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p62/SQSTM1- Dr. Jekyll and Mr. Hyde that prevents oxidative stress but promotes liver cancer

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Abstract

p62/SQSTM1 is a multifunctional signaling hub and autophagy adaptor with many binding partners, which allow it to activate mTORC1-dependent nutrient sensing, NF-κB-mediated inflammatory responses and the NRF2-activated antioxidant defense. p62 recognizes polyubiquitin chains via its C-terminal domain and binds to LC3 via its LIR motif, thereby promoting the autophagic degradation of ubiquitinated cargos. p62 accumulates in many human liver diseases, including non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC), where it is a component of Mallory-Denk bodies and intracellular hyaline bodies. Chronic p62 elevation contributes to HCC development by preventing oncogene-induced senescence and death of cancer-initiating cells and enhancing their proliferation. In this review, we discuss p62-mediated signaling pathways and their roles in liver pathophysiology, especially NASH and HCC.

Introduction

p62, also known as sequestosome 1 (SQSTM1)/A170/ZIP/STAP, is a signaling hub and an autophagy substrate and adaptor, first identified as a 62 kDa protein which binds lymphocyte-specific protein tyrosine kinase (Lck), a Src family member [1-3]. Later, p62 was also reported to be a binding partner of atypical protein kinase C (aPKC) [4, 5].

p62 harbors multiple functional motifs, including an N-terminal Phox1 and Bem1p (PB1) domain, a zinc finger (ZZ), a tumor necrosis factor receptor-associated factor (TRAF) 6 binding (TB) motif, microtubule-associated protein 1 light chain 3 (LC3) interacting region (LIR), kelch-like ECH-associated protein 1 (Keap1) interacting region (KIR) and a C-terminal ubiquitin associated (UBA) domain [6-15]. p62 forms oligomers via the PB1 domain and binds aPKC and extracellular signal-regulated kinase (ERK) 1 via PB1, receptor interacting protein (RIP) 1 via ZZ, TRAF6 via TB, Raptor via the region between ZZ and TB, LC3 via LIR and Keap1 via KIR, and activates mechanistic target of rapamycin

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complex (mTORC) 1, nuclear factor-κB (NF-κB) and nuclear factor erythroid 2-related factor (NRF) 2 [6-8, 10, 16-21] (Figures 1 and 2a). p62 also functions as a selective autophagy receptor/adaptor via its UBA and LIR domains and shuttles damaged proteins and organelles for autophagic clearance [9, 13, 22, 23]. p62 also binds polyubiquitinated caspase-8 via the UBA domain and can promote apoptosis [24]. *SQSTM1* (p62) mutations in the UBA domain were reported in Paget's diseases and considered to be one of the main causes of familial (20-50%) and sporadic (5-15%) Paget's disease of bone (PDB) [25-28]. p62 accumulation induced by impaired autophagy was suggested to promote tumorigenesis by activating p62-regulated pathways [29].

2. Regulation of p62 expression

p62 is ubiquitously expressed in various cell types [30], mainly in the cytoplasm, but is also present in the nucleus, autophagosomes and lysosomes [15, 31]. p62 is overexpressed in many types of human cancer, including hepatocellular carcinoma (HCC) [29, 32-38], intrahepatic cholangiocarcinoma [39], pancreatic cancer [40], lung cancer [41, 42], oral, head and neck cancer [43, 44], esophageal cancer [45], gastric cancer [40, 46], colon cancer [40, 47], breast cancer [48-51], prostate cancer [52, 53], melanoma [54], endometrial cancer [55], ovarian cancer [56] and kidney cancer [57], as well as chronic liver diseases, such as alcoholic and non-alcoholic steatohepatitis (ASH, NASH), that increase HCC risk [34, 37, 58-60]. Therefore, a p62-encoding DNA plasmid vaccine may be useful for cancer immunotherapy [61-63]. However, p62 expression is reduced in the stroma of several cancers, including prostate cancer, breast cancer and colon cancer, which promotes inflammation and tumorigenesis [64].

p62 expression is regulated transcriptionally and post-translationally [13, 65], whereas its activity is controlled by phosphorylation [15]. *SQSTM1* gene transcription is induced by NRF2 and NF-κB [20, 66-68], both of which are also activated by p62, thus establishing two interlocked positive feedback loops. *SQSTM1* mRNA expression is also regulated by Ras-ERK and JNK signaling [69-72] as well as by miR-372 [73] and the nuclear hormone receptor farnesoid X receptor (FXR) [74]. In addition to inflammation and oxidative stress, which activate NF-κB and NRF2, respectively, *SQSTM1* mRNA expression is induced by endoplasmic reticulum (ER) stress [75]. p62 protein is rapidly and constantly degraded by autophagy and elevated p62 expression in the absence of major changes in *SQSTM1* mRNA is used as an indicator of autophagic impairment [76-78]. Hypoxia accelerates p62 degradation by activating autophagy [79], whereas ethanol exposure induces p62 accumulation through regulation of autophagy [80-84].

3. p62-mediated signaling

3.1 p62 and NRF2 signaling

The Keap1-NRF2 pathway is critical for activation of the protective antioxidant response and is also involved in metabolic regulation and cancer chemoresistance [85-87]. NRF2 is a member of the CNC-bZIP family, that also includes the basic region leucine zipper (bZIP) transcription factors, NRF1, NRF3 and NF-E2 [88]. NRF2 heterodimerizes with small Maf (sMaf) bZIP proteins and protects cells from oxidative stress by inducing antioxidant and

detoxifying enzymes. NRF2:sMaf heterodimers recognize antioxidant response elements (ARE) in the promoter regions of genes involved in glutathione (GSH) synthesis (*Gclc, Gclm*), reactive oxygen species (ROS) elimination (*Txnrd1, Prdx1*), detoxification (*Nqo1, Gst*), glucuronidation (*Ugt1a1, Ugdh*), drug excretion (*Mrp*) and NADPH synthesis (*G6PD, ME1*) [38, 89, 90].

NRF2 expression is mainly regulated post-translationally via an adaptor for a Cul3-based E3 ubiquitin ligase, Keap1 [91, 92], although oncoproteins, such as K-Ras^{G12D} and B-Raf^{V619E}, induce *Nrf2* mRNA accumulation to enhance basal NRF2 expression [93]. Keap1 binds NRF2 and promotes its degradation by p62-independent (proteasome-dependent), and p62-dependent (autophagy-dependent) pathways [92], whereas Keap1 itself is degraded through autophagy on p62 binding [94]. But, impairment of autophagy leads to p62 accumulation which results in removal of Keap1 from NRF2, stabilization of the latter and induction of ARE-containing genes [10, 19]. NRF2 can also be activated via a p62-independent pathway, that is triggered by exposure to ROS or electrophiles, which cause oxidation of Keap1 that prevents its binding to NRF2, leading to stabilization of the latter [89, 91]. Gankyrin, encoded by an NRF2 target gene, is an oncoprotein overexpressed in human HCC, that can activate NRF2 in HCC cells in a similar way to p62 [95]. Gankyrin interacts with Keap1 to prevent its binding to NRF2, leading to NRF2 activation. Gankyrin and NRF2 cooperatively provide HCC cells with resistance to oxidative stress, and gankyrin and NRF2 overexpression in HCC correlates with poor prognosis [95]. Gankyrin overexpression can also activate mTORC1 signaling by accelerating degradation of tuberous sclerosis complex (TSC) 2 in colorectal cancer [96]. The cyclin-dependent kinase inhibitor p21$^{WAF-1/CIP1}$, PALB2/FANCN and Wilms tumor gene on X chromosome (WTX) also activate NRF2 by competing with Keap1 for NRF2 binding [97-99].

Dysregulation of the Keap1-NRF2 pathway is associated with many human diseases, especially cancer [85, 100]. However, it is somewhat controversial whether NRF2 functions as an oncogene or a tumor suppressor [87, 101-104]. Initially, NRF2 was postulated to be a tumor suppressor in liver and other organs by virtue of its ability to detoxify electrophiles and stimulate GSH synthesis [105, 106]. Transient NRF2 activation can inhibit chemical carcinogenesis in a variety of mouse models [107-113] but other studies show the opposite [34, 114, 115]. NRF2 ablation enhances diethylnitrosamine (DEN)-initiated rat liver carcinogenesis [107] and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)-induced hepatocarcinogenesis in mice [109]. *Nrf2*$^{-/-}$ mice are also susceptible to colitis-associated colorectal cancer induced by azoxymethane (AOM) [113]. However, human cancer genome sequencing revealed *KEAP1* inactivating mutations in lung, breast, ovarian, gallbladder and liver cancers [115-124] and *NFE2L2* (NRF2) activating mutations in lung, esophageal, skin and liver cancers [114, 118, 119, 122, 124, 125], suggesting that NRF2 functions as an oncoprotein, at least in late stages of tumor progression. NRF2 activation may inhibit chemically-induced tumor initiation, but accelerates tumor progression in a mouse model of lung cancer [126, 127], suggesting that NRF2 is tumor suppressive early on, but pro-oncogenic in later stages. In addition to *KEAP1* and *NFE2L2* mutations, NRF2 is activated upon p62 accumulation in human HCC [33]. NRF2 was postulated to promote liver tumorigenesis by suppressing the death of initiated hepatocytes undergoing oxidative stress or by inhibiting senescence [128]. Recent studies on the role of p62 in liver carcinogenesis

strongly support this hypothesis [34]. NRF2 is also reported to induce metabolic reprogramming which supports cell proliferation and promotes drug resistance [38, 129, 130]. Interestingly, recent papers reported that ROS accumulation limits distant metastasis of human melanoma cells, while antioxidant treatment increases melanoma metastasis in mice [131-134]. NRF2 activation by antioxidant antidiabetic agents also accelerates metastasis [135].

Hepatitis B virus X protein (HBx) binds p62 via the UBA and PB1 domains and augments the interaction between p62 and Keap1, leading to NRF2 activation [136]. Hepatic iron accumulation has been observed in patients with chronic hepatitis C [137, 138] and iron overload induces mitochondrial injury and increases the risk of HCC development in transgenic mice expressing the hepatitis C virus (HCV) polyprotein [139]. NRF2 prevents iron-induced hepatocyte cell death and liver injury [140]. Ferroptosis is a recently recognized form of regulated cell death caused by an iron-dependent accumulation of lipid ROS [141]. The p62-Keap1-NRF2 pathway plays a central role in protecting HCC cells against ferroptosis [142]. In general, NRF2-induced genes promote chemo- and radio-resistance [90, 143], and p62-induced NRF2 activation is involved in sorafenib and cisplatin resistance in human HCC and ovarian cancer cells [38, 144] and survival of cancer stem cells and anticancer drug resistance in sphere-forming breast carcinoma cells [145]. *SQSTM1* and *NQO1*, a well-studied NRF2 target gene, are the eleventh and ninth genes identified to be strongly associated with radiation resistance in human cancer cell lines [146]. It was also reported that NRF2 and the Notch signaling are regulated reciprocally [147-154]. Namely, NRF2 induces Notch1 expression and NRF2 is a Notch target [147, 148, 150, 151]. This NRF2-Notch crosstalk plays an important role in a mouse model of liver regeneration [147, 150].

3.2 p62 and NF-κB signaling

NF-κB is mainly activated by inflammation. i.e. inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin (IL)-1β, or Toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS), and plays important roles in innate and adaptive immune responses [155, 156]. NF-κB is considered to be a central player in linking inflammation and cancer [157]. NF-κB signaling is activated in human HCC [158], and both activation or inhibition of NF-κB in hepatocytes enhance hepatocarcinigenesis in mice [159-162]. Inhibition of NF-κB signaling can also result in non-alcoholic steatohepatitis (NASH)-like pathology in mice [159].

p62 binds TRAF6 via its TB domain and promotes NF-κB activation and the interaction between p62 and RIP1 links the aPKCs to TNF-TRAF6- and IL-1β-TRAF6-mediated NF-κB activation [7, 8, 163]. The *TRAF6* gene is amplified in human lung cancer [164], and p62 is important for development of Ras-induced lung adenocarcinomas in mice by activating IκB kinase (IKK) through TRAF6 polyubiquitination [69]. NF-κB activation induced by p62 also promotes mammary and pancreatic tumorigenesis [66, 165] and myeloid malignancies with chromosome 5q deletions [166].

In addition, p62 plays an important role in CD40-TRAF6- and nerve growth factor (NGF)-TRAF6-mediated NF-κB activation [167, 168] as well as receptor activator of NF-κB ligand

(RANKL)-TRAF6-mediated NF-κB activation during osteoclastogenesis which is critical in the pathogenesis of PDB [27, 169, 170]. In addition to the TB domain, the UBA domain plays an important role in RANKL-induced activation of NF-κB as well as nuclear factor of activated T cells (NFAT) and ERK [170-172]. However, p62 also inhibits NF-κB activation in osteoblasts at several levels and is required for hematopoietic stem cell/progenitor retention in bone marrow [173]. p62 also acts downstream to T-cell receptor (TCR) activation and promotes allergic airway inflammation [174-176]. p62 promotes keratinocyte inflammatory responses via NF-κB activation [177], and enhances NF-κB activity in specific tissue macrophages by sequestering A20 in autophagosomes [178]. However, it is also reported that NF-κB-induced p62 expression in macrophages inhibits inflammasome-dependent sterile inflammation and IL-1β production [67] and p62 attenuates cytokine gene expression in activated macrophages by suppressing TRAF6-NF-κB activation [179]. Undoubtedly, p62 is a multifunctional protein and its exact function is context dependent.

3.3 p62 and mTORC1 signaling

The mTORC1 complex senses and integrates diverse environmental cues to regulate a variety of processes that generate or use large amounts of energy and nutrients [180]. mTOR interacts with Raptor and Rictor to form two functionally distinct large complexes: mTORC1 and mTORC2, respectively. mTORC1 is activated by growth factors and nutrients, especially amino acids. Chronic mTORC1 activation is closely associated with human diseases, such as cancer, obesity, type 2 diabetes, and neurodegeneration. mTORC1 activation has been observed in 40-50% of HCC and is associated with poor prognosis [181, 182]. However, thus far, mTORC1 inhibitors (rapalogs) were not found to improve the overall survival of HCC patients [183], consistent with mouse data [184].

p62 binds Raptor and thereby can control mTORC1 activity [17]. Under nutrient-rich conditions, p62 is phosphorylated by p38δ at threonine 269 and serine 272 in response to amino acids in a MEKK3-dependent manner [185]. This results in recruitment of TRAF6 to p62 through its TB, and the interaction between p62 and TRAF6 enhances mTORC1 lysosomal translocation and activation [17, 185, 186]. mTORC1, transforming growth factor β-activated kinase 1 (TAK1) and other unknown kinase(s) also phosphorylate p62 at serine 349 in humans or serine 351 in mice and this enhances the KIR-mediated binding of p62 to Keap1, thereby augmenting NRF2 activation [187, 188]. Importantly, this was found to occur in liver tumors in both mice and men [36, 38, 187]. Interestingly, phosphorylated p62 is accumulated in tumor regions positive for HCV, but is barely detectable in tumor regions positive for hepatitis B virus (HBV) [38]. This may be because HCV, but not HBV, interferes with autophagy and utilizes autophagosomes for its RNA replication, although both HCV and HBV infections induce autophagosome formation [38, 189-195]. mTORC1 activation induced by hypernutrition also causes phosphorylation and inhibition of Unc-51-like protein kinase (Ulk) 1, resulting in attenuation of autophagy and p62 accumulation [196-198]. Therefore, mTORC1 and p62 are also engaged in reciprocal regulation via a positive feedback loop. Furthermore, liver-specific *Tsc1*$^{-/-}$ mice, which develop HCC due to chronic mTORC1 activation [199], exhibit massive p62 accumulation and p62 ablation in their hepatocytes completely inhibits HCC development [34]. c-Myc expression in both mouse and human HCC, which is thought to be driven by mTORC1 activation, is also diminished

upon p62 ablation [34]. p62 is also reported to promote breast cancer stem-like properties by stabilizing *Myc* mRNA [200].

3.4 p62 and autophagy

Autophagy plays an important role in both liver homeostasis and pathophysiology, e.g., nutrient and energy metabolism, clearance of misfolded proteins, lipid and alcohol metabolism, degradation and recycling of damaged organelles and the pathogenesis of viral hepatitis [201-204]. Complete inhibition of autophagy in hepatocytes, induced by deletion of the essential autophagy genes, autophagy-related (*Atg*)*5* or *Atg7*, causes spontaneous liver injury, hepatomegaly and appearance of liver tumors [205]. These tumors, however, are benign adenomas, which is consistent with other experiments showing that autophagy is required for development of pancreatic carcinomas [206]. Liver adenomas in liver-specific $Atg7^{-/-}$ mice show strong accumulation of p62 and subsequent NRF2 activation due to autophagy inhibition, and both tumor growth and liver injury are markedly suppressed by p62 deletion [23, 205]. Liver injury is alleviated by NRF2 ablation and exacerbated by Keap1 deletion in liver-specific $Atg7^{-/-}$ mice [10]. Liver inflammation, fibrosis and tumorigenesis are also completely rescued by NRF2 deletion in liver-specific $Atg5^{-/-}$ mice [207]. However, NRF2 activation induced by impaired autophagy causes liver injury independently of p62 accumulation [94].

Autophagy is a more selective degradation process than originally anticipated and p62 functions as a cargo receptor in selective autophagy [208]. Other cargo receptors include NBR1 (neighbor of BRCA1 gene 1) and NDP52 (nuclear dot protein 52), OPTN (optineurin) and TAX1BP1 (Tax1-binding protein 1) [209, 210]. Curiously, ablation of p62 in hepatocytes or pancreatic acinar cells does not result in any obvious autophagy defects [34, 211], suggesting that its function is redundant with that of other cargo receptors. p62, however, is essential for the mitophagic clearance of mitochondria that were damaged by exposure of macrophages to various NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome activators [67]. p62 also regulates autophagic removal of excess hepatic ER in mice [212]. Interestingly, depletion of p62 dramatically enhances the efficiency of gene delivery in various mammalian cells, including embryonic stem (ES) cells, by suppressing autophagosome formation at the site of exogenous DNAs [213].

The role of autophagy in tumorigenesis (i.e. tumor promoter or tumor suppressor) depends on cellular context and stage [214-216]. During early stages of malignant development, autophagy seems to suppress tumor growth by enhancing degradation of defective proteins and inhibiting accumulation of genotoxic free radicals [217]. In contrast, at later stages, autophagy helps cancer cells in the central areas of the tumor survive under local low-nutrient and low-oxygen conditions [218]. p62 is constantly degraded by autophagy, whose impairment results in its accumulation, which causes further impairment of autophagy through mTORC1 activation, although it was reported that LPS-induced p62 accumulation promotes clearance of autophagy-like aggregates in hepatocytes [68]. Of note, p62 accumulation in hepatocytes promotes HCC development and recurrence in mice and humans [33, 34, 38]. p62 accumulation caused by insufficient autophagy also promotes cell proliferation and migration through stabilization of the oncogenic transcription factor Twist1

[219]. In the absence of TAK1, p62 recruits RIP1 and mediates necrosome assembly in association with the autophagic machinery for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced necroptosis [220].

Casein kinase 2 (CK2) directly phosphorylates p62 at serine 403 within its UBA domain and regulates the selective autophagic clearance of ubiquitinated proteins by increasing p62 affinity to polyubiquitin chains [221]. p62 is also phosphorylated at serine 403 by a Sestrin2-ULK1 complex [222] or TANK-Binding Kinase 1 (TBK1) [223]. ULK1 also phosphorylates p62 at serine 407, which destabilizes the UBA dimer interface and increases the affinity of p62 to ubiquitin [224]. ULK1 was reported as potential prognostic biomarker for HCC [225].

3.5 p62 and endoplasmic reticulum (ER) stress

The ER is not only the site for synthesis, folding, and transport of secreted proteins, but it is also where sterols and other complex lipids are made and calcium is stored [226]. If protein and lipid biosynthesis exceed the processing capacity of the ER, three ER-linked defensive processes, including inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor (ATF) 6, which constitute the unfolded protein response (UPR), are activated [226]. Phosphorylated PERK which is induced by ER stress phosphorylates NRF2, leading to its dissociation from Keap1 and activation of the antioxidant response [227]. In addition, activated PERK-eIF2α increases expression of ATF4, which is an NRF2 dimerization protein [228] and also contributes to activation of the antioxidant response, as well as regulation of amino acid metabolism and autophagy, including p62 to promote cell survival [229, 230]. JNK activation also induces expression of p62 [231]. ER stress also results in induction of the antioxidant protein Sestrin2, which halts protein synthesis by inhibiting mTORC1 activation [232, 233]. N-terminal arginylation of immunoglobulin heavy chain binding protein (BiP, also known as GRP78) is induced by various stress signals, such as cytosolic misfolded proteins, together with autophagy, and arginylated BiP binds p62 via its N-terminal arginine which interacts with the ZZ domain of p62 [234]. This induces self-oligomerization and aggregation of p62 and promotes its interaction with LC3, resulting in targeting to autophagosomes and selective lysosomal degradation of both arginylated BiP and p62 and their associated cargos.

Abnormal protein aggregates, which commonly include p62, are pathognomonic features of chronic liver diseases, including HCC [235] and induce ER stress [236, 237], while ER stress induces p62 expression [75]. Functional CRISPR screening revealed that impairment of protein ufmylation elicits an ER stress response and increases p62 expression [238]. ER stress is also induced by high fat diet (HFD) or in leptin-deficient obese mice, resulting in p62 accumulation in mouse liver due to inhibition of selective autophagy at the autophagosome-lysosome fusion step [239, 240]. However, ER stress generally induces autophagy through regulation of mTORC1 and AMP-activated protein kinase (AMPK) signaling and expression of autophagy-related genes (*Atg, Becn1*), and ER stress-induced autophagy is considered as an essential protective mechanism during ER stress [241, 242]. HCV infection induces chronic ER stress and UPR in hepatocytes, which is associated with steatosis, cell death, and immune escape [190, 195, 243-249]. Upregulation of the UPR is

often observed in cancers, including HCC [250, 251], and may play important roles in HCC initiation and promotion [252]. However, due to the dual role of ER stress and the UPR in cell survival, apoptosis and autophagy, it is not clear whether these processes promote or inhibit tumor growth [253].

4. p62 and liver diseases

4.1 p62 and liver regeneration/injury

Autophagy plays an essential role in the preservation of cellular quality control and homeostasis during liver regeneration, which is severely suppressed after partial hepatectomy in liver-specific *Atg5*$^{-/-}$ mice [254]. NRF2 is highly activated in *Atg5*$^{-/-}$ mice due to p62 accumulation [255] and constitutive NRF2 activation in hepatocytes delays proliferation and induces apoptosis during liver regeneration [256]. So p62 may inhibit liver regeneration by activating NRF2, however, it is also reported that p62 expression is reduced after partial hepatectomy of mouse steatotic livers, causing more liver damage from oxidative stress and delayed regeneration mainly due to NRF2 inactivation [257]. Correspondingly, liver regeneration is significantly delayed after partial hepatectomy in *Nrf2*$^{-/-}$ mice [258]. Therefore, the role of p62 in control of liver regeneration after partial hepatectomy is still controversial, and needs to be further investigated.

In contrast to its deleterious effect on liver regeneration after partial hepatectomy, liver-specific *Atg5* deletion protects mice from acetaminophen (APAP)-induced liver injury by promoting NRF2 activation [255]. Conversely, *Nrf2*$^{-/-}$ mice are more sensitive to APAP-induced hepatotoxicity than wild-type mice [259], whereas liver-specific *Keap1*$^{-/-}$ mice, in which NRF2 is constitutively activated, are more resistant to APAP than control mice [260]. Constitutive NRF2 activation in myeloid cells also results in decreased liver damage, necrosis, apoptosis, inflammation, and oxidative stress in a mouse model of liver ischemia and reperfusion injury [261]. However, constitutive liver-specific NRF2 activation does not protect from carbon tetrachloride (CCl_4)-induced liver injury and fibrosis [256]. The role of p62 in liver injury remains to be investigated although p62 ablation in liver-specific *Tsc1*$^{-/-}$ mice prevented liver inflammation and fibrosis [34] and p62 plays a protective role against APAP-induced hepatocyte necrosis [262].

TRIM21, a RING finger domain-containing ubiquitin E3 ligase, ubiquitylates p62 at K7 via a K63-linkage, and prevents p62 dimerization and Keap1 sequestration [263]. Yet, *Trim21* null liver is protected from arsenic-induced liver damage [263]. Sestrin1 and Sestrin2 bind p62 and Keap1 and prevent oxidative liver damage by activating NRF2 [264].

4.2 p62 and non-alcoholic steatohepatitis (NASH)

Non-alcoholic fatty liver disease (NAFLD), which is closely associated with obesity, has become one of the most prevalent liver diseases worldwide. NAFLD includes simple hepatic steatosis and NASH, characterized by hepatic steatosis with inflammation, hepatocyte ballooning, presence of Mallory-Denk bodies (MDB) and fibrosis [265]. NASH-related HCC is significantly increasing and is a major indication for liver transplantation in the U.S. [266]. The ‘two-hit’ or ‘multiple-hit’ hypothesis was proposed to explain NASH

pathogenesis, according to which additional stress beyond the initial steatosis triggers fatty liver to NASH progression [267, 268]. In general, oxidative stress promotes NAFLD [269-275]. ER stress is also involved in NAFLD progression by regulating hepatic lipid metabolism [269, 276-279]. It was shown that some stresses, such as oxidative stress, lead to cell injury and death via apoptosis or necrosis, and that this is an important driver of inflammation and fibrosis in NASH [280-282]. Lipophagy, a specific type of macroautophagy that regulates lipid storage and metabolism in hepatocytes, is impaired in NAFLD as well as under conditions that predispose to NAFLD such as obesity and aging, suggesting a protective role of autophagy in the pathogenesis of NAFLD [60, 204, 283-288].

p62 aggregates are present in human NASH and mouse NASH models [34, 59, 60, 252]. p62 expression is much higher in NASH patients compared with patients with simple hepatic steatosis [60], and similar differences were found in mice [34]. Whole-body *Sqstm1*$^{-/-}$ mice develop mature-onset obesity due to ERK activation and enhanced adipogenesis [16, 289] although it was also reported that the absence of p62 in the brain causes hyperphagia and mature-onset obesity by modulating leptin signaling [290]. However, liver-specific *Sqstm1*, *Keap1* or *Nrf2* null mice or liver-specific constitutively active NRF2 transgenic mice show no apparent metabolic abnormalities despite altered expression of NRF2 target genes, suggesting that the p62-Keap1-NRF2 axis in hepatocytes does not control basal metabolism [38, 256, 260, 291]. Liver-specific *Sqstm1*$^{-/-}$ mice show no obvious liver abnormalities even if they are fed with HFD [291], although when this deficiency is introduced into a strain that develops NASH in response to HFD, it attenuates ballooning degeneration, ROS accumulation and fibrosis [34].

NRF2 suppresses expression of key enzymes involved in fatty acid synthesis [292-300]. p62-induced NRF2 activation protects cells from lipotoxicity induced by palmitic acid [301], which is the most abundant fatty acid in human serum [302] and an inducer of pro-inflammatory cytokines, including TNF, IL-6 and IL-8, and ROS production [303-305]. Together with the results described above, it appears that the p62-Keap1-NRF2 axis slows down NASH development (Figure 2b). Indeed, NRF2 activation protects mice from steatohepatitis [292, 293, 298, 300, 306-311]. NRF2 activators, such as the thiol-reactive agent oltipraz (OPZ) and NK-252 (1-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)-3-(pyridin-2-ylmethyl)urea), also attenuate the progression of NASH-related fibrosis in a rat model [312]. Liver-specific *Nrf1*$^{-/-}$ mice showed decreased expression of ARE-containing genes and developed hepatic steatosis and liver cancer [313].

p62 is also involved in insulin signaling [314]. p62 interacts with insulin receptor substrate (IRS)-1 upon insulin stimulation and promotes AKT activation, GLUT4 translocation to the membrane, and glucose uptake [314]. However, this remains to be established in liver-specific *Sqstm1*$^{-/-}$ mice. Growth factor receptor-bound protein 14 (Grb14), which binds p62 and is an inhibitor of insulin signaling, promotes liver lipogenesis through inhibition of p62-mediated NRF2 activation [315]. p62 increases hypoxia inducible factor (HIF)-1α levels and transcriptional activity by regulating mTORC1 and NF-κB signaling, thereby modulating glucose metabolism [316].

4.3 p62 and liver cancer

Adult primary liver cancer includes HCC and cholangiocellular carcinoma (CCC), and HCC is mainly caused by HBV or HCV infections, as well as NASH and alcoholic liver disease [266]. As mentioned above, p62 expression is elevated in HCC [29, 32-38] and high p62 in the surrounding non-tumor tissue is a strong predictor of HCC recurrence after radiofrequency ablation [34]. High NRF2 is also a potential prognostic marker for HCC [317]. *NFE2L2* (NRF2) and *KEAP1* mutations occur in approximately 5-15% of HCCs [118, 119, 123, 124] [318], but no *SQSTM1* mutations have been detected. Of note, *NFE2L2* or *KEAP1* mutations are not detected in human premalignant lesions that accompany liver cirrhosis [319], although *Nfe2L2* or *Keap1* mutations already occur in 70% of early chemically-induced premalignant lesions in rat liver [320]. Since p62 is overexpressed in most of human premalignant lesions of HCCs, due to autophagy inhibition and/or increased *Sqstm1* mRNA expression [33], p62 accumulation is probably a main driver of NRF2 activation in early HCC, rather than *NFE2L2* or *KEAP1* mutations.

As mentioned above, complete inhibition of autophagy by *Atg5* or *Atg7* deletion in mouse liver causes only benign liver adenoma [205]. By contrast, liver-specific $Tsc1^{-/-}$ mice develop spontaneous HCC due to mTORC1 activation, subsequent partial inhibition of autophagy and p62 accumulation [199], suggesting that a low level of basal autophagy is needed for malignant conversion of hepatic adenomas. Partial inhibition of autophagy also occurs in simple hepatic steatosis and NASH [60]. HCC development in liver-specific $Tsc1^{-/-}$ mice is suppressed by p62 ablation or by treatment with rapamycin, an mTOR inhibitor [34, 199].

In normal adult liver, hepatocytes divide only rarely and therefore inflammation and repetitive liver injury are needed for HCC development by inducing hepatocyte regeneration and compensatory proliferation [156]. Many HCC-initiating cells usually die due to excessive oxidative stress during the process and cannot keep accumulating driver mutations. We found that p62 overexpression by adenovirus-associated virus (AAV) induces activation of NRF2 and mTORC1, as well as c-Myc and is sufficient for HCC initiation in mice [34]. A point mutation in the p62 KIR domain, which prevents Keap1 binding, blocks NRF2 activation and abolishes p62 oncogenic activity, underscoring the importance of NRF2 activation for HCC development. Using four different mouse HCC models of distinct etiologies, DEN-induced HCC, mTORC1-driven HCC in liver-specific $Tsc1^{-/-}$ mice and NASH-induced HCC in *MUP-uPA* transgenic mice or streptozotocin (STZ)-HFD-treated mice, we found that p62 accumulation in hepatocytes promotes HCC development and NRF2 activation [34]. p62-promoted NRF2 activation in HCC-initiating cells allows these cells to survive oxidative stress and continuously accumulate oncogenic mutations, leading to HCC initiation and progression (Figure 2c). However, overexpression of constitutive active NRF2 in liver or NRF2 activation by liver-specific *Keap1* deletion does not induce HCC without further support [256, 260]. In addition to NRF2 activation, other pathways induced by p62, such as NF-κB, mTORC1, phosphoinositide-3-kinase (PI3K)-AKT or Wnt-β-catenin might synergize with NRF2 to promote HCC development [57, 69, 124, 165, 321, 322].

p62 as well as keratins 8 and 18 (K8/18) and ubiquitin are major components of MDB, intracellular hyaline bodies (IHB) and hybrid inclusions [37, 58], which are hallmarks of chronic liver diseases, including viral hepatitis (HBV, HCV), NASH, ASH, Wilson disease, hemochromatosis and primary biliary cirrhosis (PBC), all of which greatly increase HCC risk [323]. In addition, p62-containing aggregates are present in nearly 50% of surgical HCC specimens [58], but their significance for HCC development had remained unknown. We discovered that overexpression of a p62 deletion mutant lacking the UBA domain (p62 ΔUBA), which cannot form any aggregates, can still induce HCC when overexpressed in mouse liver [34], suggesting that formation of MDB, IHB and hybrid inclusions is not the cause of HCC development.

Tribbles homolog 3 (TRB3), a stress and metabolic sensor, is highly expressed in human HCC and its expression is positively correlated with poor prognosis [324]. TRB3 interacts with p62 and inhibits p62 binding to LC3 and ubiquitinated substrates, leading to p62 accumulation and tumor promotion [324]. Interestingly, a recent paper reported that both mTORC1 and autophagy regulate the stochastic phase of somatic cell reprogramming to induced pluripotent stem (iPS) cells by regulating p62 levels [325]. NRF2 also regulates the metabolic shift from oxidative to glycolytic energy production during iPS cell reprogramming by promoting HIF-α activation [326] and induces expression of the rate-limiting pentose phosphate pathway (PPP) enzyme glucose-6-phosphate dehydrogenase (G6PD) in HCC [327]. Since reprogramming of somatic cells to iPS cells shares much similarity with cancer initiation [328], it is possible that cancer-initiating cells use the same pathways to acquire malignant properties. Indeed, phosphorylated p62 promotes malignancy of HCV-positive HCC through NRF2-dependent metabolic reprogramming [38]. A more recent paper reported that nuclear-localized p62 which accumulates due to autophagy deficiency directly binds to and inhibits nuclear RNF168, an E3 ligase, which is essential for H2A ubiquitination and DNA damage responses, resulting in impairment of DNA double-strand breaks (DSBs) repair [329, 330]. This represents a new mechanism through which p62 can initiate and promote tumorigenesis.

5. Conclusions and future prospects

p62 expression is elevated in response to a variety of cellular stresses, especially those that result in impaired autophagic flux. By activating NRF2, accumulation of p62 triggers the protective antioxidant response. However, chronic p62 accumulation and NRF2 activation play important roles in the initiation and progression of HCC [34]. Therefore, future research should be focused on pharmacological methods to inhibit p62 accumulation and/or chronic persistent NRF2 and mTORC1 activation in HCC. NRF2, NF-κB and/or mTORC1 inhibitors may suppress p62 accumulation in addition to inhibiting their targets because *SQSTM1* expression is regulated by both NRF2 and NF-κB and mTORC1 activation induces p62 accumulation. Since the interaction between phosphorylated p62 and Keap1 is very important for NRF2 activation and HCC induction [33, 38, 187], inhibitors of this interaction may be useful in HCC prevention in high-risk individuals. Such inhibitors may alter the binding of p62 to Keap1 or prevent its phosphorylation. Recently, it was reported that K67, a specific inhibitor for Keap1 and phospho-p62 interaction, suppresses cell proliferation and drug resistance in HCC [38]. Another important pro-tumorigenic pathology

activated by p62 is mTORC1, which stimulates cancer cell anabolism and leads to upregulation of c-Myc [34, 64]. The interaction between p62 and Raptor may also be targeted as another HCC preventive strategy. Inhibitors of p38δ kinase or the interaction between phosphorylated p62 and TRAF6 may also be able to prevent HCC initiation and p62-induced mTORC1 activation. However, p62 expression in epithelial cells promotes tumorigenesis, while p62 expression in stromal fibroblasts inhibits tumorigenesis [64]. Therefore, ample consideration should be given when p62-targeted therapies are developed.

More importantly, both p62 and NRF2, as well as mTORC1, play key homeostatic roles. Whereas transient activation of NRF2 protects cells, especially hepatocytes, from oxidative stress and environmental toxins, its persistent activation upon dysregulated p62 accumulation, due to long-term impairment of autophagy, promotes development of HCC and numerous other cancers. Any future therapeutic or preventive strategy should take this into account and include inhibition of persistent NRF2 and mTORC1 activation, without reducing their basal activity.

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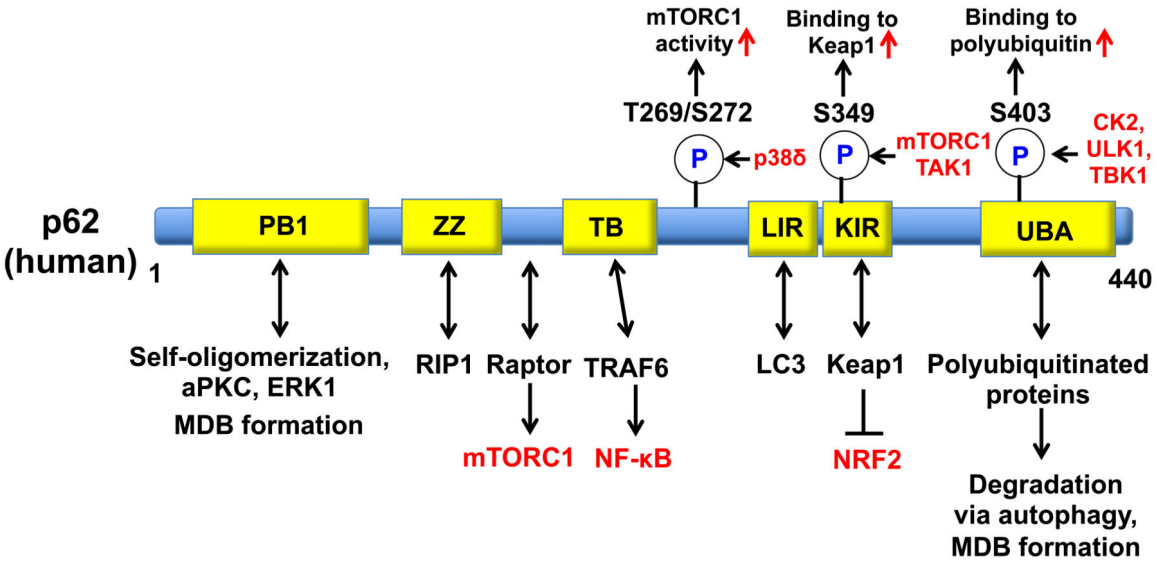

Figure 1. Schematic representation of p62 structure and functional organization

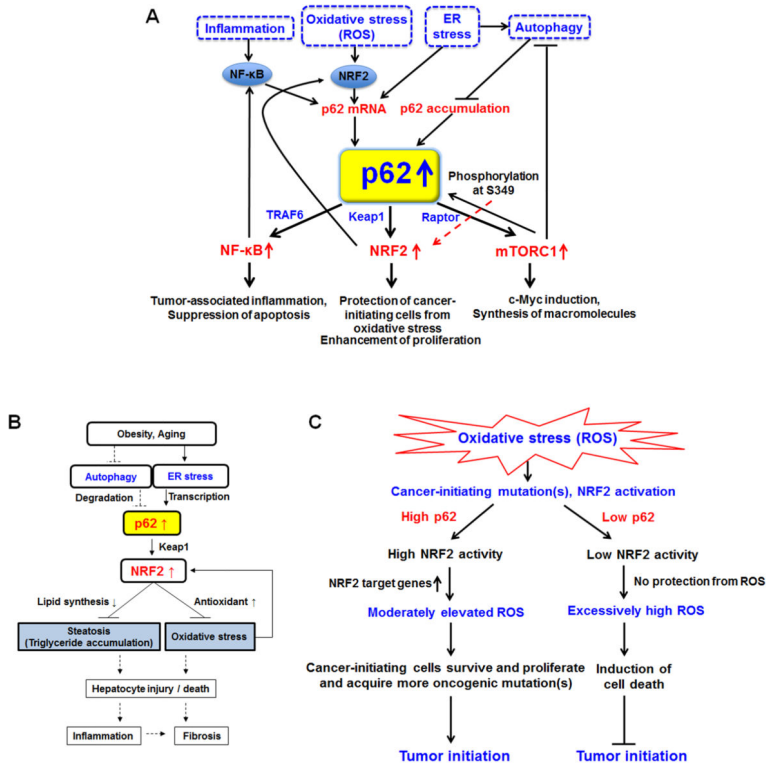

Figure 2. (a-c) Pathways through which p62 promotes liver tumorigenesis (a, c) and regulates NASH (b)