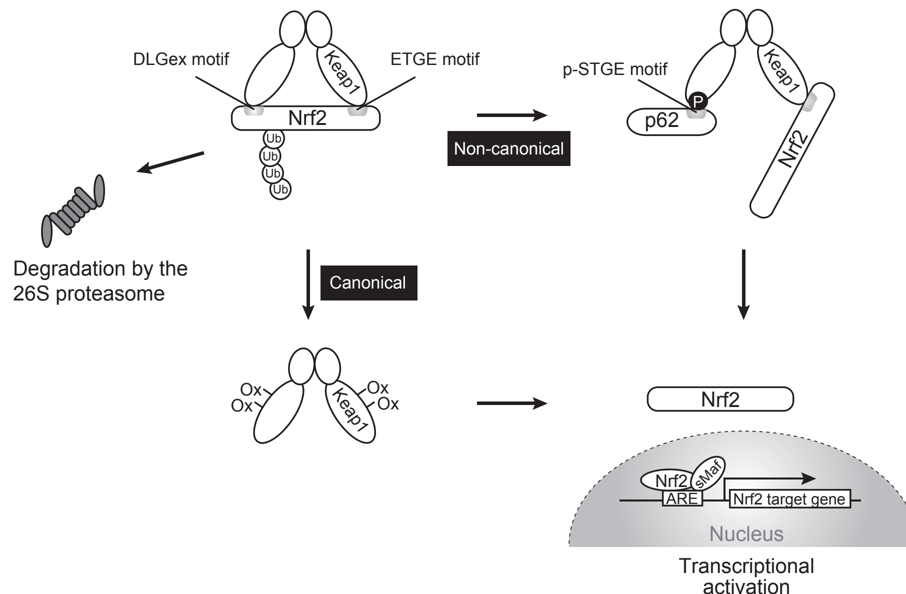
molecule binds to the Keap1 homodimer. The DLGex and ETGE binding motifs in the Neh2 domain of Nrf2 bind individually to the same binding pocket located at the bottom surface of Keap1 [19,20,28]. The two-site substrate recognition mechanism is crucial for the rapid ubiquitination of lysine residues located between the DLGex and ETGE motifs [19,20,28]. ETGE binds tightly to Keap1, whereas the Keap1–DLGex interaction is characterized as "fast-on and fast-off" [20]. X-ray crystal structural analysis of double glycine repeat and C-terminal region (DC) domain of Keap1 in complex with the p62 KIR peptide showed that the KIR peptide also binds to the bottom of the β-propeller structure [25**]. Remarkably, p62-KIR binds to Keap1 in a manner very similar to the Nrf2-ETGE. p62-KIR forms hydrogen bonds with eight amino acid residues of Keap1-DC. These eight residues and two additional Keap1 residues participate in Keap1 recognition of the Nrf2-ETGE. Therefore, the binding-dissociation constant of Keap1-p62 is lower than that of Nrf2-ETGE, accounted for by the difference in corresponding amino-acid residues, glutamic acid in Nrf2-ETGE and serine in the case of mouse p62-KIR (S351). Importantly, this serine residue is phosphorylated in response to selective autophagy conditions such as impaired proteostasis, bacterial infection, and mitochondrial depolarization [29**–31]. X-ray crystal structural analysis of Keap1-DC in complex with S351-phosphorylated p62-KIR peptide showed that the phosphorylated KIR interacts with the same number of amino acids as Nrf2-ETGE. The affinity of phosphorylated p62-KIR for Keap1-DC is 5-fold lower than that of Nrf2-ETGE, but higher than that of Nrf2-DLGex, indicating that phosphorylation of p62 at S351 is sufficient for disruption of Keap1-mediated Nrf2 ubiquitination (Figure 2).

3. Physiological role of the p62-Keap1–Nrf2 axis

What stimuli activate the p62-mediated Nrf2? p62 serves as an adaptor protein for selective autophagy: when selective autophagic cargos such as damaged mitochondria appear in the cytoplasm, they are ubiquitinated [4,5]. As a result, adaptor proteins including p62 that bind to both ubiquitin-chain and autophagosome-localized ATG8 family proteins assemble around the cargos [4,5]. At the same time, some core ATG proteins such as FIP200, indispensable for early autophagosome biogenesis, also recognize the ubiquitinated targets [32], initiating the process of autophagosome formation around the targets. During this process, Ser407, located at the UBA domain of p62, is initially phosphorylated by ULK1 kinase, which is necessary in the early steps of autophagosome biogenesis [33]. This phosphorylation destabilizes the UBA dimer interface [34] and subsequently casein kinase 2 (CK2), TANK-binding kinase 1 (TBK1), or ULK1 phosphorylates Ser403 of the UBA domain [33,35,36], which increases the binding affinity of p62 to the ubiquitin-chain. Consequently, p62 is translocated to the ubiquitinated cargos and then phosphorylated at Ser349 (corresponding to the mouse S351-phosphorylated form) by mTORC1, and Keap1 is sequestered into the ubiquitinated cargos, resulting in Nrf2-activation [29**,30]. Finally, the ubiquitinated cargos, together with phosphorylated p62 and Keap1, are degraded by autophagy [27,29**,37**]. Sestrin2 forms a ternary complex with p62 and Keap1, acting as a scaffold protein for the p62-mediated autophagy of Keap1 [38*]. The major stress response pathways, the Keap1–Nrf2 system and selective autophagy, are coupled to each other through the phosphorylation of p62 (Figure 3). Notably, because *p62* is a target of Nrf2 [39,40**], a feedback loop is present in the p62-Keap1–Nrf2 axis. Recently, it was reported that Lys7 of PB1 domain of p62 is ubiquitinated by TRIM21, which prevents p62-aggregation and subsequent Keap1-sequestration onto the aggregate structures [41**]. Thus, TRIM21 negatively regulates the p62-Keap1–Nrf2 axis.

Most autophagy-related genes are well conserved among eukaryotes, but p62, Nrf2, Keap1, and TRIM21 are not. p62 is conserved among metazoans but not in plants and fungi, and KIR appears only from vertebrates. In *Caenorhabditis elegans*, accumulation of damaged or superfluous mitochondria causes oxidative stress that activates SKN-1, a homolog of mammalian Nrf2, to induce

Figure 2



Canonical and non-canonical Keap1–Nrf2 pathways. Keap1 dimers interact with the DLGex and the ETGE of Nrf2 through their Kelch β-propeller domains. Nrf2 is therefore constantly ubiquitinated and degraded by the 26S proteasome. Under stress conditions, certain cysteine residues of Keap1 are modified by reactive oxygen species and electrophiles in the canonical Keap1–Nrf2 pathway, followed by the dissociation of Nrf2 from Keap1. In non-canonical Keap1–Nrf2 pathway, Ser349 phosphorylated p62 (p-STGE) competitively binds to Keap1, thereby dissociating the Nrf2 from Keap1. Consequently, stabilized Nrf2 translocates to the nucleus by each pathway, and then together with a small Maf protein, induces its targets, which include various cytoprotective genes. ARE: antioxidant response element.

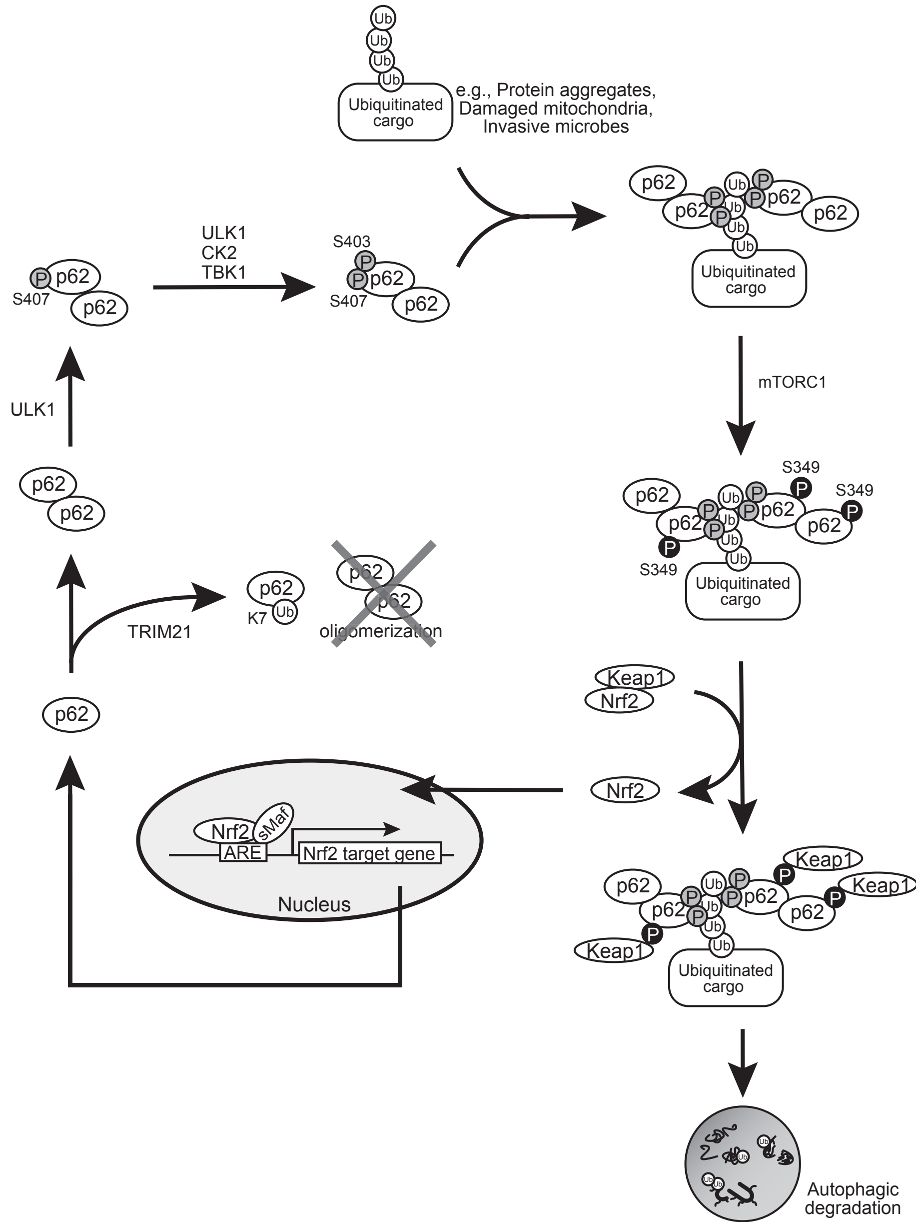
the coordinated expression of mitochondrial biogenesis and mitophagy genes to preserve mitochondrial quality [42]. Besides the SKN-1 function committed to antioxidant response and autophagy, Keap1 is absent in *C. elegans*. In *Drosophila melanogaster*, the gene expressions of both *Atg8a* and *Ref(2)P*, which code mammalian homologs LC3 and p62, are regulated by CncC, a mammalian Nrf2 homolog, and therefore activation of CncC induces autophagy [43]. Remarkably, while Ref(2)P does not have the ability to interact with Keap1, Atg8a interacts directly with Keap1 to modulate its levels through autophagy, regulating the CncC activity [43]. *TRIM21* is conserved in vertebrates, and the gene expression is regulated by interferons, a family of secreted proteins that exert antiviral and immunomodulatory activities [44]. These growing lines of evidence imply that generation of *de novo* gene products and interaction networks during evolution have provided autophagy with a sophisticated p62 mediated Keap1—Nrf2 pathway.

4. Pathophysiological role of p62-Keap1–Nrf2 axis in autophagy-deficient mouse livers

Liver-specific autophagy-deficient mice (*e.g.*, $Atg7^{flox/flox}$; Albumin-*Cre*) exhibit severe liver enlargement and liver injury, accompanied by hepatic ballooning and monocyte infiltration [45]. Due to defective autophagy, aggregate structures positive for S351-phosphorylated p62 and Keap1 form in the cytoplasm of mutant livers [29**,46*]. As a result, loss of autophagy usually causes constant Nrf2-activation, which is abrogated by simultaneous loss of *p62* [25**]. Remarkably, phenotypes observed in single *Atg7*- or *Atg5*-deficient livers are almost recovered by additional loss of *p62* and of *Nrf2*, even with defective autophagy [11*,47*], implying that constitutive activation of Nrf2 is a primary cause of liver impairment. However, because loss of *Keap1* in mouse hepatocytes leads to only mild liver abnormality [48], the phenotypes detected in autophagy-deficient livers cannot be attributed solely to Nrf2 activation. A feasible explanation is that the collapse of the balance between the synthesis (Nrf2-dependent robust protein synthesis) and degradation of cellular proteins (global turnover of cytoplasmic proteins through autophagy) could result in the appearance of destructive phenotypes.

Long-term suppression of autophagy in mouse livers causes tumorigenesis at 100% incidence [49*,50*]. Microtumors developed randomly throughout the liver of 7—9-month-old mutant mice, increasing in size and number with age. The tumors in autophagy-deficient livers are in most cases well-demarcated with little fibrous capsule, composed of irregular hepatic plate with tumor cells showing large nuclear cytoplasmic

Figure 3



Physiological role of the p62-Keap1–Nrf2 axis. Upon selective autophagy, oligomerized p62 undergoes phosphorylation at Ser residues (S407, S403) in the UBA domain and increases the binding affinity of p62 to ubiquitin, followed by sequestration of polyubiquitinated autophagic cargos (protein aggregates, damaged mitochondria, invasive microbes, etc.). Then, mTORC1 phosphorylates S349 of p62 and increases binding affinity of p62 to Keap1, resulting in the escape of Nrf2 from the Keap1 interaction. Free Nrf2 enables activation of various target genes. p62 is a target of Nrf2, indicating that a feedback loop is present in the p62-Keap1–Nrf2 axis. Keap1 is degraded together with the autophagic cargo. Note that TRIM21, a ubiquitin E3 ligase, negatively regulates the p62-Keap1–Nrf2 axis by undoing p62 oligomerization following ubiquitination at K7 in the p62 PB1 domain. ARE: antioxidant response element.

ratio and occasional nuclear atypia, which are pathologically diagnosed as hepatocellular adenoma. Such tumorigenesis is probably triggered not only by mitochondrial dysfunction followed by genome instability, but also chronic inflammation, all of which are observed in liver-specific autophagy-deficient mice [49*,50*]. The p62-Keap1–Nrf2 axis is also likely to participate in tumor development in autophagy-deficient mouse livers. While tumor development in *Atg7*$^{flox/flox}$; Albumin-*Cre* is suppressed markedly by concomitant loss of *p62*, tumorigenesis is still observed [50*]. Meanwhile, the fibrosis and tumorigenesis observed in *Atg5*$^{flox/flox}$;

Albumin-*Cre* is completely abrogated by additional loss of *Nrf2* [47*]. These lines of evidence imply that while the p62-Keap1–Nrf2 axis is involved in tumor progression, the canonical Keap1–Nrf2 pathway plays an important role in tumorigenesis under an autophagy-deficient background.

5. Pathophysiological role of p62-Keap1–Nrf2 axis in hepatocellular carcinoma

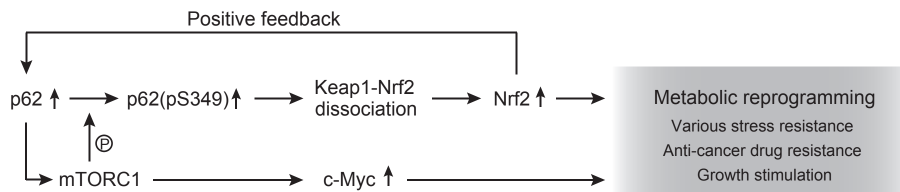
Like autophagy-deficient mouse livers, p62-positive structures called Mallory–Denk bodies and hyaline bodies have been identified in several cases of human hepatocellular carcinoma (HCC) [13,14], and they contained both the S349-phosphorylated form and Keap1 [29**]. In particular, prominent accumulation of the phosphorylated form as well as Nrf2-activation was observed in HCC positive for Hepatitis C virus (HCV) [46*]. Knockout of *p62* in hepatocellular carcinoma cells, Huh1 cells, which express S349-phosphorylated p62 at very high level, decreased their proliferative activity in xenograft assay, which was restored by introduction of S349-phosphorylation mimetic (S349E) p62, but not the phosphorylation defective (S349A) p62 [29**]. Nrf2-activation engages in not only redox signaling but also cancer metabolism. Nrf2 positively regulates the gene expression of enzymes involved in pentose phosphate pathway, purine-nucleotide syntheses, glutathione syntheses, and glutaminolysis, and thus this transcription factor's activity can redirect glucose and glutamine into anabolic pathways, in particular in lung cancer cells harboring activated phosphatidylinositol 3-kinase (PI3K)-Akt, which promotes proliferation [51]. The p62-Keap1–Nrf2 axis also promotes malignancy of HCC through similar, but not the same, metabolic reprogramming, namely enhanced UDP-glucuronate and glutathione production, which stimulate growth of HCC and provide HCC cells with tolerance to anti-tumor drugs [46*]. p62 overproduction in cancer precursor cells of mouse livers also drives tumorigenesis of HCC via activation of both mTORC1 and Nrf2 [52*] (Figure 4).

How does p62 accumulate in HCC cells? While autophagosome formation is induced by infection of HCV, fusion between autophagosomes and the lysosome is suppressed to promote viral replication on or around autophagosomes [53], resulting in decreased autophagy-flux, and followed by accumulation of p62. Even in other liver diseases including alcoholic liver disease, nonalcoholic fatty liver and nonalcoholic steatohepatitis, autophagy seems to be impaired, leading to p62 accumulation [54]. Because p62 is a stress-inducible protein whose expression is regulated by Nrf2 [39,40**], chronic inflammation in patients would also cause up-regulation of p62. Several cancers, including HCC, frequently have somatic mutations of either *Nrf2* or *Keap1* [55–57], which cause alteration or impairment of the Keap1–Nrf2 interaction, followed by persistent activation of Nrf2. Even in the absence of these somatic mutations, Nrf2 is activated in certain types of cancer and utilized for growth. For instance, Keap1 is succinylated by the fumarate accumulated in type 2 papillary renal cell carcinomas that carry mutations in fumarate hydratase [58,59], leading to hyperactivation of Nrf2. Therefore, p62 protein should accumulate in tumor cells harboring mutations of either *Nrf2* or *Keap1*, as well as in the renal cell carcinomas with succinylated Keap1, because Nrf2 positively regulates the gene expression of *p62*. Considering that the overproduction of p62 proteins is enough to stimulate S349-phosphorylation [29**], p62 would inactivate normal Keap1 protein synthesized from the Keap1-allele without the mutation, leading to a vicious circle of Nrf2 activation in the tumor cells. Accordingly, it is plausible that p62-mediated Nrf2 activation occurs in various types of tumor cells in addition to HCV-positive HCC.

6. Concluding remarks

Various mutations of *p62* have been identified in patients with Paget's disease of bone (PDB), amyotrophic

Figure 4



Role of the p62-Keap1–Nrf2 axis in hepatocellular carcinoma. In p62-accumulated HCC cells, mTORC-dependent phosphorylation of S349 (pS349) increases binding affinity of p62 to Keap1, resulting in the Keap1–Nrf2 dissociation and Nrf2 activation. The p62-Keap1–Nrf2 axis positive feedback loop chronically enables activation of target genes. At the same time, p62-mediated mTORC1 activation causes hyper activation of c-Myc. The activation of both Nrf2 and c-Myc is accompanied by cancer-linked metabolic reprogramming and thus contributes to not only tumorigenesis in HCC progenitor cells but also tumor progression of HCC.

lateral sclerosis (ALS), and frontotemporal lobar degeneration (FTLD) [60]. Moreover, p62 has been identified as a pathogenic target of 5q copy number gains in kidney cancer [61]. In addition to HCC, the accumulation of S349-phosphorylated p62 was also recently found in brains of patients with Alzheimer's disease [62,63] and in endothelial cells infected with Kaposi's sarcoma-associated herpesvirus [64]. These mutations and modification may be accompanied by alteration or ablation of p62-binding partner(s) such as Keap1 and LC3, raising the possibility that Nrf2-activity is dysregulated in the aforementioned diseases. Further analysis is needed into whether pathogenesis in patients with *p62* mutations and modification is driven by Nrf2-dysregulation.

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** of outstanding interest

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