

**Dissertation**

**Functional analysis of the role of p62/Sequestosome-1 in  
protein aggregation diseases**

Submitted by

**M.Res**

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# Declaration

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I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Parts of this thesis are based on the following original publication that are referred to in the text wherever required:

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25<sup>th</sup> October 2016

Pooja Lahiri

*To*

*My Grandfather*

*Shri. Sisir Kumar Bhattacharya*

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# Zusammenfassung

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Der Umgang von Zellen mit fehlgefalteten Protein ist von signifikanter Bedeutung in einer Vielzahl von Erkrankungen, die durch die Akkumulierung abnormaler Proteinaggregate gekennzeichnet und zusammenfassend als "Proteinaggregationserkrankungen" bezeichnet werden. Diese beinhalten unter anderem neuronale Lewy-Körper in der Parkinson'schen Krankheit, neurofibrilläre Ablagerungen in der Alzheimer'schen Krankheit, TDP-43-Aggregate in amyotropher Lateralsklerose und Mallory-Denk-Körper (MDBs) in Steatohepatitis und anderen chronischen Lebererkrankungen. Als Ausdruck ihrer gemeinsamen Pathogenese zeigen diese Einschlüsse, neben dem krankheitsspezifischen Protein-“Rückgrad”, häufig Analogien in ihrer molekularen Zusammensetzung, insofern sie alle das multifunktionale Stressadaptorprotein p62/Sequestosome-1 und Ubiquitin als konstante Bestandteile enthalten. Darüber hinaus teilen diese pathologischen Aggregate die strukturelle Gemeinsamkeit einer (amyloid-ähnlichen) Cross- $\beta$ -Faltblatt-Konformation. Der Fokus dieser Studie liegt auf der Funktion von p62 in der Bildung von MDBs. Diese sind die am besten studierten, hepatozellulären Proteineinschlüsse in zahlreichen chronischen Lebererkrankungen, insbesondere Steatohepatitis, chronischer Cholestase und hepatozellulärem Karzinom.

Um die Rolle von p62 in der Bildung von MDBs zu erforschen, haben wir Knockout-Mäuse generiert, bei denen p62 im gesamten Körper (p62<sup>-/-</sup>) oder spezifisch in Hepatozyten (p62<sup>hep</sup><sup>-/-</sup>) nicht exprimiert wird, und diese DDC ausgesetzt, um die Bildung von MDBs zu induzieren. Die untersuchten Schlüsselfragen waren, ob p62 die Bildung von MDBs initiiert, verstärkt oder inhibiert und deren Stabilität und Degradation beeinflusst. Ein weiterer Aspekt dieser Studie war es zu verstehen, wie p62 ähnlich wie bei Autophagy-Defizienz die Leberschädigung im DDC-Modell moduliert. Außerdem wurde untersucht, wie die Domänen von p62 die Bindung unterschiedlicher Komponenten der Aggregatbildung regulieren.

In Abwesenheit von p62 entstanden kleine und granuläre MDBs, die nicht in der Lage waren, zu größeren und kompakteren MDBs heranzureifen. Das Fehlen von p62 beeinträchtigte die Bindung anderer Proteine wie NBR1, Hsp25 und K17 an die MDBs. Darüber hinaus veränderte

der Verlust von p62 die zellulären Verteidigungsmechanismen durch das Herunterregulieren von Nrf2-Zielgenen während chronischer Schädigung. Während der Erholung sank die Anzahl an p62-freien MDBs rapide mit einer gleichzeitigen Abnahme vernetzter, ubiquitinierten Proteine. Weiters haben wir eine funktionell relevante zweite Isoform des humanen p62 mit fehlender PB1-Domäne identifiziert. Das Fehlen der SH2-Region der PB1-Domäne in dieser zweiten Isoform führt zur Ausbildung einer Cross- $\beta$ -Faltblatt-Struktur, die es p62 ermöglicht, ohne Ubiquitin progressiv Aggregate mit primären Komponenten von Proteininklusionen zu bilden.

Die Ergebnisse deuten auf eine essentielle Rolle von p62 in der Reifung und Stabilisierung von MDBs hin. Die Daten zeigen, dass Keratine die zentralen Proteine in der Bildung von MDBs sind, die über zwei unterschiedliche Wege zur Bildung p62-enthaltender Proteinaggregate erfolgen kann. Der erste Weg steht in Verbindung zu Steatohepatitis in Menschen und Modellen medikamenteninduzierter Hepatotoxizität, bei denen Keratin-Aggregation die Bildung von MDBs initiiert und die Bindung von p62 dann weiter zu Reifung und Stabilisierung der Aggregate führt. Der zweite Weg beinhaltet die Transition von p62-hältigen intrazellulären Hyalinkörpern (IHBs) zu MDBs durch die Aufnahme unüblicher Keratine in eine bereits bestehende Matrix aus aggregierten p62, wie dies in idiopathischer Kupfertoixikose und einigen Fällen hepatozellulärer Karzinome beobachtet werden konnte. Weiters bieten die interessanten neuen Erkenntnisse, dass eine natürliche Variante von p62 eine (amyloid-artige) Cross- $\beta$ -Faltblatt-Konformation annehmen und in Abwesenheit von Ubiquitin progressiv Aggregate bilden kann, einen neuen Ansatzpunkt zur Erforschung der Rolle von p62 in unterschiedlichen Proteinaggregationserkrankungen.

# Abstract

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How cells deal with misfolded proteins gained significant relevance in a variety of diseases characterized by accumulation of abnormal protein aggregates collectively known as “protein aggregation diseases”. They include among others, neuronal Lewy bodies in Parkinson’s disease, neurofibrillary tangles in Alzheimer’s disease, TDP-43 aggregates in amyotrophic lateral sclerosis, and Mallory-Denk bodies (MDBs) in steatohepatitis as well as other chronic liver disorders. As a reflection of a common pathogenesis, these inclusions often display, in addition to their disease-specific protein “backbone”, an analogous molecular composition, namely they all contain the multifunctional stress adaptor protein p62/Sequestosome-1, and ubiquitin as constant constituents. Moreover, these pathological aggregates share the common structural property of cross  $\beta$ -sheet (amyloid-like) conformation. Therefore, the investigation of these common structural and molecular composition are of paramount importance as they may provide new evidence on how misfolded proteins and the factors involved in aggregation machinery regulates protein aggregate formation, its clearance, and stress-induced cellular toxicity. In this study, we focused on the function of p62 in the formation of MDBs. MDBs are the best studied hepatocytic protein inclusions found in several chronic liver diseases, especially in steatohepatitis, chronic cholestasis, and hepatocellular carcinoma.

To delve into the role of p62 in MDB formation, we have generated p62 total (p62<sup>-/-</sup>) and hepatocyte-specific (p62<sup>hep</sup><sup>-/-</sup>) knockout mice and intoxicated them with DDC to induce MDBs. Key questions which are investigated are whether p62 initiates, enhances or inhibits MDB formation and influences their stability and degradation. Another aspect of this study was to comprehend how p62 modulates liver injury in the DDC model similar to that observed in autophagy deficiency. Furthermore, how p62-domains regulate the binding of various components for the formation of aggregates were also evaluated.

In the absence of p62, small and granular MDBs appeared, which failed to mature to larger and compact MDBs. The lack of p62 impaired the binding of other proteins such as NBR1, Hsp25 and K17 to MDBs. Moreover, loss of p62 altered the cellular defense mechanism by

downregulation of Nrf2 target genes during chronic injury. Upon recovery, there was a rapid numerical reduction of MDBs lacking p62 with a parallel decrease of cross-linked ubiquitinated proteins. Additionally, we identified a functionally relevant second isoform of human p62 lacking the PB1 domain. The absence of SH2 region of PB1 domain in the second isoform led to the acquisition of cross  $\beta$ -sheet structure that enabled p62 to progressively form aggregates with primary components of protein inclusions without ubiquitin.

The results indicate that p62 is essential for the maturation and stabilization of MDBs. The data highlight that keratins are the central proteins in MDB formation that proposes two different pathways of p62 containing protein aggregate formation in the liver. The first pathway is linked to human steatohepatitis and drug-induced hepatotoxicity models where keratin aggregation initiates MDB formation, and attachment of p62 then leads to maturation and stabilization of the aggregates. The second pathway involves a transition of p62-containing intracellular hyaline bodies (IHBs) into MDBs by incorporation of abnormal keratins in a pre-existing matrix of aggregated p62 as observed in idiopathic copper toxicosis and a few cases of hepatocellular carcinomas. Furthermore, the interesting new finding that a natural variant of p62 acquire cross  $\beta$ -sheet conformation (amyloid-like), and can progressively form aggregates in the absence of ubiquitin provide a new approach to study the role of p62 in various protein aggregation diseases.

# Abbreviation

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|                                                 |    |
|-------------------------------------------------|----|
| AD                                              |    |
| Alzheimer's disease .....                       | 98 |
| AP-1                                            |    |
| Activator protein.....                          | 15 |
| ARE                                             |    |
| Antioxidant-response element .....              | 22 |
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# Introduction

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## 1.1. Overview of protein folding and misfolding

Proteins are polymeric chains of amino acids synthesized on ribosomes based on genetic information. They are responsible for almost all biological processes inside the cell and the most fundamental function of a living cell is to fold the proteins into three-dimensional structures to make it functionally active (Figure 1) (Dobson, 2003, Aguzzi and O'Connor, 2010). This folding process of a protein is a high precision event orchestrated by biological macromolecules called molecular chaperones that are present in all types of cells and cellular compartments. The majority of the proteins undergo folding in the cytoplasm, and a small fraction of proteins fold in specific compartments of endoplasmic reticulum or mitochondria. Mostly, chaperones work cohesively at each and every step of folding to ensure efficient folding process (Tyedmers et al., 2010). This results in the formation of a myriad of highly specific structures which brings key functional molecules to work together leading to selective trafficking of molecules to specific cellular locations for maintaining cellular growth and differentiation (Dobson, 2003, Jahn and Radford, 2005).

Conversely, proteins do not always fold correctly despite the presence of quality control mechanisms. Adverse conditions such as proteotoxic stresses, mutations in the newly synthesized proteins or translational errors can cause protein misfolding. The quality control mechanism can adapt to the severity of protein damage by increasing the production of more chaperones and proteases. However, when the levels of misfolded proteins exceed the capacity of protein repair mechanism and degradative capacity of cell, protein aggregates start to accumulate (Tyedmers et al., 2010). Based on electron microscopy, a protein aggregate can be classified into amorphous and amyloid-like aggregates. Computer simulations and biophysical measurements reveal that  $\beta$ -sheet conformation is a common feature of both types of aggregates. The degree of  $\beta$ -sheet arrangement varies from aggregate to aggregate (with highest one present in amyloid fibrils) and is an indirect measure of the time required for the folding process (Dobson, 2003, Chiti and Dobson, 2006, Fandrich, 2007, Nelson and Eisenberg, 2006).

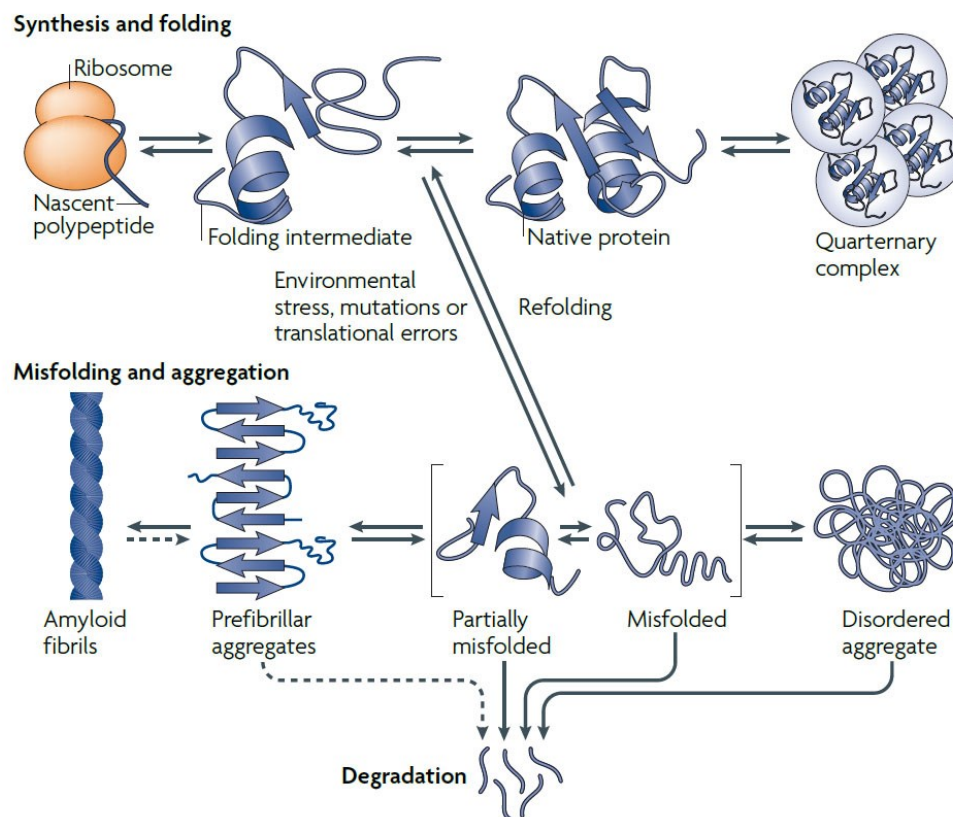


**Figure 1. Overview of protein aggregation process.**

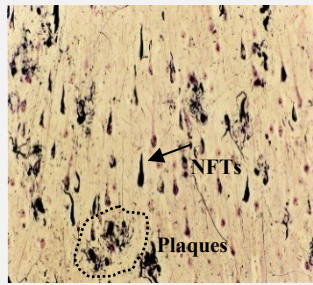
Newly synthesized proteins from ribosomes folds through various intermediates to its functional three-dimensional structure. Certain types of stress such as translational errors, mutations in the newly synthesized proteins and environmental stress leads to protein misfoldings. Misfolded proteins can be refolded to its native state through quality control mechanism generally orchestrated by chaperones or degraded by proteasomal machinery. Once the quality control mechanisms are overwhelmed, protein aggregates start to accumulate. These protein aggregates can acquire various conformations ranging from mostly disordered aggregates to prefibrillar species and highly ordered  $\beta$ -sheet-rich amyloid fibrils. Arrows indicate a process that can include several single steps.

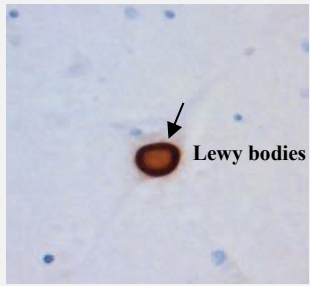
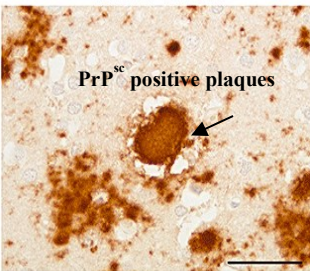
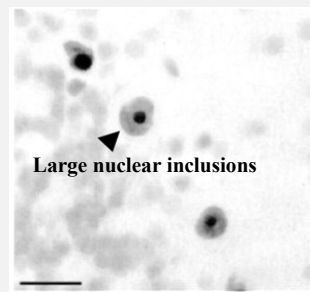
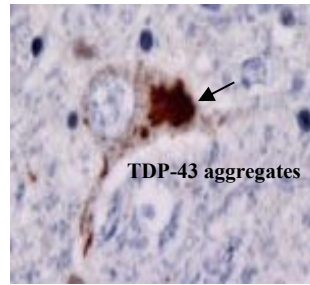
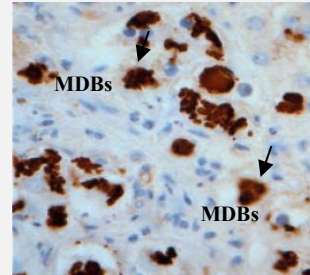
Adapted from (Tyedmers et al., 2010)

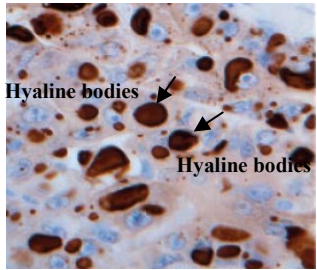
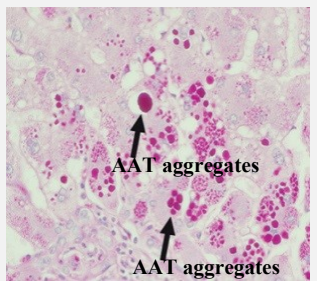
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It is now becoming apparent that a huge variety of previously unrelated diseases, share the pathological feature of aggregated misfolded protein deposits (Dobson, 2001) and collectively known as “protein aggregation diseases”. They range from dementia and age-related diseases such as Alzheimer’s disease and Parkinson’s disease to metabolic syndromes such as Steatohepatitis and Type-II diabetes (Zatloukal et al., 2007). The main proteins/peptides associated with this variety of protein deposits have no obvious similarities in primary amino acid sequence and compositions, size or structure (Dobson, 2003, Kopito and Ron, 2000). A summary of protein aggregates found in various disease is illustrated in Table 1. Most of these primary proteins seem to potentially fold into amyloid fibrils containing highly organized cross  $\beta$ -sheet conformation and acquire marked similarities in their internal structure and external morphology. Such findings support the link between the occurrence of protein aggregation and clinical manifestation of the disease (Sherman and Goldberg, 2001, Chiti and Dobson, 2006). Although, a great deal of diagnostic information regarding these diseases has emerged in the last decades but much remains to be investigated on the “proteins” which aggregate and how they affect the functions of associated organs.

**Table 1. Summary of protein aggregation in various diseases**

| <b>Disease</b>                                   | <b>Protein</b>                  | <b>Locus</b>                                                            |                                                                                       |
|--------------------------------------------------|---------------------------------|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| <b>Alzheimer's disease</b>                       | Amyloid $\beta$ -protein<br>Tau | Extracellular<br>plaques,<br>Neurofibrillary<br>tangles in<br>cytoplasm |  |
| <b>Frontotemporal dementia with Parkinsonism</b> | Tau                             | Neurofibrillary<br>tangles<br>in cytoplasm                              |                                                                                       |

|                                                                                             |                                                  |                                                             |                                                                                       |
|---------------------------------------------------------------------------------------------|--------------------------------------------------|-------------------------------------------------------------|---------------------------------------------------------------------------------------|
| <b>Parkinson disease, dementia with Lewy bodies</b>                                         | $\alpha$ -syncuclein                             | Lewy Bodies in neuronal cytoplasm                           |    |
| <b>Creutzfeldt-jakob disease, mad cow disease</b>                                           | Prion Protein (PrP) <sup>sc</sup>                | Extracellular plaques, oligomers inside and outside neurons |    |
| <b>Polyglutamine expansion diseases, e.g. Huntington's disease, spinocerebellar ataxias</b> | Long glutamine stretches within certain proteins | Neuronal nuclei and cytoplasm                               |   |
| <b>Amyotrophic lateral sclerosis</b>                                                        | TDP-43                                           | TDP-43 aggregates; Neuronal cytoplasm                       |  |
| <b>Steatohepatitis</b>                                                                      | Keratin + p62                                    | Mallory-Denk bodies; cytoplasmic inclusions of hepatocytes  |  |

|                                                                                     |                         |                                                       |                                                                                     |
|-------------------------------------------------------------------------------------|-------------------------|-------------------------------------------------------|-------------------------------------------------------------------------------------|
| <b>Hepatocellular carcinoma, Idiopathic copper toxicosis</b>                        | p62                     | Hyaline bodies; cytoplasmic inclusions of hepatocytes |  |
| <b><math>\alpha</math>-1 antitrypsin (AAT) deficiency-associated liver diseases</b> | $\alpha$ -1 antitrypsin | AAT aggregates; Hepatocytes                           |  |

Aggregates of various diseases are represented by arrows followed by the aggregate name. Image for neurofibrillary tangles is taken from (Janssen et al., 2000). Image for polyglutamine expansion aggregates is taken from (Michalik and Van Broeckhoven, 2003). Image for TDP-43 aggregates is taken from (Mackenzie and Rademakers, 2008). The images of Lewy bodies, Mallory-Denk body and Hyaline bodies are taken from (Zatloukal et al., 2002). Image for AAT aggregates is taken from (Strnad et al., 2013). Image for PrP<sup>sc</sup> positive plaques is taken from (Wadsworth et al., 2010). All the cited images have copyright license for reuse.

.....

The formation of protein aggregates with high degree of amyloid fibrils are often associated with enhanced cellular toxicity and cell death in various diseases, whereas the sequestration of such assemblies into compact aggregates ready for degradation might serve to counteract the cellular toxicity (Nilsberth et al., 2001, Bergeron et al., 1996, Sousa et al., 2001). This sequestration of protein aggregates are carried out by various cellular processes such as ubiquitination of aggregated proteins by stress-adaptors or microtubule-dependent transport, routing them to proteasomal/autophagic clearance (Moscat and Diaz-Meco, 2009b, Tyedmers et al., 2010). Hence, to better understand the cellular reactions elicited by the accumulation of protein aggregates, it is necessary to search for common proteins that might have the ability to directly interact with aggregated proteins of interest.

p62/Sequestosome-1 is one such stress and adaptor protein, which is found to be a constant component of various protein aggregates across diseases such as neuronal Lewy bodies in Parkinson's disease, neurofibrillary tangles in Alzheimer's disease, TAR DNA-binding protein-43 (TDP-43) aggregates in amyotrophic lateral sclerosis, and Mallory-Denk bodies (MDBs) in steatohepatitis as well as other chronic liver disorders (Figure 2) (Zatloukal et al., 2002, Tanji et al., 2012). Despite the emerging view of common mechanisms, wide gaps in our understanding of protein aggregation phenomena remain, especially the chasm between in vitro-based models and observations made in human diseases. In this thesis, we have investigated the functional relevance of p62 in the context of MDB formation using drug-induced mouse models of liver toxicity.

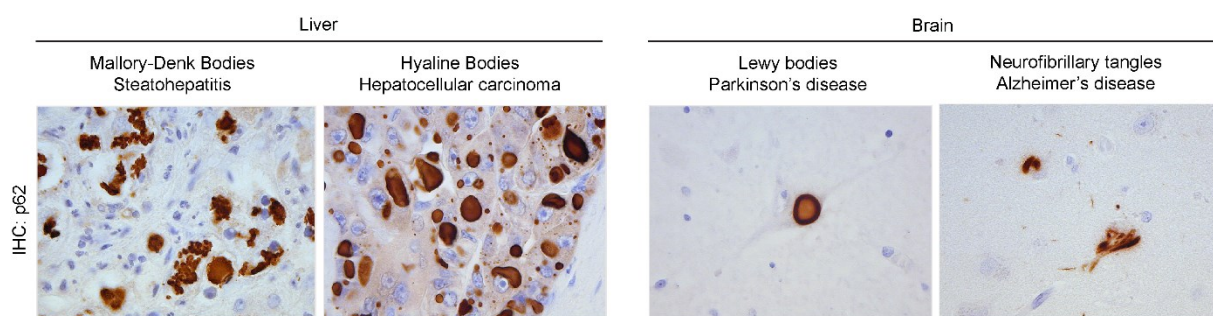


**Figure 2. p62 is a common denominator of cytoplasmic inclusions in the liver and neurodegenerative diseases.**

Immunohistochemical detection of p62 in MDBs of steatohepatitis, hyaline bodies of hepatocellular carcinoma, Lewy bodies of Parkinson's disease and neurofibrillary tangles of Alzheimer's disease. Modified from (Zatloukal et al., 2002).

## 1.2. p62/Sequestosome-1: Expression and Regulation

p62 is a conserved, ~62 KDa protein, which was originally identified as a phosphotyrosine ligand of src homology 2 (SH2) domain of p56<sup>lck</sup> from humans (Joung et al., 1996, Park et al., 1995). In the following years, the biological function of p62 has been studied in diverse systems and processes such as osteoclastogenesis, inflammation, protein aggregation and obesity. The official approved gene name of p62 is SQSTM1 (sequestosome-1). Alternative common names

of p62 are “aPKC-interacting protein p62”, “ubiquitin-binding protein p62”, “zeta-interacting protein” (Sqstm1 ZIP), and “A170” (Sqstm1 A170). The latter two is associated with rat and mouse p62 respectively (McGarvey et al., 2015).

In human, mouse and rat genome, p62 mRNA is transcribed from a single gene (Human SQSTM1; Gene ID: 8878, Mouse Sqstm1; Gene ID: 18412, Rat Sqstm1; Gene ID: 113894) consisting of 8 exons. p62 mRNA is ubiquitously expressed in humans and mouse as consistent with the study in rodents (Joung et al., 1996, Puls et al., 1997, Gong et al., 1999, Okazaki et al., 1999, Croci et al., 2003). p62 acts as an immediate early response gene and the expression is rapidly upregulated by various stimuli including oxidative stress (Ishii et al., 1996, Ishii et al., 1997), proteasomal inhibitors (Ishii et al., 1997) and differentiation inducing factors like nuclear growth factor (NGF), phorbol 12-myristate 13-acetate (PMA),  $\text{Ca}^{2+}$  ionomycin and interleukin 3 (IL3) (Okazaki et al., 1999, Gong et al., 1999, Lee et al., 1998). The proximal promoter region of p62 extending into its first exon has high content of CpG islands, a characteristic feature of highly regulated genes essential for cellular endurance (Bird, 1986, Vadlamudi and Shin, 1998). Furthermore, the 5' promoter region of p62 in human and mouse genes contain binding sites for transcription factors including activator protein (AP-1), specificity protein 1 (Sp1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), c-myc, Ets-1 family, nuclear factor erythroid factor 2 (NRF2), myosin differentiation (MyoD), and C/EBP (CCAAT-enhancer-binding proteins), possibly accounting for the sensitivity of p62 expression to the above-mentioned inducing factors (Vadlamudi and Shin, 1998, Okazaki et al., 1999). Also, the rapid expression of p62 seems to be co-regulated by transient mRNA stabilization and continuous activation of transcription (Lee et al., 1998).

### 1.3. Functions of p62

The eight exons encode for a full-length p62 protein of 440 aa for humans, 442 aa for mouse and 439 aa for rodents. The p62 protein is highly conserved across various species consisting of many functional domains and motifs. At its N-terminal, p62 consists of an evolutionary conserved Phox and Bem1 (PB1; aa. 21-103) domain followed by a zinc finger (ZZ; aa. 128-163) domain, that aids p62 to interact with proteins involved in various essential signaling pathways. On the other hand, the C-terminal harbors two very important domains, the LC3

interacting region (LIR; aa. 321-345) and ubiquitin associated (UBA; aa. 321-345) domain. These domains contribute to the non-covalent binding of p62 to ubiquitinated proteins and helps p62 to function as an adaptor between autophagy and protein ubiquitination (Figure 3) (Geetha and Wooten, 2002, Moscat et al., 2007, Seibenhener et al., 2004).

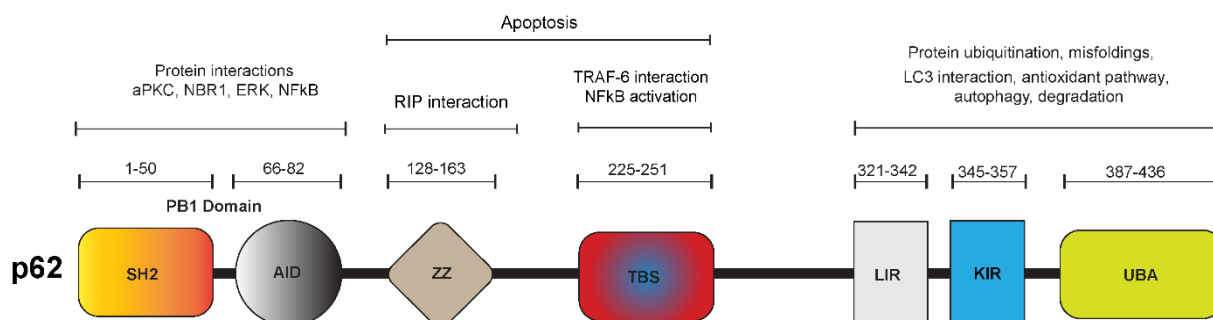


**Figure 3. Structural organization of p62 protein.**

Protein structure of p62 showing its various domains and their positions in the sequence. N-terminal of p62 consists of PB1, ZZ and TBS domains that are required for protein-protein interaction, whereas, C-terminal UBA domain and, LIR and KIR motifs place aids p62 to play a regulatory role in protein aggregation and degradation pathways. Functions of the domain are indicated above the domains. PB1, Phox-1 and Bemp-1 domain; SH2, serine homology 2; AID, acid interacting region; ZZ, zing finger domain; TBS, TRAF-6 binding sequence; LIR, LC3 interacting region; KIR, keap-1 interacting region; UBA, ubiquitin-associated domain.

### 1.3.1. Cross talk between cellular strategies of controlling protein aggregation in a p62-dependent manner

The transition of an unfolded protein to its folded states often results in partially folded intermediates that have a strong propensity to aggregate. The misfolded proteins once deposited in the cytoplasm or extracellular space could cause cell damage and enhance the deleterious effects of stress situations. Hence, modulation of stress responses and efficient management of misfolded protein aggregates is essential for cell survival and maintenance. The protein quality control system works at multiple levels. Firstly, the **chaperone machinery** (Figure 4) generally including the heat shock proteins Hsp 25, 60 and 70 augments the transformation of unfolded proteins into their functional 3-D structure and promotes the clearance of proteins which fails

to refold (Fink, 1999). The formation of pathogenic protein aggregates suggests a lack of molecular chaperones usually due to the formation of long-lived misfolded intermediates or rapid rate of protein synthesis or combination of both. These misfolded proteins are then recognized and gets polyubiquitinated by specific E3 ubiquitin ligases and finally ends up in proteasome for degradation (Cyr et al., 2002).

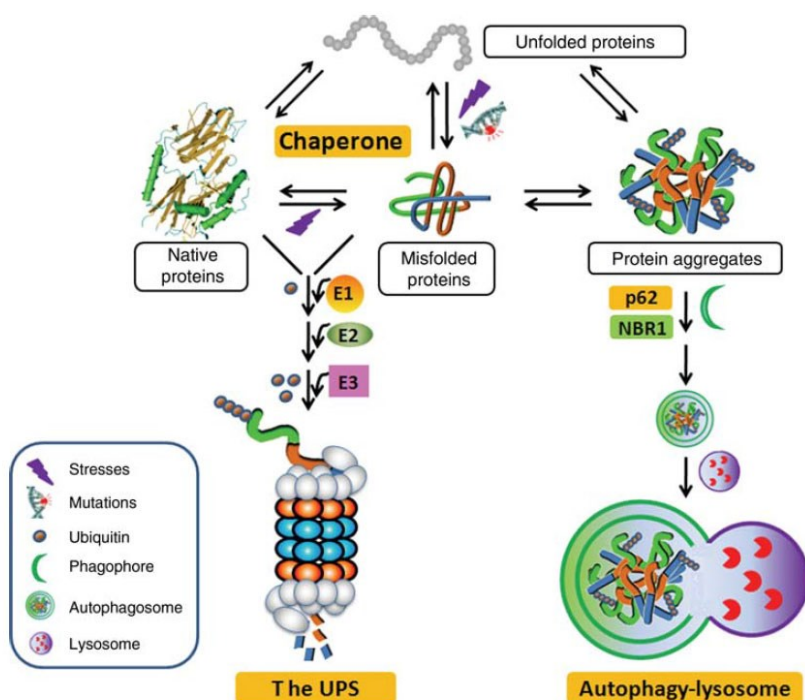
**Proteasomal degradation or ubiquitin proteasomal system (UPS)** (Figure 4) is a highly selective process of degradation. The covalently linked ubiquitinated substrates (misfolded proteins) serve as recognition motifs which enable their delivery to 26S proteasome subunit. 26 proteasome consists of a 20S proteolytic cylinder and a 19S regulatory particle. The multimerization of ubiquitin moieties mostly occurs through linkages between their lysine residues (e.g. K48) bound to the 19S particle. This leads to the unfolding of the substrate and removal of ubiquitin (Lecker et al., 2006). Often, the misfolded proteins are not efficiently cleared/unfolded by the proteasomal system. It becomes difficult for the large misfolded proteins to pass through the narrow barrels of proteasomes where polypeptides are digested. As a matter of fact, the accumulation of larger amounts of misfolded proteins may obstruct proteasomal activities by congesting the proteasomes, resulting in further accumulation of misfolded proteins rendering the cells more prone to aggregation and cellular damage (Snyder et al., 2003, Yao, 2010). In recent studies, p62 was shown to bind non-covalently to K63 linked substrates via its UBA domain and proteasomal subunits Rpn10/S5a and Rpt1p62's via PB1 domain (Seibenhener et al., 2004). This controversial observation suggests that p62 can act as a shuttling factor for K63 polyubiquitinated substrates for degradation. There are also several reports which suggest that p62 not only binds to the ubiquitinated substrates but also competes for the binding with ubiquitinated substrates with other proteins (e.g., p97) (Korolchuk et al., 2009, Bjorkoy et al., 2005). In certain cases, p62 can get self-ubiquitinated and form a cargo with already ubiquitinated aggregates, may promote the aggregation of large mature aggregates which surpass proteasomal degradation. The biological significance of this aggregate formation remains unclear, although aggregates could have a potential cytoprotective function by sequestering otherwise toxic misfolded oligomers. Moreover, p62 act as “trash collector” and further present the aggregates to the phagophore for autophagic degradation (selective autophagy) providing a **cross-talk between the proteasomal system and autophagy** (Korolchuk et al., 2009, Bitto et al., 2014).

In the most characterized version of **autophagy** (Figure 4), macroautophagy, nutrient depletion induces the formation of double-membrane structures called autophagosomes containing the sequestered components such as large organelles including ribosome and mitochondria. Autophagosomes later fuse to lysosomes and the sequestered products get cleared by lysosomal enzymes. Autophagy was always considered as a random and not selective process of degradation of macromolecules (Mizushima et al., 2008, Ohsumi, 2001). Interestingly, growing lines of evidence on the involvement of the specific ubiquitin binding factors in the selective selection of polyubiquitinated proteins and detection of protein aggregates inside the autophagosome suggested a selective form of autophagy (Johansen and Lamark, 2011). Recent studies on adaptor proteins indicate that p62 transport the ubiquitinated cargo to autophagosomes and can itself get degraded by autophagy (Bjorkoy et al., 2005, Pankiv et al., 2007). Johansen's and Komatsu's group showed that p62 directly interacts with microtubule-associated protein 1A/1B-light chain 3 (LC3) during the formation of the autophagosomes (via LIR present between the zinc finger domain and UBA domain of p62 (Bjorkoy et al., 2005) (Kirkin et al., 2009, Komatsu and Ichimura, 2010, Komatsu et al., 2007). Hence, ablation of autophagy leads to increase in the levels of p62 and accumulation of p62-positive inclusions. In this context, p62 proteins containing a mutation in LIR are not efficiently degraded by autophagy leading to the formation of p62-containing aggregates even in the presence of functional autophagy. Conversely, although there is a marked attenuation of autophagic degradation of the PB1 mutants (which are defective in oligomerization), inclusion formation does not occur. These observations suggest that both accumulation and oligomerization of p62 is required for the formation of p62 containing aggregates (Bjorkoy et al., 2005, Pankiv et al., 2007, Komatsu and Ichimura, 2010, Komatsu et al., 2012, Komatsu et al., 2007).

Furthermore, majority of the inclusions found in autophagy-deficient cells are positive for both ubiquitin and p62. In cases where both autophagy and p62 are absent, there is a severe reduction in the formation of ubiquitin inclusions in several model organisms (Nezis et al., 2008, Komatsu et al., 2007). These observations form the hypothesis that ubiquitinated proteins first recognizes p62, then the accumulation of the p62 containing ubiquitinated proteins causes aggregation which finally gets degraded by autophagy. It is postulated that deficiency of p62 leads to the accumulation of ubiquitinated proteins and also attenuates autophagy. Interestingly, the levels of ubiquitinated proteins in p62 deficient tissues was less than that of autophagy-deficient

livers (Komatsu and Ichimura, 2010, Komatsu et al., 2007). A possible explanation for this discrepancy could be the presence of a structurally similar protein of p62 called Neighbor of BRCA1 (NBR1).

Recently, NBR1 is found to be associated with the ubiquitinated cargo. NBR1 and p62 have conserved PB1, LIR, and UBA domains (Kirkin et al., 2009). Hence, apart from p62 and NBR1's involvement in sequestration of ubiquitinated cargo, they can also interact with each other via PB1 and compete for the binding among each other with ubiquitinated substrates. Recent in-vitro data indicated that that NBR1 could alone initiate the selective autophagy process in the absence of p62 and thereby suggest that NBR1 might compensate for the role of p62. Interestingly, NBR1 is also emerging as an active component of various pathogenic inclusions of different etiologies such as Lewy bodies of the brain and MDBs of liver. However, the binding preference of p62 and NBR1 differs between aggregates associated with different protein aggregation diseases. p62 binds more strongly to protein aggregates containing tau and TDP-43 of amyotrophic lateral sclerosis whereas NBR1 prefers binding to  $\alpha$ -synuclein aggregates (Johansen and Lamark, 2011, Kirkin et al., 2009, Odagiri et al., 2012).



#### **Figure 4. Overview of pathways controlling protein aggregation and degradation.**

Molecular chaperones are responsible for the refolding of various newly synthesized protein intermediates. Alternatively, it facilitates the recognition of misfolded protein by proteasomal ubiquitin system (UPS), leading to their ubiquitination by CHIP, E3, and their degradation by the 26S proteasome. Overloading of proteasomal system during chronic stress leads to the ubiquitination of protein aggregates, which triggers binding of the adaptor proteins p62 and NBR1 to the protein aggregates. The newly formed cargo is then transferred to the autophagosomes for degradation.

Adapted from (Su and Wang, 2010).

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#### **1.3.2. p62 in receptor-mediated signaling**

p62 participates in the regulation of numerous signaling pathways as a scaffold protein but, is not always phosphorylated or modified by the components of signaling cascades and does not have any catalytic activity of its own. p62 is studied extensively for its ability to bind atypical protein kinase C (PKC $\zeta$  and PKC $\lambda$ ) via its N-terminal PB1 domain (Sanz et al., 1999, Jin et al., 2009). In tumor necrosis factor (TNF) signaling, heterodimerization of p62 with atypical PKC $\lambda$  and binding of RIP (receptor interacting protein) kinase through ZZ domain is crucial for the activation of NF $\kappa$ B. The binding of the receptors to p62 is followed by recruitment of E3 ubiquitin ligase TRAF-6 (TNF receptor-associated factor 6) followed by ubiquitination and oligomerization of the p62-TRAF-6 receptor complex through p62-UBA domain. This results in the opening of various docking sites for NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  kinase IKK (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha kinase) phosphorylation and subsequent NF- $\kappa$ B activation by dissociation of I $\kappa$ B $\alpha$ -NF- $\kappa$ B complex (Sanz et al., 1999, Moscat et al., 2007). Also, NF $\kappa$ B activity is regulated by p62-PB1's interaction with the IL-1 $\beta$ R (interleukin-1 $\beta$  receptor) (Lee et al., 2012) and the (NGF) nerve growth factor receptors TrkA (tropomyosin receptor kinase A) and p75<sup>NTR</sup> (neurotrophin receptor) (Wooten et al., 2001). In

NGF signaling, p62 facilitates the TRAF6-mediated ubiquitin conjugation of TrkA by stabilizing the TrkA and p75<sup>NTR</sup> complexes leading to activation of NF- $\kappa$ B (Geetha et al., 2005, Geetha et al., 2008). Furthermore, the cellular levels of p62 are required for proper functioning of cytokine signaling. Increased degradation of p62 by UPS or autophagy tends to hamper IL-1 $\beta$ R signaling and exhibit anti-inflammatory effects to the cells (Lee et al., 2012), that are mediated by Atg16L and the E3 ligase Cullin-3 (Lee et al., 2012).

p62 is also linked to the extrinsic apoptosis pathway via Cullin-3 activity. p62 regulates the optimal functioning of death receptors DR4 and DR5. Cell activation by TRAIL (TNF-related apoptosis-inducing ligand) is dependent on cullin-3 activity and further promotes the polyubiquitination of caspase-8. Of note, the polyubiquitination of caspase 8 occurs via p62, which further recruits the cargo to oligomeric p62-speckles leading to the activation of the apoptotic pathway (Jin et al., 2009). Based on these results, Moscat and Diaz-Meco suggest that the p62-puncta's are signaling hubs that could determine cell survival by activating the TRAF6-NF- $\kappa$ B pathway, or death, by inducing the aggregation of caspase-8 and its downstream effector caspases.

This intricate signaling network of p62 which decides whether cells live or die is orchestrated by p62's multiple domains has attained remarkable degree of specificity. p62's presence as a constant component of protein inclusions, role in the extrinsic apoptosis pathway, the modulation of autophagy by p62 are important factors in various interrelated diseases and may provide therapeutic solutions.

### **1.3.3. Role of p62 in metabolic dysfunction**

Another important role of p62 via its PB1 domain is its involvement in the betterment of metabolic syndrome. Metabolic syndrome (MetS) encompasses a multitude of metabolic risk factors such as abdominal obesity, insulin resistance, and glucose tolerance. The prevalence of metabolic syndromes are increasing dramatically, and individuals are at risk of developing diabetes and associated fatty-liver diseases (Bonomini et al., 2015). It was proven that aging worsen MetS and it has been linked to increase the chances of developing neurodegenerative disorders (Grundy, 1999). In a study with p62-knockout mice, it was shown that absence of p62 leads to mature-onset obesity and several features of MetS including deposition of fat in the adipose and liver, increased plasma triglycerides and cholesterol levels, decreased energy

expenditure and sensitivity towards insulin. p62 deficiency led to an abnormal extracellular signal-regulated kinase 1 (ERK1) activity in-response to insulin, resulting in the development of metabolic symptoms. Under normal conditions, ERK1 signaling is checked by p62/SQSTM1, which sequesters the kinase into p62 bodies through its PB1 domain, thereby counteracting the excessive proliferation and differentiation of white adipocytes (Muller et al., 2013, Rodriguez et al., 2006). Also PKC $\zeta$ , another kinase regulated by p62 also blocks glucose intolerance by reducing the production of pro-inflammatory cytokines in white adipose tissue of mice (Zhang et al., 2009). Hence, reduction in the expression of p62 with age may contribute to the increased fat accumulation and metabolic dysfunctions seen in the elderly. Recently, a pharmaceutical composition consisting of p62 protein is contemplated for therapeutic supplementation of the metabolic pathway (US patent 7608412 B2; owner, Jorge Moscat).

#### **1.3.4. Involvement of p62 in Cellular Defense Mechanism**

One of the main modes of transcriptional regulation of p62 is via transcription factor Nrf2 (Nuclear erythroid factor-2) (Figure 5). Under basal conditions, Nrf2 is sequestered in the cytoplasm by binding to its interaction partner Keap1, followed by ubiquitination and proteasomal degradation by E3 ubiquitin ligase cul3 (Cullinan et al., 2004). Oxidative stress leads to a conformation change in Kelch-like ECH-associated protein 1 and Cullin 3 (keap1-Cul3) association, inhibiting the enzymatic activity of cul3, leading to Nrf2 dissociation. Free Nrf2 translocate to the nucleus and initiates the transcription of various antioxidant genes such as NAD(P)H quinone dehydrogenase 1 (Nqo1) and glutathione synthetase (Gst), thereby, reducing the cellular toxicity (Kobayashi et al., 2004, Osburn and Kensler, 2008). Alternatively, in response to oxidative stress, Nrf2 binds to the ARE motif (antioxidant-response element) located in the p62 promoter and stimulate the expression of p62 transcript. p62 competitively inhibits the Keap1-Nrf2 interaction, leading to the stabilization of Nrf2 allowing nuclear translocation and expression of cytoprotective genes (Komatsu et al., 2010). This process contributes to the maintenance of the positive feedback loop in which p62 activates Nrf2 to drive its own transcription. It explains the increased reactive oxygen species (ROS) production in tau positive neuronal cells in the absence of p62. However, these observations do not provide an explanation why Nrf2 remains inactivated by keap1 in p62-deficient mice with impaired autophagy. As p62 is constantly degraded by autophagy, it may be assumed that any defect in

autophagy should increase the Nrf2 transcriptional activity but, this non-canonical mechanism would strictly have to rely on the constant degradation of p62 by autophagic machinery. These findings are in line with Atg7-deficient mouse model of hepatocellular carcinoma (HCC), where autophagy deficiency triggers the formation of p62-positive aggregates in 25% of HCC cases with constant Nrf2 activity. It can be concluded from the recent reports that there is a link between p62-dependent autophagic activity and Nrf2 transcription in carcinogenesis.

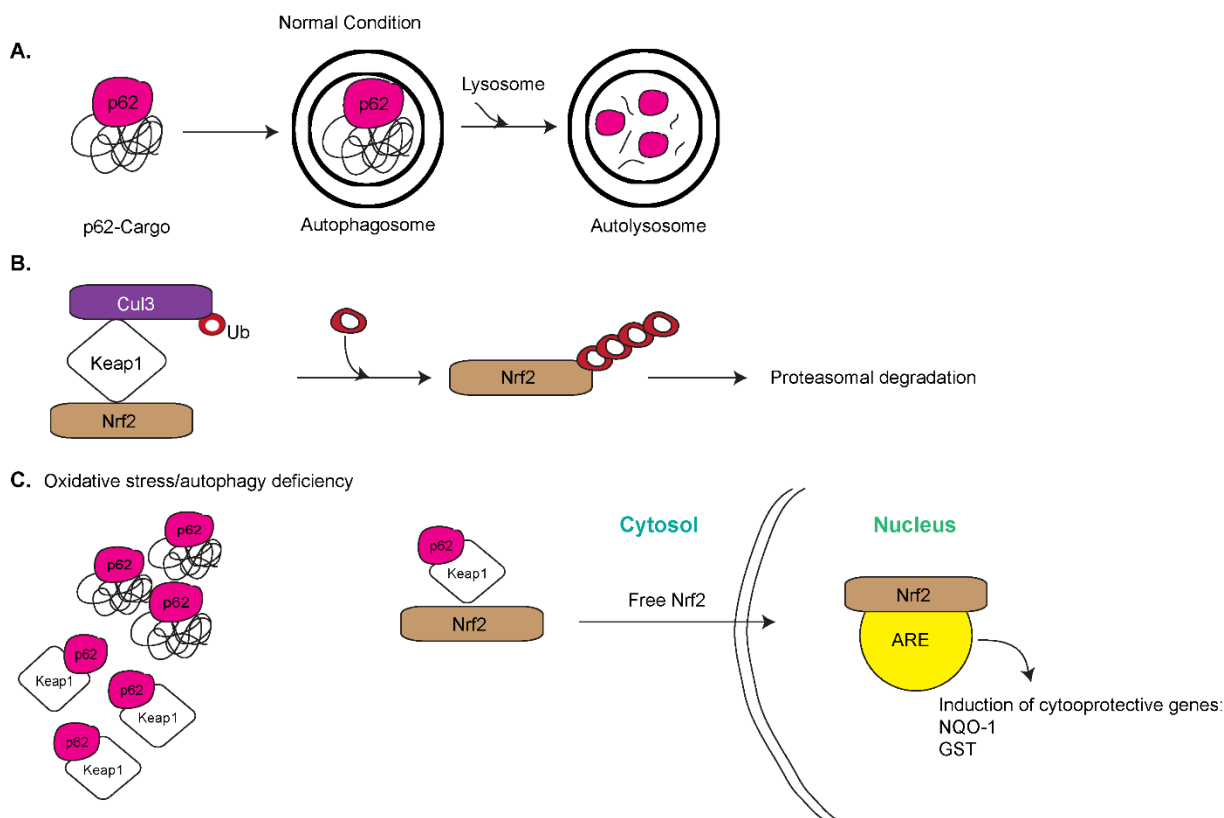


**Figure 5. Nrf2-keap1-p62 signaling.**

(A) Under normal conditions, basal autophagy continuously clears p62 and associated cargo (such as toxic aggregate-prone proteins) from the cytoplasm. (B) Nrf2 is ubiquitinated by the Cul3 ubiquitin ligase complex through the adaptor Keap1 and degraded by the proteasome. (C) Under conditions of oxidative stress or autophagy deficiency, p62 and associated cargo accumulates in the cytoplasm. Excess p62 binds Keap1, disrupting ubiquitination and ensuing degradation of Nrf2. This causes cytoplasmic Nrf2 levels to increase, allowing Nrf2 to enter the nucleus, and activate transcription from ARE containing promoters. Modified from (Rusten and Stenmark, 2010).

## **1.4. p62 and Pathogenic Protein Aggregates across Diseases**

### **1.4.1. p62 as a component of protein aggregates of liver**

The first demonstration of a possible functional role of p62 came in the context of hepatocytic protein aggregates, where Denk and colleagues (Stumptner et al., 1999) identified p62 to be a primary and constant component of intracellular hyaline bodies (IHBs) in hepatocellular carcinoma. IHBs are round or ovoid, eosinophilic protein aggregates ranging from barely visible globules to large inclusions of fibrillar morphology (Denk et al., 2006). So far, the pathogenesis of IHBs are unclear however, based on the ultrastructure; they are considered to be related to another liver inclusion known as MDBs. Eventually, p62 was also identified as a major component of MDBs and several other inclusions of neurodegenerative diseases including Lewy bodies of Parkinson's disease, neurofibrillary tangles in Alzheimer's disease and Rosenthal fibers in astrocytoma (Zatloukal et al., 2007).

MDBs are found in a variety of liver disorders including Steatohepatitis of both alcoholic and non-alcoholic etiology and hepatocellular carcinoma (Denk et al., 2000, Muller et al., 2004, Zatloukal et al., 2007). Unlike IHBs, MDBs are primarily composed of abnormal and misfolded keratins. The growing MDBs seemed to incorporate hyperphosphorylated p62 as an early response stress adaptor that associates with aggregated keratins likely via binding to ubiquitin chains in the aggregates (Stumptner et al., 2002). Many aspects of MDB pathogenesis is still unknown. However, it was speculated that the upregulation of p62 in MDBs is a defensive response against misfolded keratins and shuttles the ubiquitinated misfolded cargo to autophagy for degradation.

The majority of this thesis will be focused on the current understanding of Mallory-Denk body pathogenesis (Introduction; Heading 2.1.). The main emphasis will be on the investigation of the functional role of p62 in Mallory-Denk body formation (Results Part I; Discussion).

### 1.4.2. p62 as a common component of protein aggregates of brain

Zatloukal et. al., in 2002 identified p62 to be a major constituent of cytoplasmic protein aggregates in various neurodegenerative disorders (NDs) namely Lewy bodies of Parkinson's disease, Neurofibrillary tangles of Alzheimer's disease and TDP43 aggregates of amyotrophic lateral sclerosis. Currently, it is difficult to understand the physiological meaning of early upregulation of p62 in hepatic inclusions as well as in brain inclusions based upon the recent literature available. During this Ph.D. work, we have partially attempted to investigate the molecular functions of p62 using an in-vitro model of "Lewy body" formation (Results: part II) based on the observations made in mouse models of MDBs (Results: part I).

Lewy bodies (LBs) are most characterized and well-studied protein inclusions of the brain and diagnostic markers of Parkinson's disease (PDs) and dementia with Lewy bodies (DLBs). LBs are classified into brainstem and cortical types (Gibb et al., 1991, Forno, 1996, Lowe, 1998). The "classical" brainstem LBs are spherical, eosinophilic inclusions with concentric layers (core surrounded by a halo) and the layers are not generally indistinguishable. The LBs in cortical regions presents a less spherical morphology. The site distribution of LBs within various regions of brain are often associated with the clinical state of the disease. In particular, the presence of LBs in the cerebral cortex is used as a diagnostic marker of DLBs, whereas the occurrence of LBs in the substantia nigra and other pigmented brainstem nuclei is a criterion for the diagnosis of PD. Electron microscopy revealed that outer layers of LB consist of haphazardly arranged radiating fibrils along with a dense granular core. Both brainstem and cortical MDBs are primarily consists of  $\alpha$ -synuclein fibrils, 5-10 nm in diameters.  $\alpha$ -synuclein is a natively unfolded protein, consisting an N-terminal domain harboring 11-amino acid repeats that bind to membranes by assuming an  $\alpha$ -helical structure, a hydrophobic intermediate region, and a C-terminal acidic region with chaperone-like activity. The functions of  $\alpha$ -synuclein are now well known, but its key function is thought to be in the regulation of synaptic vesicular transport. It is often reported in the literature that abnormal fibrillization of  $\alpha$ -synuclein is responsible for pathological protein aggregation in PD (Baba et al., 1998, Spillantini et al., 1998, Goedert, 2001). So far, the pathogenic relevance of structural changes of  $\alpha$ -synuclein in LB formation is not well investigated, possibly due to the lack of good animal models of PD representing Lewy Body morphology. Further, various immunohistochemical studies shows less consistent

presentation of ubiquitinated species and phosphorylated neurofilaments (Dev et al., 2003, Iwatsubo et al., 1996). However, p62 expression was found to be consistently upregulated with the development of LBs.

Despite the differences in distribution, morphology and primary proteins responsible for the deposition of protein aggregates in various NDs, majority of them shares common component p62 and ubiquitin (ub). Protein aggregation in NDs are associated with cell loss in the same brain areas, indicating a close interconnection between the events underlying cell death and inclusion formation.

## **1.5. Mallory-Denk Bodies and Steatohepatitis**

Steatohepatitis is a type of fatty liver disease characterized by inflammation and concurrent fat accumulation in the liver. Steatohepatitis is often observed in alcoholics (alcoholic steatohepatitis or ASH) but, also frequently diagnosed in people with obesity and diabetes (non-alcoholic steatohepatitis or NASH). Steatohepatitis of either etiology are undoubtedly progressive leading to cirrhosis or hepatocellular carcinoma and can be a potential cause of unexplained cirrhosis (Wree et al., 2013, Michelotti et al., 2013).

The prevalence of NASH is 2.1-6.3% among the general population and 9-40% in obese individuals with a body mass index of 30kg/m<sup>2</sup> or more. NASH is diagnostically characterized by hepatocellular injury, steatosis (fat accumulation in liver) and lobular inflammation (Michelotti et al., 2013). Hepatocellular injury refers to ballooning/degeneration of hepatocytes along with the presence of apoptotic and necrotic bodies (Sakhuja, 2014, Wree et al., 2013). The enlarged hepatocytes having a reticulated appearance are observed to contain cytoplasmic protein aggregates known as “Mallory-Denk bodies” (Denk et al., 2000, Zatloukal et al., 2007, Mallory, 1911, Mallory, 1914). Loss of intracytoplasmic deposition of keratins 8/18 immunostaining are also observed and considered as an objective marker for histological recognition of MDBs (Denk and Franke, 1981, Zatloukal et al., 2007).

MDBs were first described by Prof. Frank B. Mallory as cytoplasmic hyaline inclusions in hepatocytes of patients with alcoholic steatohepatitis (Mallory, 1911, Mallory, 1914). Subsequently, they became one of the important morphological markers of ASH and NASH.

MDBs are also observed in chronic cholestasis, copper storage and intoxication diseases such as Wilson diseases, idiopathic copper toxicosis, and Indian childhood cirrhosis. MDBs coexist with IHBs or as intermediate hybrid inclusions in hepatocellular carcinoma (Denk et al., 2006, Zatloukal et al., 2007) (Figure 6). However, they are not yet reported in viral hepatitis and acute cholestasis suggesting that occurrence of MDBs is a specific cellular reaction of injury upon oxidative stress in a subset of chronic liver diseases.

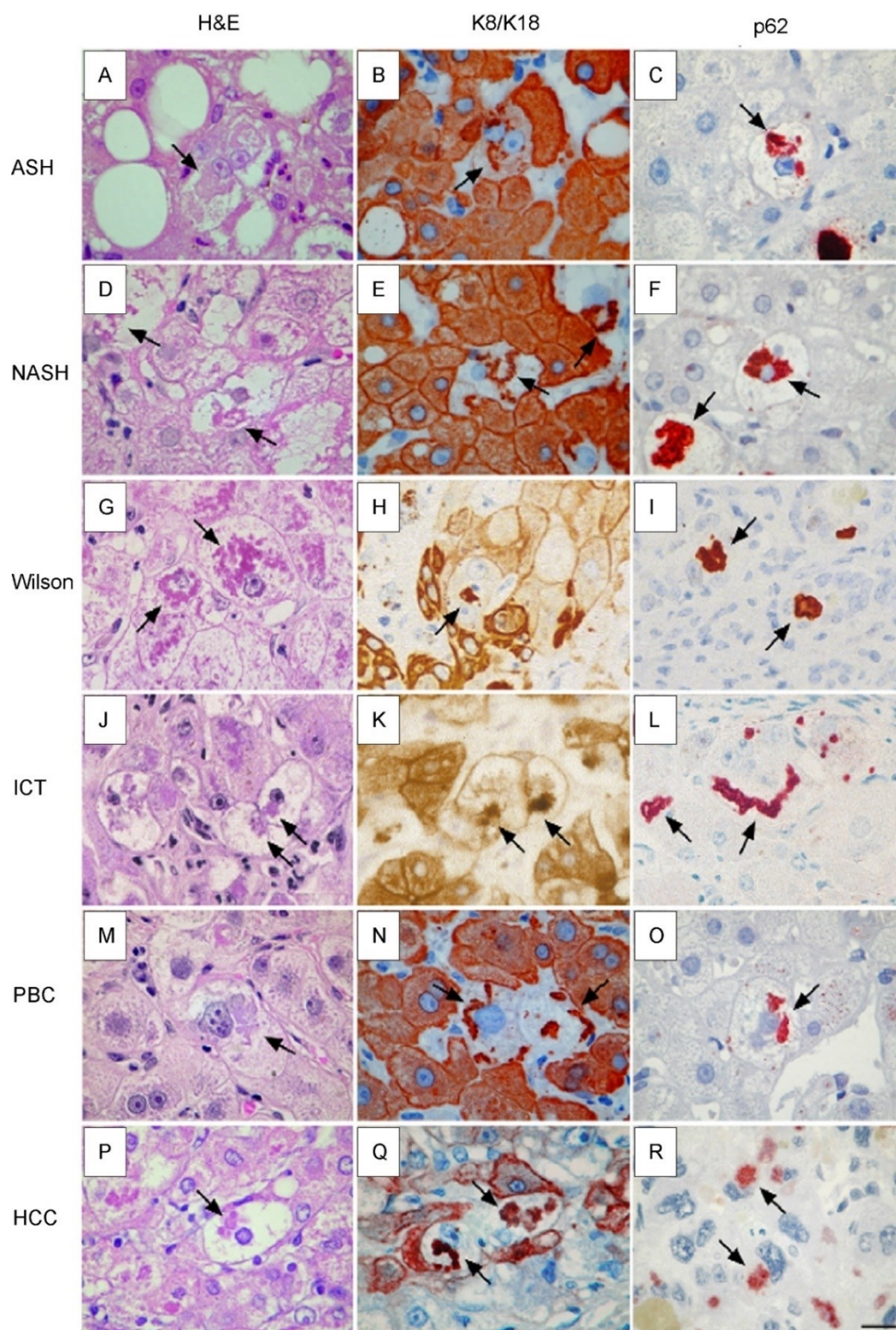


**Figure 6. MDBs in human liver diseases.**

MDBs (indicated by arrows) are visualized by hematoxylin–eosin staining (H–E) or by immunohistochemistry using antibodies to keratins 8 and 18 (K8/18) and to p62. K8/18 and p62 are singly stained and shown in red, followed by counterstaining of the tissues with hematoxylin (light blue). The stained human liver samples are from patients with alcoholic steatohepatitis (ASH, A–C), non-alcoholic steatohepatitis (NASH, D–F), Wilson disease (Wilson, G–I), idiopathic copper toxicosis (ICT, J–L), primary biliary cirrhosis (PBC, M–O) and hepatocellular carcinoma (HCC, P–R). Note that MDBs are preferentially seen in enlarged (ballooned) hepatocytes with reduced staining of the keratin intermediate filament (IF) cytoskeleton. Adapted from (Zatloukal et al., 2007).

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### **1.5.1. Structural characteristics and composition of MDBs**

MDBs have a constant biochemical composition and morphological appearance irrespective of their presence in various chronic liver disorders of different origin. MDBs are morphologically characterized as dense, rosy, eosinophilic intracytoplasmic perinuclear inclusion present in ballooned hepatocytes (Denk et al., 2000, Franke et al., 1979, Jensen and Gluud, 1994a, Jensen and Gluud, 1994b). Occasionally, MDBs are recognized as small globular structures distributed throughout the cytoplasm associated with hepatocyte keratin intermediate filaments. Furthermore in few cases, MDBs are observed in cell periphery where they colocalize with desmosomes (Denk et al., 2000, Jensen and Gluud, 1994a, Jensen and Gluud, 1994b).

Based on electron microscopy, MDBs are filamentous in structure coated with the amorphous material and filament diameter ranging from 3-24nm (vs. the typical 10nm sized intermediate filaments). MDBs are ultra-structurally divided into three types. Type-I MDBs are documented to have parallel filament arrangements whereas Type-II MDBs have randomly oriented filaments. Type III MDBs are found in association with Type II MDBs at the cell periphery and consist of electron dense granular amorphous material (Franke et al., 1979, Strnad et al., 2007, Wiggers et al., 1973) (Figure 7).

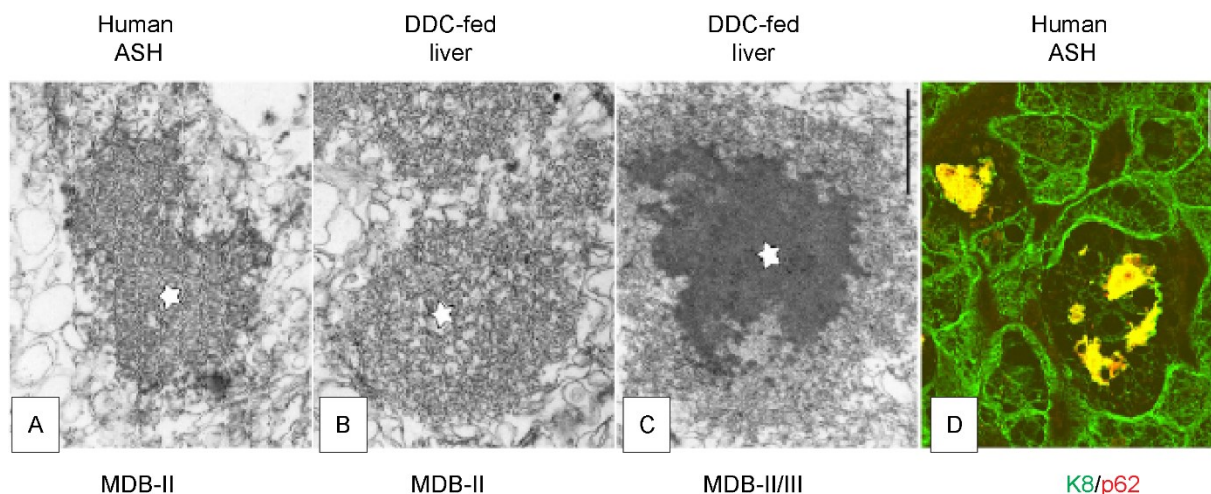


**Figure 7. Ultrastructure and immune staining of MDBs.**

Transmission electron micrographs of MDBs in human liver in a patient with ASH (A) and in DDC-fed mouse livers (B and C). Panels A and B illustrate type-II MDBs, while Panel c shows a type-III MDB surrounded by type-II MDB filaments (MDBs are indicated by stars). Scale bar=1  $\mu$ m. Fluorescence staining to identify MDBs in a patient with ASH (D) is shown by double-staining for K8/18 (green) and p62 (red) which when colocalized appear yellow. Adapted from (Zatloukal et al., 2007).

Electron microscopy studies suggested that MDBs are composed of cytoskeletal proteins. Subsequent, immuno-staining and biochemical analysis revealed that Keratins are the main component of MDBs. Keratin 8 and 18 (K8 and K18) are the primary keratin polypeptides found in MDBs (Zatloukal et al., 2000). Studies on keratins found in MDBs have shown that K8 and K18 are the targets of various post-translational modifications (Lahiri et al., 2016, Stumptner et al., 2000, Tsunoo et al., 1987, Zatloukal et al., 2000). K8/K18 within MDBs are hyperphosphorylated at various sites, and K8, in particular, serves as a preferred substrate for transamidation by transglutaminase-2 (TG2) followed by ubiquitination (Zatloukal et al., 1992, Strnad et al., 2007, Strnad et al., 2008b). These modifications form high molecular weight complexes. Another important class of proteins found in MDBs are stress proteins p62 and ubiquitin which are hypothesized to be upregulated after keratin modifications (Nan et al., 2004, Stumptner et al., 2002). Except for the presence of higher levels of p62 in MDBs, very little is

known about its molecular potential in MDB formation process. Furthermore, chaperones such as heat-shock proteins Hsp70 and Hsp25 were also detected in MDBs. In response to external stimuli or stress, Hsp25 attaches to misfolded proteins and prevents any further alterations. On the other hand, high molecular weight chaperones like Hsp70 and Hsp90 minimize aggregation of newly synthesized proteins by unfolding them at the expense of adenosine triphosphate (ATP) (Riley et al., 2003, Zatloukal et al., 2002).

### 1.5.2. Pathogenesis of MDBs

The current understanding of the pathogenesis of MDBs are facilitated by the studies on mouse models. MDBs are induced in mice by feeding them with griseofulvin (GF) for 4-5 months or 3,5 diethoxycarbonyl-1,5 dihydrocollidine (DDC) for two months (Denk et al., 1975, Yokoo et al., 1982). Upon recovery from DDC-intoxication for four weeks with a normal diet, liver cytoskeleton gets restored, and the majority of the MDBs disappear (Stumptner et al., 2001a). Interestingly, the recovered mice retain the molecular memory of the chronic DDC or GF assault and hence, proactively form MDBs within a very short period if re-challenged with DDC or GF (Strnad et al., 2008a). The discovery of keratins as intrinsic proteins in MDBs led to the generation of K8 and K18 knockout or transgenic mouse models which provided considerable information on the functions of K8 and K18 in MDB formation process. Upon stress, both K8 and K18 undergo various alterations during the formation of MDBs. An unbalanced keratin expression with an excess of K8 over K18 ( $K8 > K18$ ) is required for the disposition of MDBs as seen in experiments with K8 and K18 knockout and transgenic mice (Stumptner et al., 2007, Strnad et al., 2008b, Zatloukal et al., 2000). In parallel, chaperones such as Hsp25, Hsp60, and Hsp70 initially reduce the effects of elevated oxidative stress by chaperone stabilization of misfolded proteins. As chaperone function becomes compromised, progressive keratin misfolding occurs together with K8 cross-linking by TG2 and ubiquitination, that ultimately leads to the formation of MDBs (Zatloukal et al., 1992, Strnad et al., 2008b, Strnad et al., 2008a). K8 also undergoes a conformational change from  $\alpha$ -helical to  $\beta$ -sheet structure conferring MDBs the conformation characteristic of diverse types of amyloid seen in neurodegenerative diseases (Mahajan et al., 2011). Along with keratins, The M<sub>M</sub>120-1 antibody recognizes a high molecular weight MDB component which so far has been exclusively

detected in MDBs and, therefore, serves as another specific marker for the MDBs (Zatloukal et al., 1990a).

Apart from keratins, another protein which is found in large quantities in MDBs is p62/Sequestosome-1. Previous cell culture studies on MDBs revealed that combined transfection with p62, K8 and ub resulted in aggregates with typical MDB ultrastructure. However, MDBs did not form when only K8, K18 and ub were transfected alone or in combination (Stumptner et al., 2007, Stumptner et al., 2001b, Stumptner et al., 2002). Additionally, DDC primed hepatocytes showed attenuation of MDB in the absence of p62. Recent reports suggest that p62 is involved in the degradation of MDBs. Unfortunately, none of the aforementioned studies provide a clear understanding of the involvement of p62 on MDB pathogenesis and biochemical and structural changes associated with the formation of MDBs (Figure 8) especially in the context of human diseases.

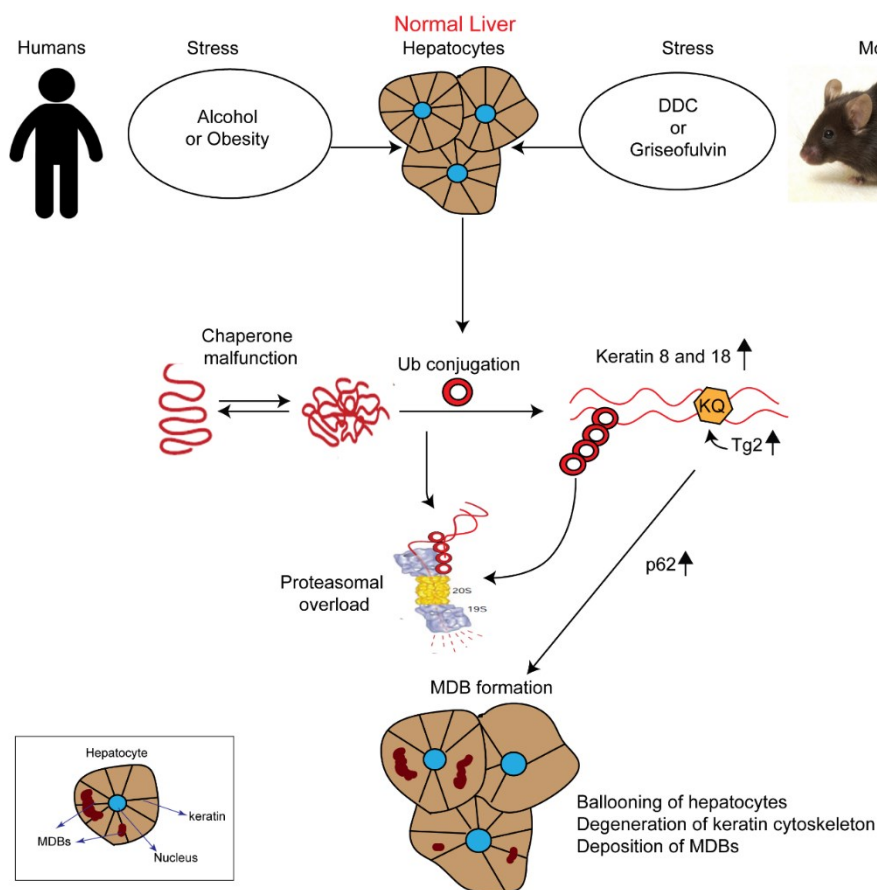


**Figure 8. Pathogenesis of MDBs.**

Unique types of stress (e.g. alcohol or obesity in susceptible patients and DDC or Griseofulvin in mice) leads to malfunction of chaperones and trigger the upregulation of keratins (K8 and K18). With increasing stress, K8 gets transamidated by TG2 at lysine (K) and glutamine (Q) residues and forms keratin crosslinks. Oxidative and other types of stress also cause the accumulation of misfolded proteins that can overwhelm the proteasome via accumulation of ubiquitinated or crosslinked proteins. The end result is the formation of MDBs. Modified from (Zatloukal et al., 2007)

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### 1.5.3. Association of Mallory-Denk bodies with intracellular hyaline bodies

Based on the literature over the last decade on primary liver inclusions, they can be categorized into three types namely, (i) p62-containing inclusions, (ii) inclusions of ER storage disorders, and (iii) porphyrin containing inclusions (Strnad et al., 2013).

**Table 2. Primary liver inclusions**

| Inclusion type                   | Inclusions                             | Etiology                   | Characteristic diseases                           |
|----------------------------------|----------------------------------------|----------------------------|---------------------------------------------------|
| <b>p62-containing inclusions</b> | Mallory-Denk bodies                    | Acquired                   | ASH, NASH, Copper storage disorders               |
|                                  | Intracellular hyaline bodies           | Acquired                   | Hepatocellular carcinoma                          |
| <b>ER storage disease</b>        | $\alpha$ -1 antitrypsin-inclusion      | Inherited                  | $\alpha$ -1 antitrypsin deficiency                |
|                                  | $\alpha$ -1 antichymotrypsin-inclusion | Inherited                  | $\alpha$ -1 antichymotrypsin deficiency           |
|                                  | Ground glass hepatocyte                | Acquired/somatic mutations | Chronic Hepatitis B                               |
|                                  | Fibrinogen-containing inclusion        | Inherited/Acquired         | Fibrinogen-deficiency/various acquired conditions |
|                                  | Pale bodies                            | Acquired                   | Hepatocellular carcinoma                          |

|                                        |                                          |          |                               |
|----------------------------------------|------------------------------------------|----------|-------------------------------|
| <b>Porphyrin containing inclusions</b> | Needle-like cytoplasmic inclusions (NLI) | Acquired | Porphyria cutanea tarda       |
|                                        | NLI-like inclusions                      | Acquired | Erythropoietic protoporphyria |

\* table is modified from (Strnad et al., 2013)

MDBs and IHBs represent the prototype of p62-containing pathogenic inclusions and are used widely to study the aggregation process in general. Previous reports document the presence of MDBs and IHBs either alone or together in hepatocellular carcinoma and few cases of idiopathic copper toxicosis (Stumptner et al., 1999, Denk et al., 2006, Komatsu et al., 2007). Immunohistochemical studies report both similarities and differences between these two liver inclusions (Figure 9). MDBs appear to be more reticular whereas IHBs are more globular with a surrounding halo but ultrastructurally, both are composed of similar filament material. Furthermore, MDB formation is a more keratin-driven process, and IHBs are considered to be equivalent of “p62-bodies” with or without ubiquitin. The relationship between MDBs and IHBs becomes more interesting as “hybrid inclusions” presenting an intermediate structural and biochemical morphology of IHBs and MDBs were also observed in certain neoplastic and non-neoplastic livers (Stumptner et al., 1999, Denk et al., 2006). Till date, very little is known about the induction of IHBs in liver diseases. Previous studies on p62-containing inclusions across diseases suggested that oxidative stress is responsible for the formation of misfolded proteins and upregulation of p62 (Janig et al., 2005, Komatsu et al., 2007). Also, oxidative stress is responsible for the imbalance of keratin ratio leading to the formation of MDBs (Janig et al., 2005, Zatloukal et al., 2000, Zatloukal et al., 2007). Hence, it is safe to assume that oxidative stress is also responsible for IHB formation. It can be hypothesized that a hybrid inclusion represents a scenario where p62 aggregation and ubiquitination precedes keratin alterations. Misfolded keratins either gets incorporated at a later stage or in minute quantities with growing intensity of oxidative stress. This leads to the changes in morphology and might be responsible for the transformation of p62 containing IHBs to MDBs. However, IHBs and MDBs are presented separately in some cells and what is the physiological function of both aggregates with respect to liver disorders are still unclear.

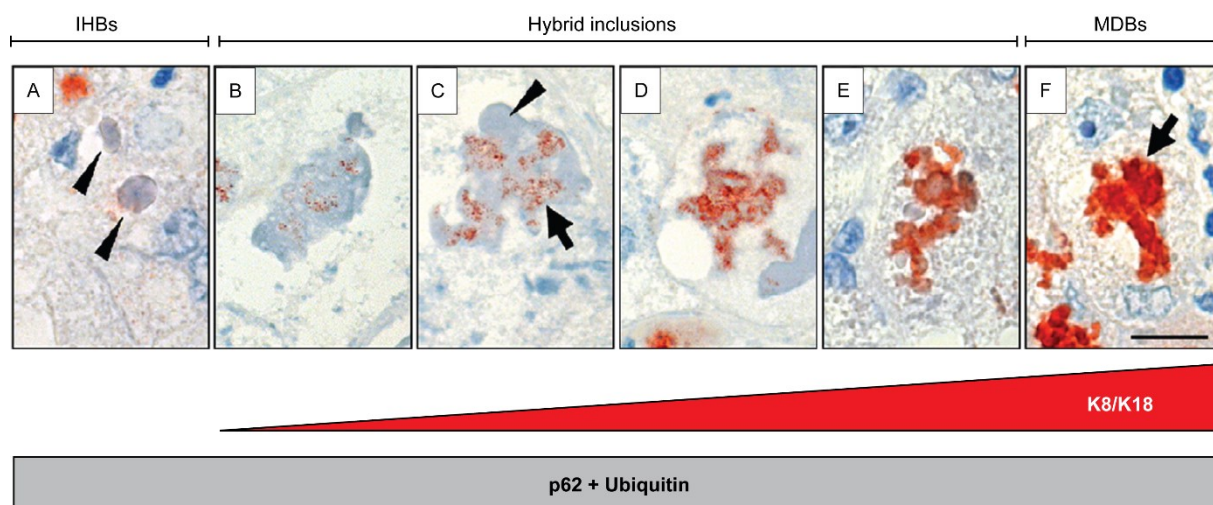


**Figure 9. Immunohistological profiles of hepatocyte MDB, IHB and hybrid inclusions.**

In A–F, different transitional stages (hybrid inclusions) between IHBs (exemplified by arrowheads in A and C) and MDBs (arrows in C and F) are shown as seen by staining of the fixed liver tissue from a child with idiopathic copper toxicosis using antibodies to K8/18 (red) and hematoxylin as counterstain (light blue). Note that the transition from IHB to MDB is characterized by increased presence of keratin whereas ubiquitin and p62 are present (this staining is not shown but represented schematically) and relatively constant constituents of IHBs and MDBs. Modified from (Zatloukal et al., 2007).

## 1.6. Protein aggregation: an epiphenomenon of injury?

The identification of p62 as a common and constant molecular component of various protein aggregates, the structural alteration of primary proteins from alpha helical to  $\beta$ -sheet structure, and the clinical interconnections of various diseases containing protein inclusions as metabolic syndromes suggests that, protein aggregation process follows the same pattern irrespective of the tissue involved (Zatloukal et al., 2002). Hence, investigating the pathogenesis of MDBs will also enhance our understanding of other hepatic and non-hepatic aggregates.

MDBs results from a complex interplay of increasing oxidative stress, drug-toxicity, genetic and epigenetic factors (Zatloukal et al., 2007, Denk et al., 2000). Over the years, studies on

various components of MDBs has improved our knowledge of MDB formation process and its effect on cellular (hepatocyte) environment. However, it is still debatable whether MDBs represents a by-product of injury or acts as a protective mechanism that leads to the advancement of liver injury (Grune et al., 2004, Bence et al., 2001, Haass and Selkoe, 2007). The notion that formation of larger aggregates are detrimental to the cells is recently counteracted by studies on Alzheimer plaques which supports that it is the small oligomers/protofibrils (precursors of large aggregates), that causes cellular toxicity (Haass and Selkoe, 2007). In fact, the formation of large aggregates are beneficial as they are often ubiquitinated and ready for degradation by proteasome or autophagy, thereby, ameliorating the deleterious effects of cellular stress. For example, injection of amyloid  $\beta$  ( $A\beta$ ) into the brain of rats resulted in toxic effects, which could be alleviated by co-injection of an oligomer-specific antibody. In Parkinson's disease, the formation of Lewy bodies per se did not lead to cellular death. Given that, administration of inclusion promoting chemical compounds alleviates the toxicity for of aggregation-prone proteins in cellular models of Huntington's and Parkinson disease. Interestingly, mutations in  $\alpha$ -synuclein progressively form protofibrils than non-pathogenic proteins which leads to dopaminergic neurotoxicity (Conway et al., 2000, Lansbury and Lashuel, 2006). Previous in-vitro studies showed no correlation between MDB formation and cellular death. Also, withdrawal of DDC in mouse models led to the disappearance of MDBs and restoration of the cytoskeleton of hepatocytes (Stumptner et al., 2007, Stumptner et al., 2001b). Therefore, utilizing the available models of MDB formation as a setting for investigating liver injury will help in sharpen the perception of aggregates as a "friend" or a "foe" of cellular homeostasis.

# Material and Methods

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## 2.1. Materials

### 2.1.1. General Technical equipment's

#### ***PCR machine***

- Gene Amp PCR System 9700 PE; Applied Biosystems, Foster city, CA, USA

#### ***Thermo block***

- Thermomixer comfort; Eppendorf, Hamburg, Germany

#### ***Vortex***

- Vortex-Genie 2, USA

#### ***Water bath***

- mgw; Lauda RMS

#### ***Incubators***

- 37<sup>0</sup> C and 65<sup>0</sup> C; Heraeus

#### ***Spectrophotometers***

- Smart spec 3000; Biorad
- NanoDrop 2000; Thermo Scientific

#### ***Shaker***

- Certomat H and U; Braun

#### ***Gel electrophoresis instruments***

- Sub-cell GT; BIO-Rad, Vienna, Austria

### ***Gel imaging and documentation system***

- FlurChrom 5500; Alpha innotech

### ***Centrifuges***

- Biofuge fresco; Heraeus, Buckinghamshire, United Kingdom
- Megafuge 2.0R; Heraeus
- Sorvall RC-5B; refrigerated superspeed centrifuge; Sorvall, Vienna, Austria

## **2.1.2. Specialized Instruments and resources used in animal dissections and sample storage**

- Surgical tools (knives, forceps and tweezers) ; VWR International, Vienna, Austria
- Surgical blades ; VWR international, Vienna, Austria
- Syringes; VWR international, Vienna, Austria
- Paraffin cassettes; VWR international, Vienna, Austria
- Cryo tube; VWR international, Vienna, Austria

## **2.1.3. Cell culture laboratory equipment's**

- CO<sub>2</sub> incubator: Forma Scientific Steri - cult 200
- Waterbath: Julabo SW-20<sup>0</sup>C; water sterilized with Prothermal; Lucerna ChemAG
- Sterile Workbench: Prettl; Laminar flow + Prozesstechnik GmbH
- Haemocytometer counting chamber: 0.200 mm depth; 0.0625mm<sup>2</sup>; Fuchs-Rosenthal, Assistant Germany
- Square coverslips: 22mm x 22mm; Assiatant Germany

## **2.1.4. Microscopes**

- Light optical microscope: Nikon, Eclipse E 600 with 10x, 20x, 40x, 60x objectives; Nikon JAPAN
- Digital Camera: Canon S3 Canon

- Immunofluorescence microscope: with 25x, 40x oil, 63x oil and 100x oil objectives; Carl Zeiss, Jena, Germany
- Confocal Laser Scanning Microscope: Zeiss LSM 510; Zeiss

### 2.1.5. Plasticwares

- 1.5 mL to 15 ml tubes; Corning, Eppendorf and Sarstedt
- PCR-softstrips (0.2ml); Biozym
- 6 well plates and 75 cm<sup>2</sup> vented culture flasks; Costar

### 2.1.6. Chemicals, Reagents and Buffered solutions

**Table 3. Recipes for Stock solutions**

| Categories           | Solutions/buffers/kits   | Recipe                                                                                                                                            | Company | Storage Conditions |
|----------------------|--------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|---------|--------------------|
| <b>General Usage</b> | 1 M TRIS, pH 7-8         | 121.1 g TRIS base<br>1 L dd H <sub>2</sub> O<br>pH adjustments with conc. HCl                                                                     |         | Room temperature   |
|                      | 0.5 M EDTA, pH 8.0       | 93.05 g of EDTA<br>500 ml dd H <sub>2</sub> O<br>pH adjustments with 5M NaOH                                                                      |         | Room temperature   |
|                      | 5 M NaOH                 | 100 g NaOH<br>500 ml dd H <sub>2</sub> O                                                                                                          |         | Room temperature   |
|                      | 5 M NaCl                 | 292.2 g NaCl<br>1 L dd H <sub>2</sub> O                                                                                                           |         | Room temperature   |
|                      | 3 M KCl                  | 93.2 g of KCl<br>500 ml dd H <sub>2</sub> O                                                                                                       |         | Room temperature   |
|                      | PBS pH 7.2 - 7.3         | 1.4625 g Na <sub>2</sub> HPO <sub>4</sub> ·H <sub>2</sub> O<br>0.2450 g KH <sub>2</sub> PO <sub>4</sub><br>8 g NaCl<br>500 ml dd H <sub>2</sub> O |         | Room temperature   |
|                      | TE buffer                | 5 ml 1M TRIS, pH 8.0<br>1ml 0.5M EDTA, pH 8.0<br>500 ml ddH <sub>2</sub> O                                                                        |         | Room temperature   |
|                      | 10 % Triton-X-100        | 5 ml Triton-X-100<br>50 ml ddH <sub>2</sub> O                                                                                                     |         | Room temperature   |
|                      | 10 % sodium deoxycholate | 5g sodium deoxycholate<br>50 ml ddH <sub>2</sub> O                                                                                                |         | 4°C                |

|                                    |                                              |                                                                                               |                    |                    |
|------------------------------------|----------------------------------------------|-----------------------------------------------------------------------------------------------|--------------------|--------------------|
|                                    | 20 % Sodium dodecyl sulphate (SDS)           | 20 g of SDS powder<br>100 ml of ddH <sub>2</sub> O                                            |                    | Room temperature   |
| <b>Antibiotic stock solutions</b>  | Kanamycin                                    | 50 mg kanamycin powder<br>1 ml dd H <sub>2</sub> O                                            | Sigma              | -20 <sup>0</sup> C |
|                                    | Ampicillin                                   | 100 g ampicillin powder<br>1 ml dd H <sub>2</sub> O                                           | Sigma              | -20 <sup>0</sup> C |
|                                    | Chloramphenicol                              | 1.7 g Chloramphenicol<br>50 ml absolute ethanol                                               | Sigma              | -20 <sup>0</sup> C |
| <b>Agarose gel electrophoresis</b> | 20X Tris-Borate buffer (TBE) buffer          | 216 g Tris<br>110 g Boric acid<br>40 ml 0.5 M EDTA, pH 8.0                                    |                    | Room temperature   |
|                                    | 5 X gel loading buffer                       | Ready to use                                                                                  | Fermentas          | 4 <sup>0</sup> C   |
|                                    | Gene Ruler 50bp ladder                       | Ready to use                                                                                  | Fermentas          | 4 <sup>0</sup> C   |
|                                    | Gene Ruler 1Kb ladder                        | Ready to use                                                                                  | Fermentas          | 4 <sup>0</sup> C   |
|                                    | Seakem ME Agarose for gel electrophoresis    | Ready to use                                                                                  | Cambrex            | Room temperature   |
| <b>RNA extraction</b>              | TRIZOL reagent                               | Ready to use                                                                                  | Invitrogen         | 4 <sup>0</sup> C   |
| <b>DNA extraction</b>              | QIAquick Gel Extraction Kit                  | Ready to use                                                                                  | QIAGEN             | Room temperature   |
|                                    | QIAfilter Plasmid Maxi Kit                   | Ready to use                                                                                  | QIAGEN             | Room temperature   |
|                                    | GeneJET Plasmid Miniprep Kit                 | Ready to use                                                                                  | fermentas          | Room temperature   |
|                                    | DirectPCR® Lysis Reagent Tail                | Ready to use                                                                                  | Peqlab             | Room temperature   |
|                                    | peqGOLD Proteinase K solution                | 20 mg of Proteinase K<br>1 ml of TE buffer                                                    | Peqlab             | Room temperature   |
|                                    | High Capacity cDNA Reverse Transcription Kit | Ready to use                                                                                  | Applied Biosystems | -20 <sup>0</sup> C |
| <b>Protein extraction</b>          | RIPA buffer                                  | 150 mM NaCl<br>1 % Triton-X-100<br>0.5 % Sodium deoxycholate<br>0.1% SDS<br>50mM Tris, pH 8.0 |                    | 4 <sup>0</sup> C   |
|                                    | Triton-X-100 buffer                          | 1 % Triton-X-100<br>5mM EDTA                                                                  |                    | 4 <sup>0</sup> C   |
|                                    | High Salt buffer                             | 10 mM Tris, pH6.8<br>1.5 M KCl                                                                |                    | 4 <sup>0</sup> C   |

|                     |                                                                     |                                                                                                                 |                  |                  |
|---------------------|---------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|------------------|------------------|
|                     |                                                                     | 140 mM NaCl<br>0.5 mM EDTA<br>0.5 % Triton-X-100                                                                |                  |                  |
|                     | PhosSTOP<br>Phosphatase<br>Inhibitor Cocktail<br>Tablets            | 1 tablet / 10 ml of<br>protein extraction buffer                                                                | Roche            | 4 <sup>0</sup> C |
|                     | cOmplete Protease<br>Inhibitor<br>Cocktail Tablets<br>in EASYpacks. | 1 tablet / 10 ml of<br>protein extraction buffer                                                                | Roche            | 4 <sup>0</sup> C |
| <b>Western Blot</b> | 10 X transfer buffer                                                | 121.1 g Tris Base<br>576 g glycine<br>4 L ddH <sub>2</sub> O                                                    |                  | 4 <sup>0</sup> C |
|                     | 1 X transfer buffer                                                 | 700 ml cold ddH <sub>2</sub> O<br>100 ml 10X Transfer<br>buffer<br>200 ml methanol                              |                  | 4 <sup>0</sup> C |
|                     | 20 X TBS                                                            | 193.6 g Tris base<br>640 g NaCl<br>4 L with ddH <sub>2</sub> O                                                  |                  |                  |
|                     | 20 X TBS-Tween-20                                                   | 2 ml Tween-20<br>100 ml of 20X TBS                                                                              |                  |                  |
|                     | 2 X sample loading<br>buffer (non-reducing)                         | 5 ml 1 M Tris, pH 7<br>25 ml 20% SDS<br>20 ml glycerol<br>2 mg bromophenol blue<br>100 ml of ddH <sub>2</sub> O |                  | 4 <sup>0</sup> C |
|                     | 2 X sample loading<br>buffer (reducing)                             | 950 µL 2x non-reducing<br>sample loading buffer<br>50 µL β-<br>mercaptoethanol                                  | 4 <sup>0</sup> C |                  |
|                     | 10 X Tris-Glycine<br>buffer (running<br>buffer)                     | 121.1 g Tris base<br>576 g glycine<br>200 ml 20% SDS<br>Bring up the volume to<br>4 L with ddH <sub>2</sub> O   |                  | 4 <sup>0</sup> C |
|                     | 1.5 M Tris, pH 8.8<br>(Resolving gel buffer)                        | 181.65 g Tris Base<br>1000 ml ddH <sub>2</sub> O<br>pH adjustments with<br>conc. HCl                            |                  | 4 <sup>0</sup> C |
|                     | 1.5 M Tris, pH 6.0<br>(Stacking gel buffer)                         | 181.65 g Tris<br>1000 ml ddH <sub>2</sub> O                                                                     |                  | 4 <sup>0</sup> C |

|  |                                            |                                                                                                                                |            |                  |
|--|--------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|------------|------------------|
|  |                                            | pH adjustments with conc. HCl                                                                                                  |            |                  |
|  | NuPAGE® MOPS SDS Running Buffer (20X)      | Ready to use                                                                                                                   | Invitrogen | 4°C              |
|  | NuPAGE® MOPS SDS Running Buffer (20X)      | Ready to use                                                                                                                   | Invitrogen | 4°C              |
|  | Mild stripping buffer                      | 15 g glycine<br>1 g SDS<br>10 ml Tween20<br>Adjust pH to 2.2<br>1 L ddH <sub>2</sub> O                                         |            | Room temperature |
|  | Harsh stripping buffer                     | 20 ml SDS 10%<br>12.5 ml Tris HCl pH 6.8<br>0.5M EDTA<br>67.5 ml ddH <sub>2</sub> O<br>0.8 ml β-mercaptoethanol under fumehood |            | Room temperature |
|  | Coomassie (Bradford) Protein Assay Reagent |                                                                                                                                | Pierce     | 4°C              |

**Table 4. List of Primers used in genotyping, Q-PCR and RT-PCR**

| Primer Name                      | Sequence                        |
|----------------------------------|---------------------------------|
| <i><b>Genotyping Primers</b></i> |                                 |
| CreA                             | 5' ATATCAGGGCCATAGGTGCTCA 3'    |
| CreB                             | 5' TGAGACAGGATCTTATAACCACA 3'   |
| LoxPa                            | 5' TATCCTTACTAAACTGTATAGCCA 3'  |
| CreF                             | 5' ACCTGAAGATGTTCGCGATTATCT 3'  |
| CreR                             | 5' ACCGTCAGTACGTGAG- ATATCTT 3' |
| <i><b>qPCR primers</b></i>       |                                 |
| K8 Fwd                           | 5' ATCAAGAAG GATGTGGACGA 3'     |
| K8 Rev                           | 5' CTGCAACTCACGGATCTC 3'        |
| Nrf2 Fwd                         | 5' CAGCATGATG GACTTGGAG 3'      |
| Nrf2 Rev                         | 5' AAATCCATGTCCTGCTGG 3'        |
| Hmox-1 Fwd                       | 5' ACATTGAGCTGTTTGAGGAG 3'      |

|                              |                             |
|------------------------------|-----------------------------|
| Hmox-1 Rev                   | 5' GGTCTTTGTGTTCTCTGTC 3'   |
| Keap1 Fwd                    | 5' CATCCACCCTAAGGTCATGGA 3' |
| Keap1 Rev                    | 5' GACAGGTTGAAGAACTCCTCC 3' |
| Nqo1 Fwd                     | 5' GACCTTGCTTTCTATCACCA 3'  |
| Nqo1 Rev                     | 5' GATGCCACTCTGAATCGG 3'    |
| Gst Fwd                      | 5' GCTGACTTTGAGAAACGGA 3'   |
| Gst Rev                      | 5' CAAGGACATCATAGACGAGG 3'  |
| $\beta$ -actin Fwd           | 5' ATGGAGGGGAATACAGCCC3'    |
| $\beta$ -actin Rev           | 5'TTCTTTGCAGCTCCTTCGTT 3'   |
| <b><i>RT-PCR primers</i></b> |                             |
| p62 Fwd                      | 5' AAGTCAGCAAACCTGACG 3'    |
| p62 Rev                      | 5' CCATCTGTTCTCTGGCT 3'     |

**Table 5. List of antibodies for immunofluorescence, immunohistochemistry and westernblot**

| Antibody Name                          | Company                                     |
|----------------------------------------|---------------------------------------------|
| <b><i>Primary antibodies</i></b>       |                                             |
| p62                                    | Progen; #GP62-C, Heidelberg, Germany        |
| K8                                     | Progen; #Ks 8.7, Heidelberg, Germany        |
| K8                                     | abcam; #ab53280, Cambridge, UK              |
| K18                                    | Progen; #Ks 18.04, Heidelberg, Germany      |
| K18                                    | abcam; #ab53118, Cambridge, UK              |
| Ubiquitin                              | Cell Signalling; #3933S, Austria            |
| Ubiquitin                              | Sigma Aldrich; #GW10073F, Vienna, Austria   |
| NBR1                                   | Novus; #NBP171703, Oxon, UK                 |
| M <sub>M</sub> 120-1                   | neat; (3)                                   |
| LC3                                    | Novus; #NB100-2200, Oxon, UK                |
| Desmoplakin                            | abcam; #ab16434, Cambridge, UK              |
| <b><i>Secondary antibodies</i></b>     |                                             |
| Alexa fluor goat anti rabbit IgG 488nm | Life technologies; #A11034, Vienna, Austria |
| Alexa fluor goat anti rabbit IgG 594nm | Life technologies; #A11012, Vienna, Austria |
| Alexa fluor goat anti mouse IgM 488nm  | Life technologies; #A21042, Vienna, Austria |

|                                         |                                             |
|-----------------------------------------|---------------------------------------------|
| Alexa fluor goat anti mouse IgM 594nm   | Life technologies; #A21044, Vienna, Austria |
| Alexa fluor goat anti chicken IgM 647nm | Life technologies; #A21449, Vienna, Austria |
| Goat anti-guineapig IgG HRP             | DAKO; #P0141, Glostrup, Denmark             |
| Goat anti-mouse IgG HRP                 | DAKO; #P0260, Glostrup, Denmark             |

## 2.2. Methods

### 2.2.1. Animal experiments

MDBs were induced in two months old male  $p62^{-/-}$ ,  $p62^{hep^{-/-}}$ ,  $p62^{f/f}$  and  $p62^{hep^{+/+}}$  mice by feeding DDC (0.1% DDC wt/wt in the standard diet; Sigma-Aldrich, Steinheim, Germany) for next 8 weeks. To study MDB disappearance, DDC-fed mice were allowed to recover for 4 weeks on the standard diet. At least five male mice per genotype and treatment condition were used whereas five untreated male mice of the same age, and sex served as controls. Mice were sacrificed by cervical dislocation and blood was collected by cardiac puncture. Serum was analyzed using automatic chemistry analyzer (Hitachi, Roche, Switzerland). Livers were immediately removed and cut into pieces that were (i) snap frozen in methyl butane precooled with liquid nitrogen for immunofluorescence staining; (ii) fixed in formalin for histological/immunohistochemical analysis or (iii) placed in liquid nitrogen for biochemical/RNA expression analyzes. Animal experiments were approved by the Austrian Federal Ministry of Science, Research and Economy in compliance with the Austrian Law for Welfare of Laboratory Animals with the license number: BMWF-66.010/0114-II/3b/2012 and BMWF-66.010/0114-ii/3b/2013.

### 2.2.2. Extraction of DNA/RNA and Polymerase Chain Reaction (PCR)

DNA was extracted from mouse tail using DirectPCR® Lysis Reagent Tail (PEQLAB) and used for all genotyping PCRs. For all cloning reactions, DNA was extracted using Maxi-prep kit (QIAGEN) or Mini-prep Kit (Fermentas). All PCR's were performed using *Taq* PCR Core Kit (QIAGEN) according to manufacturer's instructions and PCR conditions were optimized depending on the amplified product size. Livers were homogenized using MagNA Lyser (Roche) for 30 seconds at 7500 rpm, and RNA was extracted with TRIZOL reagent (Life Technologies) following manufacturer's instructions. 2 µg of RNA was transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To check the

efficiency of p62 deletion, semi-quantitative reverse-transcriptase PCR (RT-PCR) was performed. To determine the expression of selected genes, quantitative real-time qPCR was performed with 7900HT Fast Real-Time Sequence Detection System (Applied Biosystems) and cDNA was amplified with Power SYBR® Green PCR Master Mix (Applied Biosystems). Samples were analyzed in triplicates using specific primers (primer table), and  $\beta$ -actin was used as an internal control. Relative expression levels were computed as a difference in the amount of the gene of interest and the internal control. 4-5 mice per group were analyzed, and results are presented as mean $\pm$ SD.

### **2.2.3. Immunofluorescence and immunoelectron microscopy**

Immunofluorescence staining of tissues was performed using 4  $\mu$ m thick liver tissue cryosections fixed in precooled acetone for 10 minutes, and immunocytochemistry was performed on cells grown on coverslips fixed in methanol for 5 minutes followed by 30 seconds in acetone at -20°C. After a brief drying period, 100  $\mu$ l of primary antibody diluted in PBS was added to the section and incubated for one hour or overnight followed by PBS washes (3X) for 5 minutes. The fluorescently conjugated secondary antibody was diluted in PBS and incubated for 30 minutes. Tissue sections were given a wash with PBS (3X) for 5 minutes and briefly dipped in absolute ethanol to dehydrate the sections (only for tissue sections) and mounted with anti-fade mounting medium (DAKO). To detect proteins with cross-beta sheet structure, acetone-fixed cryosections or methanol: acetone fixed cells were incubated with the luminescent conjugated oligothiophene h-HTAA (1:10,000) for 20 minutes before the addition of primary antibodies and rest of the procedure was same as immunofluorescence for tissues and cells. Following primary antibodies were used: p62 (#GP62-C; Progen), K8 (#Ks 18.04, Progen and #ab53118; Abcam) and K18 (#Ks 18.04, Progen and #ab53118; Abcam), Mm120-1 (made in house; paper reference), ubiquitin (#3933S; Cell signaling and #GW10073F; Sigma Aldrich;) and Nbr1 (#NBP171703; Novus). Secondary antibodies were Alexa Fluor goat anti-rabbit IgG 488nm (#A11034) and 594nm (#A11012), goat anti-mouse IgM 488nm (#A21042) and 594nm (#A21044) and goat anti-chicken 647nm (#A21449; all from Life Technologies).

Stained liver sections were analyzed on an LSM 510 META (Carl Zeiss, Jena, Germany) confocal laser scanning microscope with Plan-Neofluar 40/0.75 objective and a 32-element

photomultiplier tube array detector (META detector accessory). Images were processed with Zeiss LSM Image Browser 4.2 software.

**Table 6. Antibody dilutions and incubation times of antibodies used in immunofluorescence experiments**

| Target Proteins         | Primary Abs              | Dilution | Incubation time | Secondary Abs                          | Dilution | Incubation time |
|-------------------------|--------------------------|----------|-----------------|----------------------------------------|----------|-----------------|
| <b>p62</b>              | p62 CT; Progen           | 1:500    | 1 hour          | Anti guniepig rhodamine                | 1:500    | 30 min          |
| <b>M<sub>M</sub>120</b> | Cell culture supernatant | neat     | 30 min          | Alexa fluor IgM 488 anti-mouse         | 1:500    | 30 min          |
| <b>Keratin 8</b>        | K8.7; Progen             | 1:100    | 30 min          | Alexa fluor IgG 488/594 anti-mouse     | 1:500    | 30 min          |
|                         | K8; Abcam                | 1:100    | 30 min          | Alexa fluor IgG 488/594 anti-rabbit    | 1:500    | 30 min          |
| <b>Keratin 18</b>       | K18; Progen              | 1:100    | 30 min          | Alexa fluor IgG 488/594/647 anti-mouse | 1:500    | 30 min          |
|                         | K18; Abcam               | 1:100    | 30 min          | Alexa fluor IgG 488/594 anti-rabbit    | 1:500    | 30 min          |
| <b>Keratin 7</b>        | K7; Abcam                | 1:200    | 2 hours         | Alexa fluor IgG 488/594 anti-mouse     | 1:500    | 1 hour          |
| <b>Keratin 17</b>       | K17; Abcam               | 1:200    | 2 hours         | Alexa fluor IgG 488/594 anti-rabbit    | 1:500    | 1 hour          |
| <b>Keratin 19</b>       | K18; Abcam               | 1:200    | 1 hour          | Alexa fluor IgG 488/594 anti-rabbit    | 1:500    | 1 hour          |
| <b>SMI-31</b>           | SMI-31; Sternberger      | 1:100    | 1 hour          | Alexa fluor IgG 488/594 anti-mouse     | 1:500    | 30 min          |
| <b>Ubiquitin</b>        | Ub; Cell signalling      | 1:100    | 1 hour          | Alexa fluor IgG 488/594 anti-rabbit    | 1:500    | 30 min          |
|                         | Ub; Sigma                | 1:100    | 2 hours         | Alexa fluor IgG 647 anti-chicken       | 1:500    | 30 min          |

Immunoelectron microscopy was performed on acetone-fixed cryosections following the same procedure as immunofluorescence with a gold labeled secondary antibody. Ultrathin sections (50 nm thick) were prepared with an ultra-microtome (Ultracut; Reichert-Jung, Nussloch,

Germany), stained with uranyl acetate and lead citrate, and examined with a CM 100 electron microscope (Philips Electronic Instruments Co., Eindhoven, Netherlands) at 80 kV.

**Table 7. Antibody dilutions and incubation times of antibodies used in immunoelectron experiments**

| Target protein            | Primary Ab                                     | Dilution | Incubation time | Secondary Ab                                                                                            | Dilution | Incubation time |
|---------------------------|------------------------------------------------|----------|-----------------|---------------------------------------------------------------------------------------------------------|----------|-----------------|
| <b>M<sub>M</sub>120-1</b> | M <sub>M</sub> 120-1; cell culture supernatant | neat     | 2 hours         | 18nm Colloidal gold affini Pure Goat anti-mouse IgM, $\mu$ chain; # 115-215-075; Jackson ImmunoResearch | 1:10     | 2 hours         |
| <b>p62</b>                | p62 CT; Progen                                 | 1:100    | 2 hours         | Goat pAB to Guniepig IgG 10nm; # ab39167; Abcam                                                         | 1:10     | 2 hours         |
| <b>K8</b>                 | K8.7; Progen                                   | 1:100    | 2 hours         | 18nm Colloidal gold affini Pure Goat anti-mouse IgG(H+L); #115-215-146; Jackson ImmunoResearch,         | 1:10     | 2 hours         |
|                           | K8; Abcam                                      | 1:100    | 2 hours         | 12nm colloidal gold affini Pure Goat anti-rabbit IgG(H+L), code 111-205-144 Jackson ImmunoResearch      | 1:10     | 2 hours         |
| <b>Keratin 18</b>         | K18; Progen                                    | 1:100    | 2 hours         | 18nm Colloidal gold affini Pure Goat anti-mouse IgG(H+L); #115-215-146; Jackson ImmunoResearch,         | 1:10     | 2 hours         |
|                           | K18; Abcam                                     | 1:100    | 2 hours         | 12nm colloidal gold affini Pure Goat anti-rabbit IgG(H+L), code 111-205-144 Jackson ImmunoResearch      | 1:10     | 2 hours         |

## 2.2.4. Histological stainings

### Haematoxylin & eosin (H&E) staining

H&E staining was performed on 4 $\mu$ m thick formaldehyde fixed paraffin embedded (FFPE) liver sections. Paraffin was removed from FFPE liver sections by incubating at 65°C for 20 minutes followed by incubation in xylene for 15 minutes. The slides were rehydrated for 2 minutes each with decreasing concentrations of ethanol (90%, 70% and 50 %). After a brief wash with dd H<sub>2</sub>O, the liver sections were incubated with hematoxylin for 2 minutes followed by 30 seconds

incubation with eosin. After that, the liver sections were washed with warm tap water for 2 minutes and mounted with entellan.

## **Immunohistochemistry**

FFPE liver sections were first deparaffinized and rehydrated as mentioned above. Antigen retrieval was done in 0.1M EDTA buffer, pH8 for p62 and 0.1M citrate buffer, pH6 for K8 labeling for 30 minutes at 240 W. Endogenous peroxidase activity was blocked by incubation in 1% H<sub>2</sub>O<sub>2</sub> in methanol for 10 minutes. Exposure to primary and secondary antibodies took place for 60 and 30 minutes, respectively. Goat anti-guinea pig IgG HRP (#P0141; DAKO) and goat anti-mouse IgG HRP (#P0260; DAKO) secondary antibodies were used for p62 and K8 staining, respectively. Subsequently, a ready to use DAKO Envision+Dual link (#K4061; DAKO) solution was added for 30 minutes and the antigens were detected using AEC substrate chromogen.

## **Oil-Red-O staining**

Oil-Red-O staining was performed on 8-10 µm thick liver cryosection fixed in formalin. After a brief incubation in 60% isopropanol, liver sections were incubated in Oil-Red-O working solution for 15 minutes. Liver sections were again rinsed in 60% isopropanol and stained with hematoxylin for 1 minute. The excess stain of the liver sections was washed off with warm tap water and mounted with glycerine gelly.

### **2.2.5. Protein isolation and western blot**

Whole liver cell homogenates were prepared using 3% Sodium Dodecyl Sulphate (SDS) containing a buffer. Alternatively, soluble proteins were extracted with Triton-X-100 buffer and keratin-enriched fractions were obtained as described previously taking advantage of their relative insolubility in Triton and high-salt buffers. Protein concentration was quantified with Bradford assay, an equal amount of proteins were separated using Nu-PAGE Bis-Tris Gels (Life Technologies) and transferred onto a PVDF membrane for immunoblotting. The antigen-antibody complexes were visualized using Pierce ECL Western Blotting Substrate (Thermo Scientific). The following antibodies were used: anti-p62 / SQSTM1 ( #GP62-C; Progen ), Keratin 8 ( #Ks 8.7, Progen and #ab53118; Abcam), Ubiquitin ( #3933S; Cell signaling ) and Nbr1 ( #NBP171703; Novus ) and LC3 antibody (#NB100-2220; Novus).

**Table 8. Optimized westernblot experimental parameters for target proteins.**

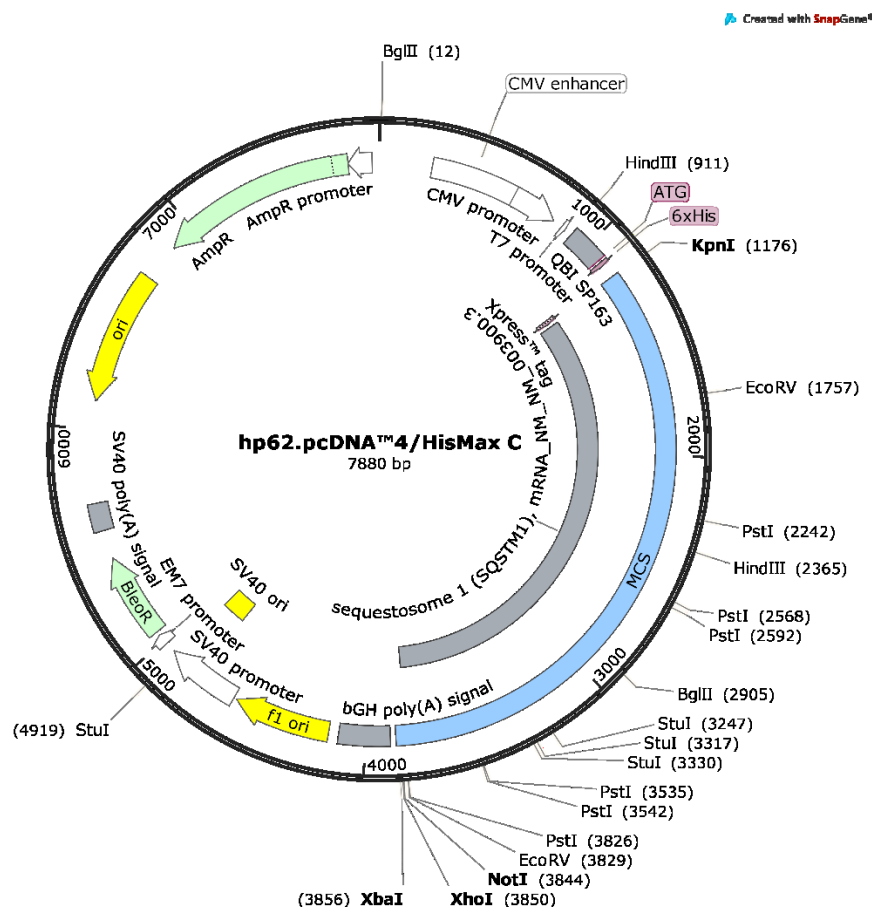
| Target Protein            | Expected bands | Transfer               | Blocking | Primary Ab; dilutions; incubation time      | Secondary Ab; dilutions; incubation time                                     | ECL visualisation                                                               |
|---------------------------|----------------|------------------------|----------|---------------------------------------------|------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| <b>p62</b>                | 48 kD & 44 kD  | 2.5 hours              | 1 hour   | p62 CT; Progen; 1:500; overnight            | DAKO anti guniepig; 1:1000; 4 hours                                          | Normal                                                                          |
| <b>K8</b>                 | 51 kD<br>91 kD | 2.5 hours to overnight | 2 hour   | K8.7 Progen or K8abcam; 1:1000; overnight   | Anti mouse IgG DAKO- 1:1000; 4hours<br>Anti rabbit IgG DAKO- 1:500; 4 hours  | Normal- untreated samples<br>Super sensitivity- DDC samples, insoluble extracts |
| <b>K18</b>                | 48 kD          | 2.5 hours to overnight | 2 hours  | K18.04 DAKO or abcam; 1:1000; overnight     | Anti mouse IgG DAKO- 1:1000; 4hours<br>Anti rabbit IgG DAKO- 1:5000; 4 hours | Normal- untreated sample<br>Super sensitivity- DDC samples, insoluble extracts  |
| <b>K7</b>                 | 51 kD          | overnight              | 4 hours  | K7; Abcam; 1:1000; overnight                | Cell signaling IgG mouse; 1:1000; 4 hours                                    | normal                                                                          |
| <b>K17</b>                | 48 kD          | overnight              | 4 hours  | K17; Abcam; overnight; 1:1000               | Cell signaling IgG rabbit; 1:1000; 4 hours,                                  | normal                                                                          |
| <b>NBR1</b>               | 99 kD          | overnight              | 4 hours  | NBR1; Novus; 1:1000; overnight              | Cell signaling IgG rabbit; 1:1000; 4 hours                                   | super sensitivity                                                               |
| <b>ubiquitin</b>          | Smears; 37 kD  | overnight              | 2 hours  | ubiquitin; cell signaling; 1:1000 overnight | Cell signaling IgG rabbit; 1:1000; 4 hours                                   | super sensitivity                                                               |
| <b>HSP70</b>              | 70 kD          | overnight              | 4 hours  | HSP70; santa cruz; 1:1000; overnight        | Cell signaling IgG mouse; 1:1000; 4 hours                                    | normal                                                                          |
| <b>HSP25</b>              | 25 kD          | 2.5 hours              | 4 hours  | HSP25; stress gene; 1:1000; 4 hours         | Cell signaling IgG rabbit; 1:1000; 4 hours                                   | normal                                                                          |
| <b>Transglutaminase 2</b> | 77 kD          | overnight              | 4 hours  | Tg2; neomarkers; 1:1000; overnight          | Cell signaling IgG rabbit; 1:1000; 4 hours                                   | normal                                                                          |
| <b>Nrf2</b>               | 65 kD          | overnight              | 4 hours  | Nrf2; santa cruz, 1:500 , overnight         | Cell signaling IgG rabbit; 1:1000; 4 hours                                   | super sensitivity                                                               |
| <b>Keap 1</b>             | 70 kD          | overnight              | 4 hours  | Keap1; Abcam;overnight ; 1:1000             | Cell signaling IgG rabbit; 1:1000; 4 hours                                   | normal                                                                          |
| <b>HO1</b>                | 32 kD          | overnight              | 4 hours  | HO1; enzolife sciences; overnight 1:1000    | Cell signaling IgG rabbit; 1:1000; 4 hours                                   | normal                                                                          |
| <b>LC3</b>                | 17 kD & 15 kD  | 2.5 hours              | 4 hours  | LC3; Novus; overnight; 1:1000               | Cell signaling IgG rabbit; 1:1000; 4 hours                                   | super sensitivity                                                               |

## 2.2.6. Expression plasmids

Expression plasmids used in this study are as follows:

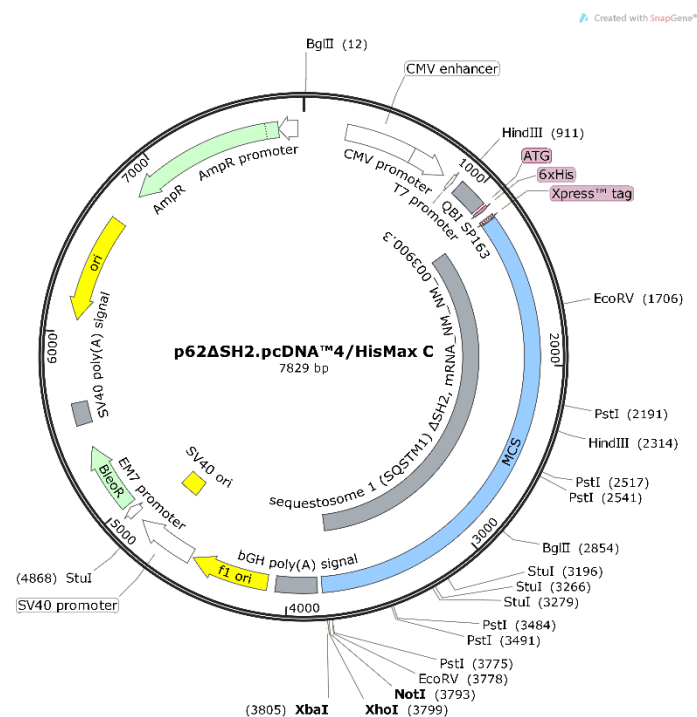
### 1. hp62.PCDNA4<sup>TM</sup>/HisMaxC

p62 Sequence: NM\_0039003 (cloned from Pos. 35 to Pos. 1360)



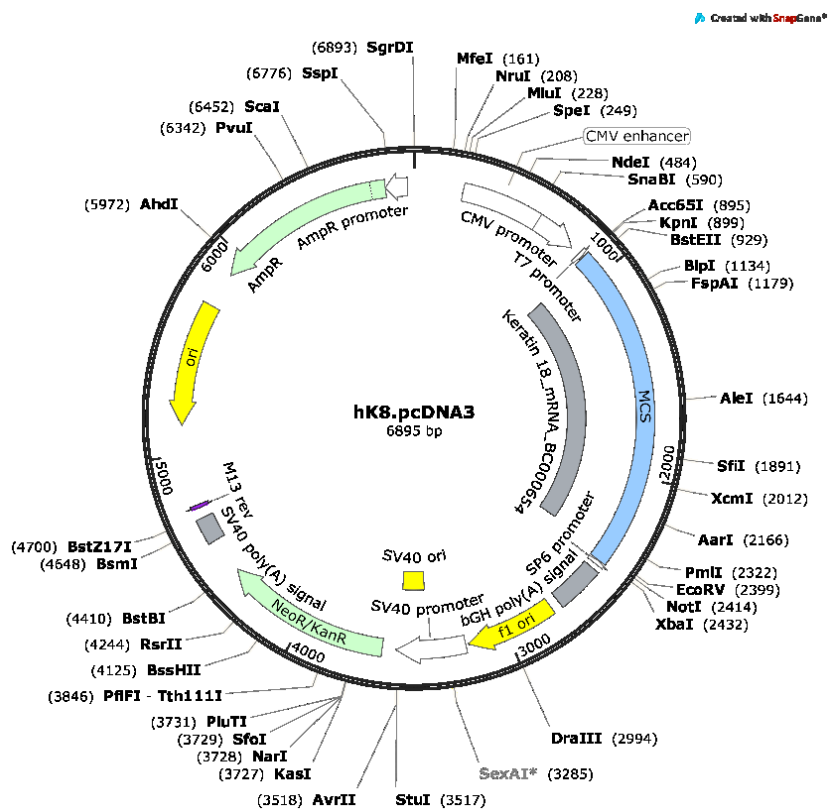
### 2. hp62 $\Delta$ SH2. PCDNA4<sup>TM</sup>/HisMaxC

p62 Sequence: NM\_0039003; Deletion 1-50 bp (cloned from Pos. 85 to Pos. 1360)



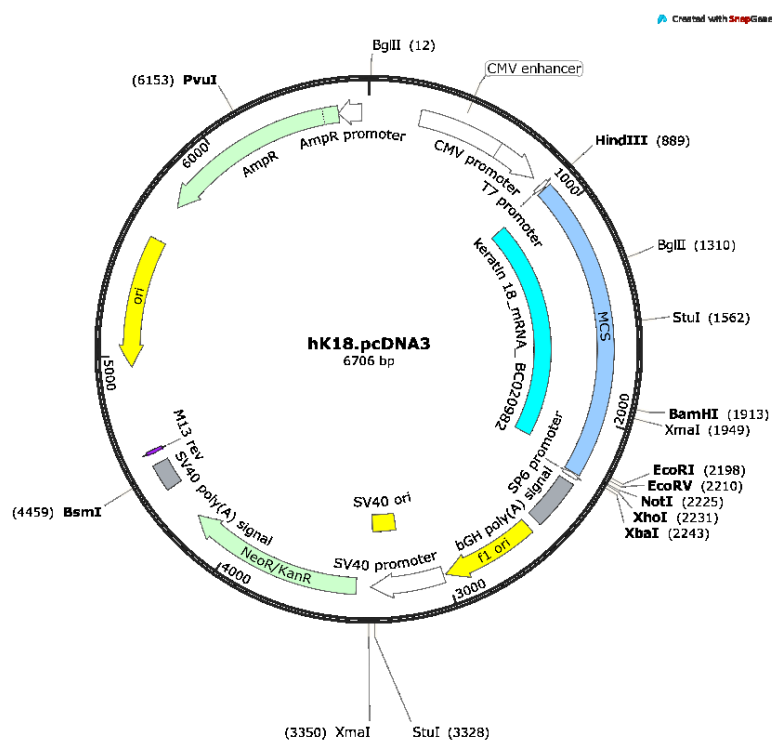
### 3. hK8.pcDNA3

K8 Sequence: BC000654 (cloned from Pos. 65 to Pos. 1529)

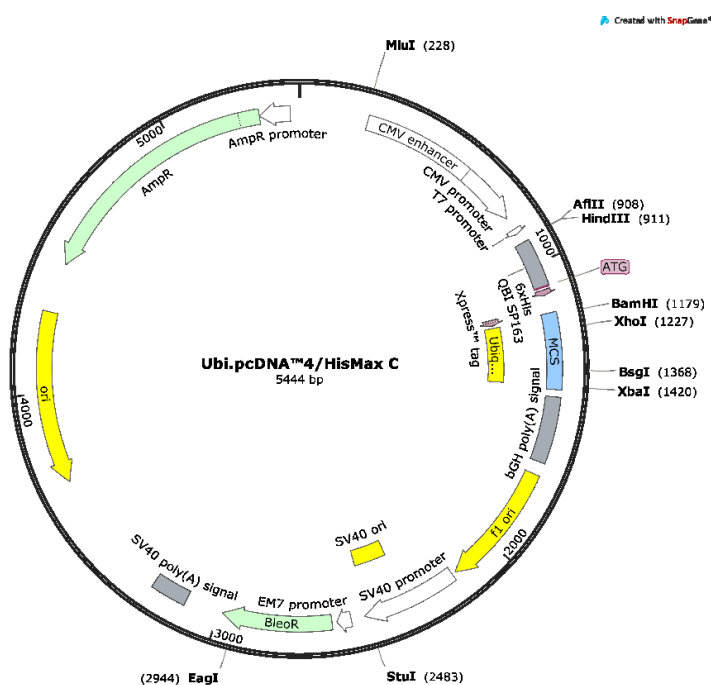


## 4. hK18.pcDNA3

K18 Sequence: BC020982 (cloned from Pos.48 to Pos. 1349)

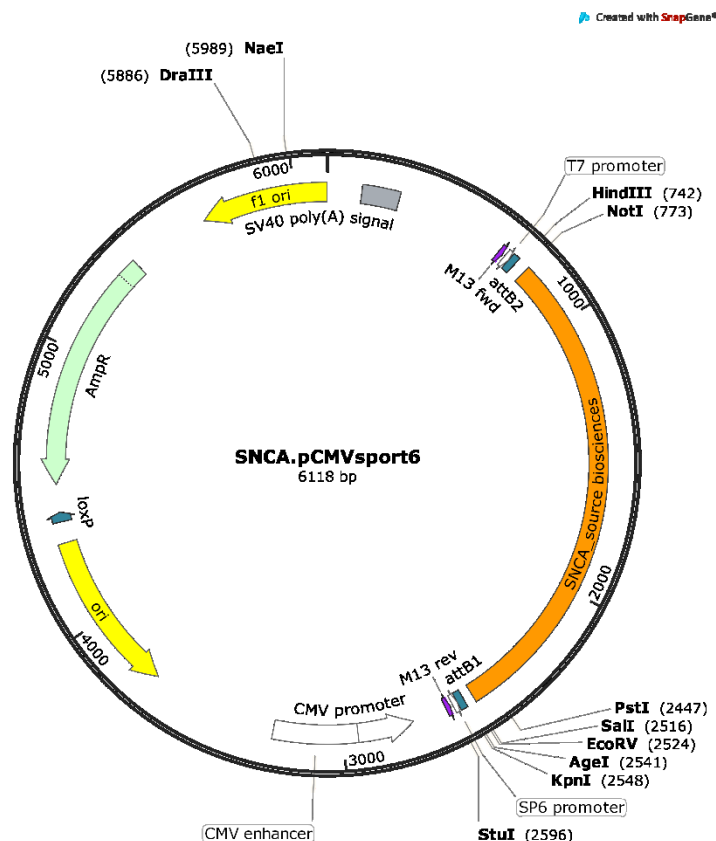
5. hUbi.PCDNA4<sup>TM</sup>/HisMaxC

Ubiquitin Sequence: M26880 (cloned from Pos. 1960 to Pos. 2193)



## 6. SNCA.pCMVsport6

Sequence: NM\_000345.3



### 2.2.7. Standard cell culture conditions

Chinese Hamster Ovary Cells (CHO-K1 -CCL-61 ATCC Manassas VA) cell lines were maintained in Ham's-F12 media supplied by SIGMA supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin/ streptomycin(1%) and kept in the incubator at 37°C and 5% CO<sub>2</sub>. Cells were split at 1:3 when 80-90% confluency was achieved.

### 2.2.8. Transfection

All the transfection experiments were performed on CHO-K1 cells or p62 knockout mouse embryonic fibroblasts using Lipofectamine 2000 transfection reagent (life technologies). 400,000 cells were seeded in 6 well plates and allowed to grow for 80% confluency in Hams-

F12 media (GIBCO) or Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 10% fetal calf serum and 1% penicillin / streptomycin antibiotics. On the day of transfection, 2 ug of DNA and 5ul of lipofectamine 2000 reagent were added to 500ul Opti-MEM reduced serum media (GIBCO) and incubated for 20 minutes for proper acclimatization of the mixture. Thereafter, the complete growth medium of 80% confluent cells was changed with fresh antibiotic-free medium required for optimum transfection. The mixture containing DNA and lipofectamine 2000 was added onto the 6 well plate prepared for transfection and incubated for 16 hours in a CO<sub>2</sub> incubator at 37°C. Transfection efficiency was determined in 3 independent experiments by counting GFP-positive cells vs. the total number of cells in 30 vision fields at 250 X magnification.

**Table 9. Experimental setup for transfections**

|                                            | DNA constructs                | DNA concentration. (ng/μl) | DNA (μl)     | LF2000 (μl) | opt-mem |
|--------------------------------------------|-------------------------------|----------------------------|--------------|-------------|---------|
| <b>Single/ Double/ Triple transfection</b> |                               |                            | 6μg in total | 15ul        |         |
| <b>exp1</b>                                | <b>p62.PCDNA4/HisMax C</b>    | 1049.22                    | <b>2</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
| <b>exp 2</b>                               | <b>p62ΔSH2.PCDNA4/hismaxC</b> | 1043.2                     | <b>2</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
| <b>exp 3</b>                               | <b>K8.pcDNA3</b>              | 1501.32                    | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
| <b>exp4</b>                                | <b>k18.pcDNA3</b>             | 1370.12                    | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
| <b>exp5</b>                                | <b>Ub.PCDNA4/HisMax C</b>     | 2481.5                     | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
| <b>exp6</b>                                | <b>SNCA.PCMV sport6</b>       | 1275.62                    | <b>2</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |
|                                            | pcDNA4_ empty vector          | 2531.38                    | <b>1</b>     | 5           | 500ul   |

|                     |                      |         |          |   |       |
|---------------------|----------------------|---------|----------|---|-------|
|                     |                      |         |          |   |       |
| <b>exp7_control</b> | pcDNA4_ empty vector | 2531.38 | <b>1</b> | 5 | 500ul |
|                     | pcDNA4_ empty vector | 2531.38 | <b>1</b> | 5 | 500ul |
|                     | pcDNA4_ empty vector | 2531.38 | <b>1</b> | 5 | 500ul |

### 2.2.9. Bioinformatics

UniGene database and ENSEMBL Genome Browser database was used for the analysis of p62/Sequestosome-1 transcript variants. For protein isoforms, UniProt database and the NCBI protein database were used. Sequence alignments of the transcript variants and protein isoforms were performed using BLAST and CLUSTAL-W interfaces.

### 2.2.10. Data Analysis

The graphical data were prepared, and statistical analysis was performed using Prism 5 software (Graph Pad software). All reported P values are two-tailed and values less than 0.05 were considered statistically significant. Densitometric analysis for protein bands was performed using FluorChem 5500 software (Alpha Innotech). Morphometric quantification of Mm120-1 labeled immunofluorescence images was carried out with ImageJ interface. Photoshop CS5 (Adobe) was used to prepare the immunofluorescence figures whereas schematics were assembled with Illustrator CS2 software (Adobe).

# Results: Part I

## **p62/Sequestosome-1 is indispensable for maturation and stabilization of Mallory-Denk bodies in a mouse model of drug induced hepatotoxicity**

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*\*Declaration: Parts of the results are taken from (Lahiri et al., 2016)*

### **3.1. Aims of the study**

The majority of the studies on the functional role of p62 in protein aggregation is based on starvation-induced autophagy or genetic inactivation of autophagy-related proteins such as Atg7 and p62. Earlier studies revealed that p62 binds to polyubiquitinated misfolded proteins via its UBA domain, shapes them into sequestosomes and recruits them to cell degradation pathways such as UPS or autophagy (Geetha and Wooten, 2002, Komatsu et al., 2007). p62 seems to enhance the development of inclusions since genetic deletion of p62 resulted in the reduction of aggregates in both mice and flies (Nezis et al., 2008, Komatsu and Ichimura, 2010) but whether p62 is essential for the formation of disease-related inclusions, such as MDBs remained unknown.

In this study, we have selected MDBs as a prototype to investigate the role of p62 in a pathogenic aggregate formation. The central components of MDBs are Keratins, p62, and ubiquitin (Ub). Keratins are studied extensively in context to their structural and molecular alteration in MDB formation process but, the physiological function of the parallel increase in the expression of p62 in MDBs, is still elusive. Previous cell culture studies showed p62's availability is a crucial factor for MDB formation when transfected with p62, K8 and Ub alone or in combination (See introduction, section 1.4). However, the molecular modifications of MDB components and its deposition to a large extent is dependent on chronic oxidative stress which cannot be mimicked in a cell-culture models. Hence, we have employed both p62 whole body knockout mice (p62<sup>-/-</sup>) and hepatocyte-specific knockout mice (p62<sup>hep</sup><sup>-/-</sup>) and fed them with DDC for 8 weeks to induce MDB formation. The specific aims of the study are:

1. How the availability of p62 influences the MDB formation process in context to keratin-crosslinking,  $\beta$ -sheet formation, ubiquitination and binding of other molecular components such as Hsp's, other keratins to MDBs.
2. Whether p62 shuttles the ubiquitinated MDBs to autophagosomes for degradation.
3. Characterization of p62's ability to influence cellular defense mechanism in response to chronic DDC-mediated injury.
4. To elucidate the morphogenesis of MDBs and their relation to IHBs and other p62-containing inclusion using p62 and ubiquitin as a window into the aggregation process.

## 3.2. Results

### 3.2.1. Generation of p62<sup>-/-</sup> and p62hep<sup>-/-</sup> mice

We generated p62 knockout mice using the Cre/loxP strategy. 5' fragment of the p62/Sqstm1 gene (NC\_000077) containing exons 1-5 was PCR-amplified and inserted via SacI into the pBluescript vector (kindly provided by C. Birchmeier; Max-Delbrueck-Centre for Molecular Medicine, Berlin, Germany) upstream of the loxP-neomycin-loxP cassette (Figure 10A). An additional loxP site was introduced before exon 5 using the QuickChange Site-Directed Mutagenesis protocol (Stratagene). To complete the construct, the PCR-amplified 3' fragment of the p62/Sqstm1 gene was ligated via SalI into the vector and the BamHI-SalI-cleaved product was introduced into a wildtype embryonic stem cell line with a 129S2/SvPas background by electroporation. Neomycin resistant colonies were screened for homologous recombination by long range PCR and the positive clones with a single integration event were microinjected into C57BL/6 blastocysts. Chimeric males were mated with C57BL/6-Tg (Meu-Cre40) animals (Postic et al., 1999, Leneuve et al., 2003) to obtain mice which either lacked the neo-selection cassette but contained floxed exons 1 to 4 of the Sqstm1 gene (Sqstm1<sup>tm1Biat</sup>, designated in the text as p62<sup>floxed/floxed</sup> or p62<sup>f/f</sup>) or lacked the complete floxed region (Sqstm1<sup>tm1.1Biat</sup>, designated in the text as p62<sup>-/-</sup>) (Figure 10A). To generate a hepatocyte-specific p62-deficient mouse line (p62hep<sup>-/-</sup>), p62<sup>f/f</sup> mice were mated with mice that express Cre-recombinase under the control of albumin promoter (Alb-Cre) (Figure 10A) (Postic et al., 1999). To obtain mice that are

susceptible to MDB formation, animals were crossed for several generations with wildtype 129S2/SvPas mice.

Genotyping was performed by PCR using genomic tail tip DNA and a combination of three sets of primers as shown in Figure 10B & C. Littermate wildtypes of the  $p62^{-/-}$  and  $p62^{hep^{-/-}}$  mouse strains were included in all experiments. To test for the presence of the Alb-Cre transgene, an additional Cre-specific PCR was performed (Figure 10C, PCR C; Table 10). The PCR conditions maintained were (i) initial denaturation at 95°C for 5 minutes, (ii) denaturation at 95°C for 30 sec (iii) annealing at 60°C for 30 seconds, (iv) elongation at 72°C for 30 seconds, (v) final elongation at 72 °C for 10 minutes for 35 cycles.

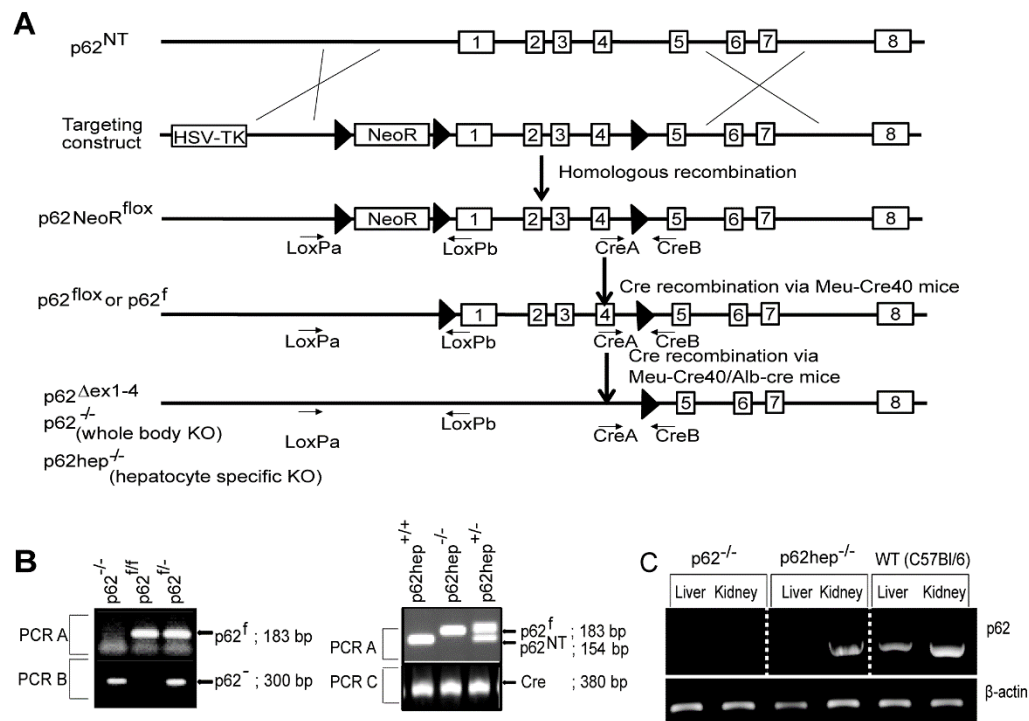


**Figure 10. Generation of total ( $p62^{-/-}$ ) and hepatocyte-specific ( $p62^{hep^{-/-}}$ ) p62-knockout mice**

(A) The scheme depicts the non-transgenic ( $p62^{NT}$ ) allele (upper diagram) consisting of 8 exons (numbered rectangles) and the targeting construct consisting of neomycin resistance selection cassette (NeoR), herpes simplex virus thymidine kinase gene cassette (HSV-TK) and three loxP sequences (represented as block triangles). Two of the loxP sites flank NeoR and the third is

located between exon 4 and 5 of the p62 gene. The p62NeoR<sup>fllox</sup> allele was generated by homologous recombination in ES cells. A deletion of NeoR and the first loxP site resulted in the conditional p62<sup>f</sup> allele while an additional ubiquitous excision of exon 1-4 via crossbreeding with Meu-CRE40 mice yielded the constitutive p62<sup>Δex1-4</sup> or p62<sup>-/-</sup> knockout. The hepatocyte-specific deletion of exon 1-4 (p62hep<sup>-/-</sup>) was achieved by breeding p62<sup>f</sup> mice with animals expressing Cre-recombinase under the control of the liver-specific albumin promoter (Alb-Cre mice). The schemes also include the localization of the genotyping primers LoxPa/b and CreA/B. **(B)** PCR genotyping of offspring using tail biopsies obtained from the breeding of p62NeoR<sup>fllox</sup> mice with Meu-Cre40 (left panel) and p62<sup>f/f</sup> mice with Alb-Cre mice (right panel). The alleles and their sizes are shown on the right side of the images. **(C)** RT-PCR detects the expression levels of p62 in livers and kidney of the highlighted phenotypes. Weak p62 band in the livers of p62hep<sup>-/-</sup> mice likely corresponds to the signal from non-epithelial cells. β-actin was used as a loading control.

**Table 10. Genotyping PCR**

| PCR | Forward Primer | Reverse Primer | p62 <sup>NT</sup> | p62 <sup>f</sup> | p62 <sup>-/-</sup> |
|-----|----------------|----------------|-------------------|------------------|--------------------|
| A   | CreA           | CreB           | 154 bp            | 183 bp           |                    |
| B   | LoxPa          | LoxPb          |                   |                  | 300 bp             |
| C   | CreF           | CreR           | 380 bp            | 380 bp           | 380 bp             |

PCR A/B was performed to distinguish between p62 non-transgenic (p62<sup>NT</sup>), p62 floxed (p62<sup>f</sup>) and p62<sup>Δexon1-4</sup> (p62<sup>-</sup> or p62hep<sup>-</sup>) while PCR C was performed to detect the presence of Cre-recombinase.

### 3.2.2. Characterization of whole body p62<sup>-/-</sup> and hepatocyte specific p62hep<sup>-/-</sup> mice.

Both p62<sup>-/-</sup> and p62hep<sup>-/-</sup> and their corresponding littermate wildtype p62<sup>f/f</sup> and p62hep<sup>+/+</sup> mice were born at the expected Mendelian ratio and had a normal life span. Mice were back-crossed with 129S2/SvPas background animals for five generations because mice with this background

showed increased MDB formation (Hanada et al., 2008). Consistent with earlier findings (Rodriguez et al., 2006, Muller et al., 2013),  $p62^{-/-}$  but not  $p62^{hep-/-}$  mice became obese at the age of 4 months (Figure 11A). In particular, liver enzymes as well as serum cholesterol and triglyceride levels were in the normal range and did not differ significantly among genotypes (Figure 11C-F). Haematoxylin and Eosin (H&E) staining of liver sections revealed normal liver morphology for 2 months old mice of all genotypes (Figure 12, panel 1). However, significant steatosis was observed in both  $p62$ -deficient lines at the age of 4 months, which was also confirmed by Oil-Red-O staining (Figure 12, panels 2-3). However,  $p62^{-/-}$  mice displayed a pronounced microvesicular steatosis concentrated in acinar zones 2 and 3 with some confluence to larger fat vesicles at the periphery (towards zone 1), whereas  $p62^{hep-/-}$  animals showed macrovesicular steatosis (scores 2) predominantly in acinar zones 2 and 3.

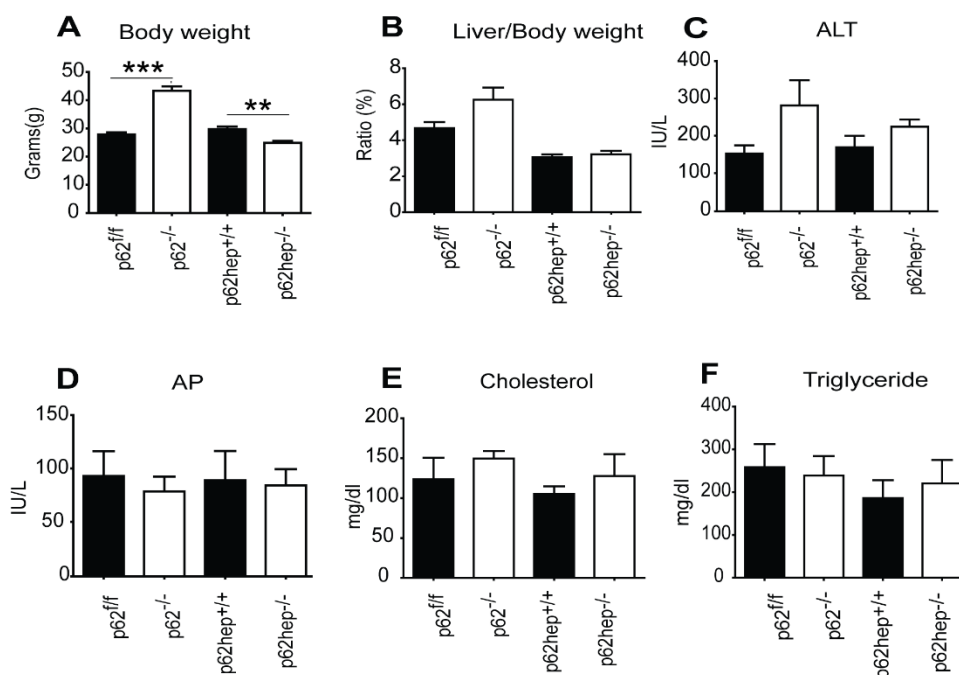


**Figure 11. Total  $p62$ -knockout ( $p62^{-/-}$ ) mice develop obesity but no changes in liver enzymes or parameters of fat metabolism.**

(A) Body weight, (B) liver-to-body weight ratio, (C) serum alanine aminotransferase (ALT), (D) alkaline phosphatase (AP), (E) cholesterol and (F) triglyceride levels were measured in 4 months old total ( $p62^{-/-}$ ) and hepatocyte-specific ( $p62^{hep-/-}$ )  $p62$ -knockout mice as well as in

their littermates with unaffected hepatic p62 expression ( $p62^{f/f}$  and  $p62^{hep+/+}$ ). All the mice were fed with a normal standard diet. Note the development of obesity in  $p62^{-/-}$  animals while the other parameters remained unaffected. Values are expressed as mean  $\pm$  SEM. At least five animals per genotype were analyzed using student's t-test. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ .

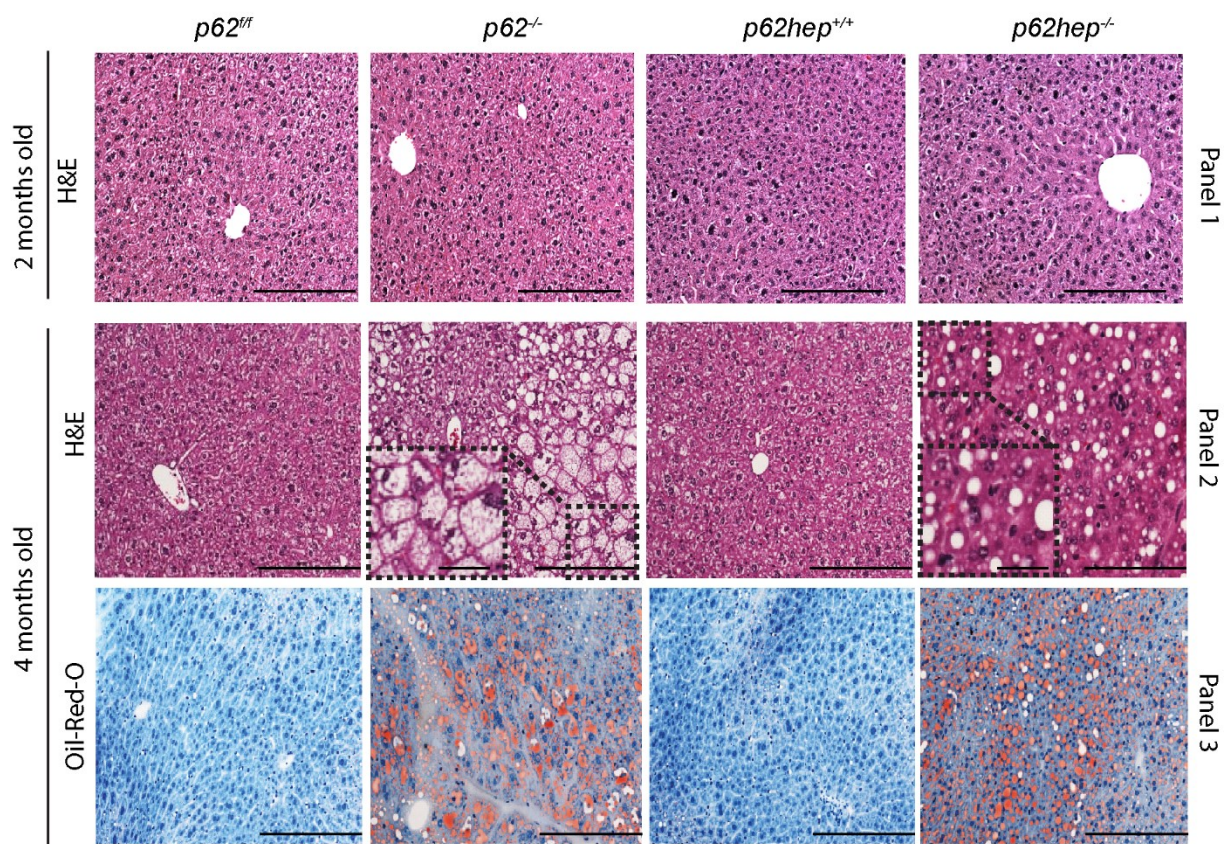


**Figure 12. Total p62 ( $p62^{-/-}$ ) and hepatocyte-specific ( $p62^{hep-/-}$ ) p62 knockout mice display liver steatosis.**

H&E staining was performed on liver sections of 2 months (panel 1) and 4 months old  $p62^{-/-}$ ,  $p62^{hep-/-}$  (panel 2) mice as well as their littermates  $p62^{f/f}$  and  $p62^{hep+/+}$  (Scale bar = 100  $\mu$ m; inset shows the higher magnification, scale bar = 50  $\mu$ m). Oil-Red-O staining was performed on 4 months old mice of indicated genotypes (panel 3). Note the development of microvesicular steatosis in  $p62^{-/-}$  and macrovesicular steatosis in  $p62^{hep-/-}$  animals. All the animals were fed with a normal standard diet.

### 3.2.3. p62 deficiency prevents the formation of mature MDBs.

To induce MDB formation, mice were fed a diet containing 0.1% DDC for 8 weeks starting at the age of 2 months. Among DDC-exposed animals, p62<sup>-/-</sup> mice displayed a significantly higher body weight than p62<sup>f/f</sup> mice whereas no difference was seen between p62hep<sup>+/+</sup> and p62hep<sup>-/-</sup> mice (Figure 13). As reported previously, DDC treatment induced a significant increase in liver-related serum enzymes and liver/body weight ratios (Stumptner et al., 2001a). However, all parameters did not differ significantly among genotypes (Figure 13B-D).

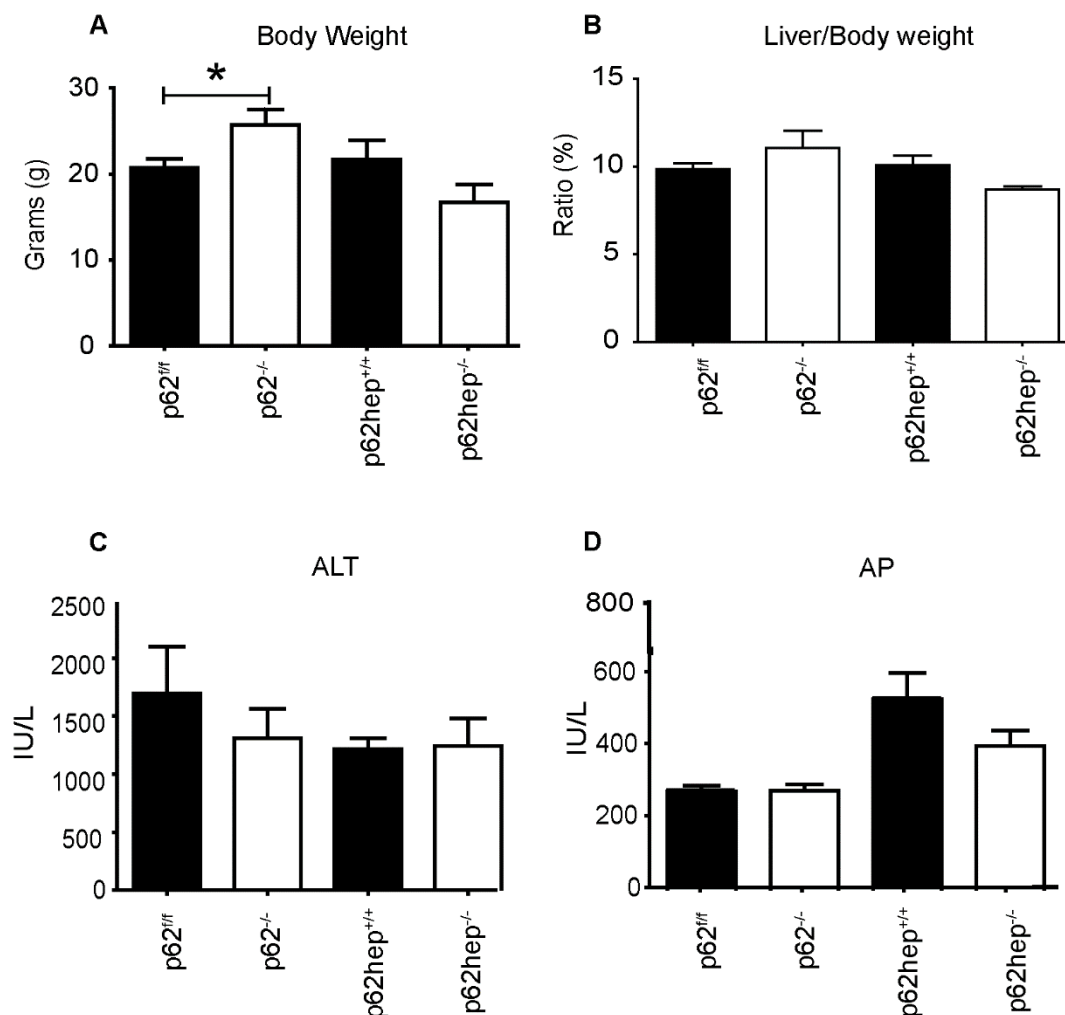


**Figure 13. The extent of DDC-induced liver injury is not affected by loss of p62.**

(A) Body weight, (B) liver-to-body weight ratio, (C) serum alanine aminotransferase (ALT) (D) alkaline phosphatase (AP) levels were measured in 8 weeks DDC-fed 4 months old p62<sup>-/-</sup> and p62hep<sup>-/-</sup> mice as well as in their littermates p62<sup>f/f</sup> and p62hep<sup>+/+</sup>. Among DDC-fed mice, p62<sup>-/-</sup>

mice displayed significantly higher body weight than  $p62^{f/f}$  animals. DDC induced a substantial increase in liver size and liver enzymes. However, none of these parameters differed between  $p62$ -deficient animals and their respective littermates. Values are expressed as mean  $\pm$  SEM. At least five animals per genotype were analyzed using student's t-test. \* $p < 0.05$ .

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Histological analysis of livers from  $p62^{f/f}$ ,  $p62^{-/-}$ ,  $p62^{hep+/+}$  and  $p62^{hep-/-}$  mice fed DDC for 8 weeks (Figure 14A, panel 1), in contrast to animals fed a normal diet (Figure 12, panel 1), revealed the occurrence of characteristic features of DDC-induced liver injury such as porphyria (brown pigment deposition), bridging fibrosis, inflammation and hepatic ballooning (Figure 14A). No differences in these morphological features were found between the different genotypes. Interestingly, the typical dense and distinct eosinophilic “classical” MDBs regarded as “mature” MDBs were detected only in  $p62^{f/f}$  and  $p62^{hep+/+}$  livers (Figure 14A, panel 1) whereas in  $p62$ -deficient livers, MDBs were small and less distinct (Figure 14A, panels 2-3). The other features of DDC-induced liver alterations such as hepatocyte ballooning were not different in  $p62$ -deficient mice and their corresponding controls. Immunohistochemical staining for K8/K18 confirmed the presence of “empty” cells lacking keratin cytoskeleton in ballooned hepatocytes in all genotypes of DDC-fed mice (Figure 14A, panel 3). Further, keratin and  $p62$  immunohistochemistry and immunoblotting confirmed the genotype of the animals as well as the formation of MDBs (Figure 14A, panels 2-3 and Figure 14B).

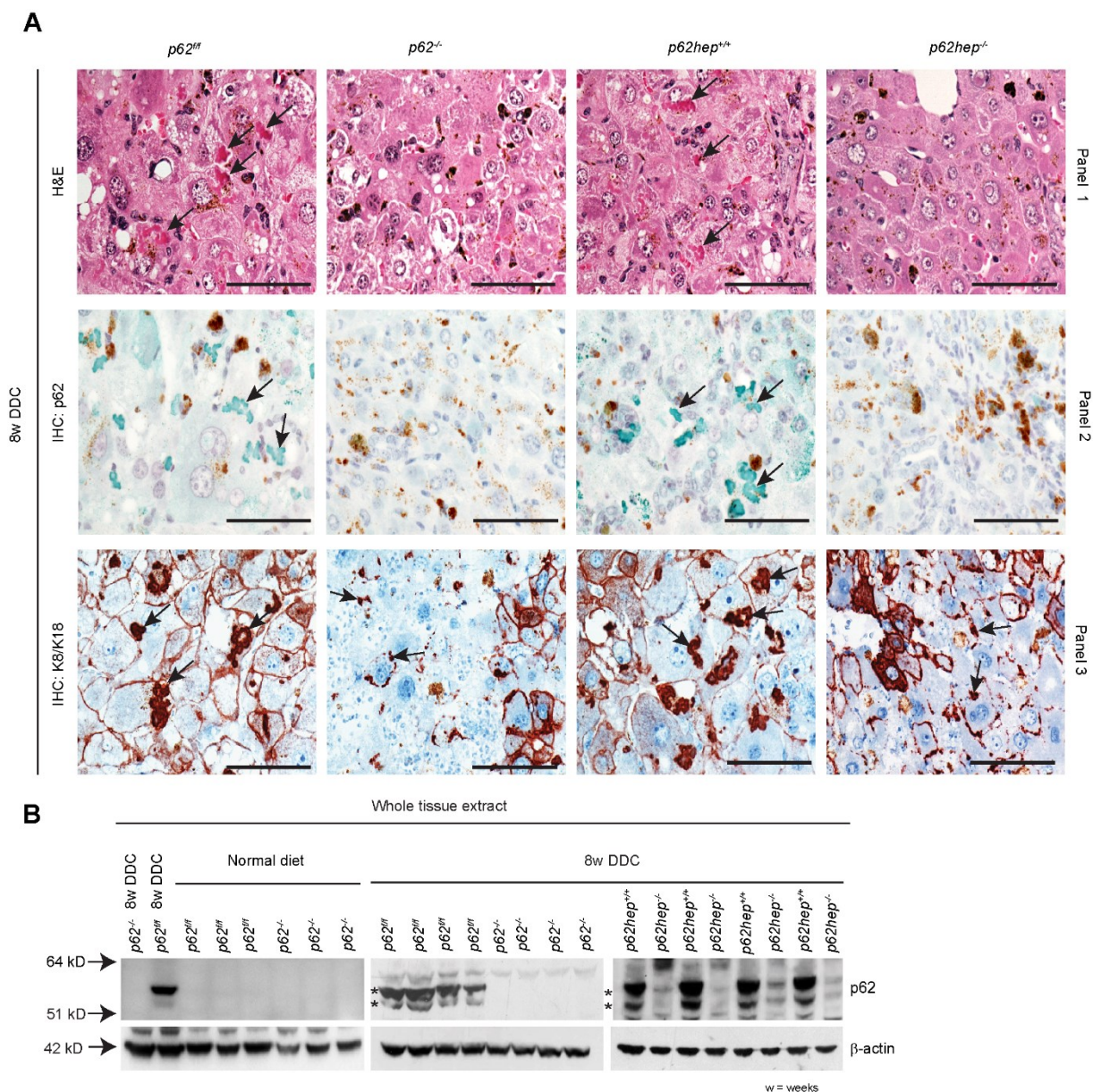


**Figure 14. Loss of p62 impairs the formation of DDC-induced MDBs.**

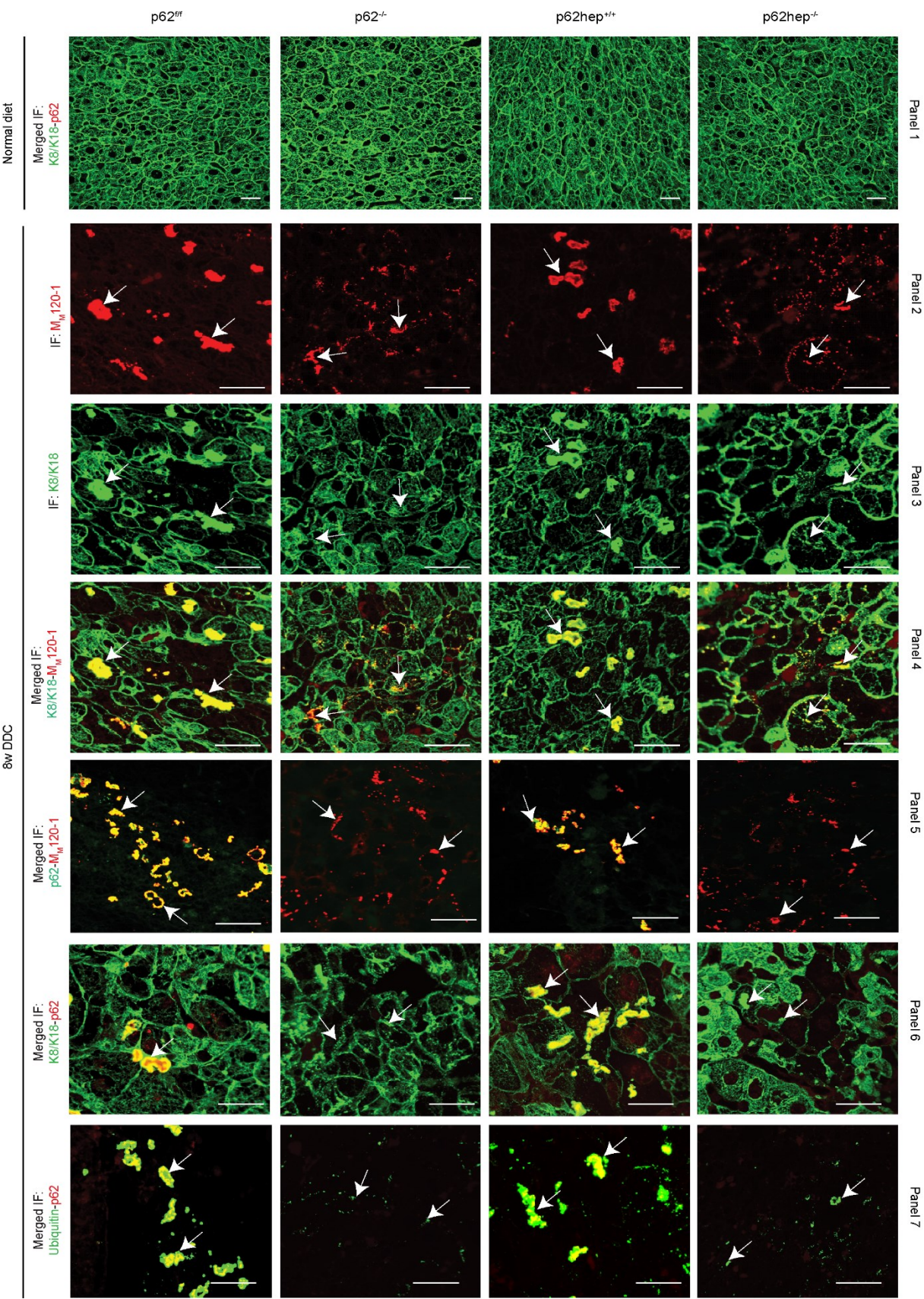
(A) H&E staining (upper panel) and immunohistochemistry (IHC) with antibodies against p62 (middle panel) and keratins 8/18 (K8/K18) (lower panel) were performed on liver sections of  $p62^{-/-}$ ,  $p62^{hep-/-}$  mice as well as their littermates with unaffected hepatic p62 expression ( $p62^{ff}$  and  $p62^{hep+/+}$ ) that were fed a 0.1% DDC-containing diet for 8 weeks. MDBs are indicated by arrows. Large and distinct MDBs are only visible in  $p62^{ff}$  and  $p62^{hep+/+}$  mice in H&E stained sections and also decorated by p62 and K8/K18 antibodies. In  $p62$ -deficient mice, MDBs are small and distinct and only decorated by K8/K18 antibodies. MDB-containing hepatocytes

display diminished or lacking keratin cytoskeleton (empty cells). Brown pigment in the livers of  $p62^{-/-}$ ,  $p62^{f/f}$ ,  $p62^{hep^{-/-}}$  and  $p62^{hep^{+/+}}$  mice resembles protoporphyrin accumulation (arrowheads). MDBs are stained in red in both IHC with antibodies against p62 and K8/K18 (Scale bar = 50  $\mu$ m). **(B)** Immunoblotting revealed abundant p62 expression in livers of DDC-fed  $p62^{f/f}$  and  $p62^{hep^{+/+}}$  mice while no p62 signal was detected in  $p62^{-/-}$  liver. Minimal p62 signal was detected in  $p62^{hep^{-/-}}$  livers likely due to preserved p62 production in non-hepatocytic cells.  $\beta$ -actin was used as a loading control.

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To further monitor the formation of MDBs, we relied on the detection of M<sub>M</sub>120-1 antigen that constitutes a constant component of MDBs (Zatloukal et al., 1990a, Stumptner et al., 2001a). Double immunofluorescence staining with M<sub>M</sub>120-1 antibody and antibodies to K8/K18 revealed predominantly smaller, granular and less distinct aggregates in DDC-intoxicated  $p62$ -deficient livers whereas both small granular as well as large MDBs were seen in  $p62^{f/f}$  and  $p62^{hep^{+/+}}$  livers (Figure 15, panels 2-5). The morphometrical analysis confirmed smaller but more numerous aggregates in DDC-intoxicated  $p62$ -deficient livers than in corresponding  $p62^{f/f}$  and  $p62^{hep^{+/+}}$  livers (Figure 16A). No alterations of the keratin cytoskeleton were observed in livers under a normal diet (Figure 15, panel 1). The absence of p62-positive aggregates in DDC-intoxicated  $p62$ -deficient animals was confirmed by performing various combinations of double immunofluorescence staining's with antibodies against MDB-specific components (Figure 15, panels 5-7). Moreover, we observed a rather weak ubiquitin staining of  $p62$ -deficient MDBs when compared to the pronounced colocalization of ubiquitin in MDBs of  $p62^{f/f}$  and  $p62^{hep^{+/+}}$  livers (Figure 15, panel 7).

Immunoelectron microscopy with M<sub>M</sub>120-1 antibody revealed that  $p62$ -deficient livers contained smaller aggregates when compared to larger MDBs in control livers, which however resembled the classical filamentous type II MDBs, occasionally with electron-dense amorphous material as described for type III MDBs (Zatloukal et al., 2007) (Figure 16B). Therefore, on the ultrastructural level, no differences existed between the filamentous material of MDBs in  $p62$ -deficient and corresponding control animals.



### Figure 15. Loss of p62 impairs the formation of “mature” MDBs.

Double immunofluorescence staining with K8/K18 (green) and p62 (red) antibodies was performed on liver sections of  $p62^{f/f}$ ,  $p62^{-/-}$ ,  $p62^{hep+/+}$  and  $p62^{hep-/-}$  mice under normal diet (panel 1). To detect MDBs, double immunofluorescence staining with M<sub>M</sub>120-1 (red) and K8/18 antibodies (green) was performed on liver sections of 8 weeks DDC-intoxicated mice ( $n \geq 10$ ). Note that MDBs were found in all genotypes. However,  $p62^{f/f}$  and  $p62^{hep+/+}$  mice developed large aggregates while only small inclusions were observed in  $p62^{-/-}$  and  $p62^{hep-/-}$  mice (panels 2-5) (scale bar = 20 $\mu$ m). To confirm the absence of p62 in MDBs present in p62-deficient livers, various combinations of double immunofluorescence stainings with p62 (green) + M<sub>M</sub>120 (red), K8/K18 (green) + p62 (red) and ubiquitin (green) + p62 (red) antibodies were performed on DDC-treated liver of the different genotypes (panels 5-7). The arrows are used to indicate few MDBs among all the MDBs positive for keratin, M<sub>M</sub>120-1 or p62 to underline the observations (panels 2-7).

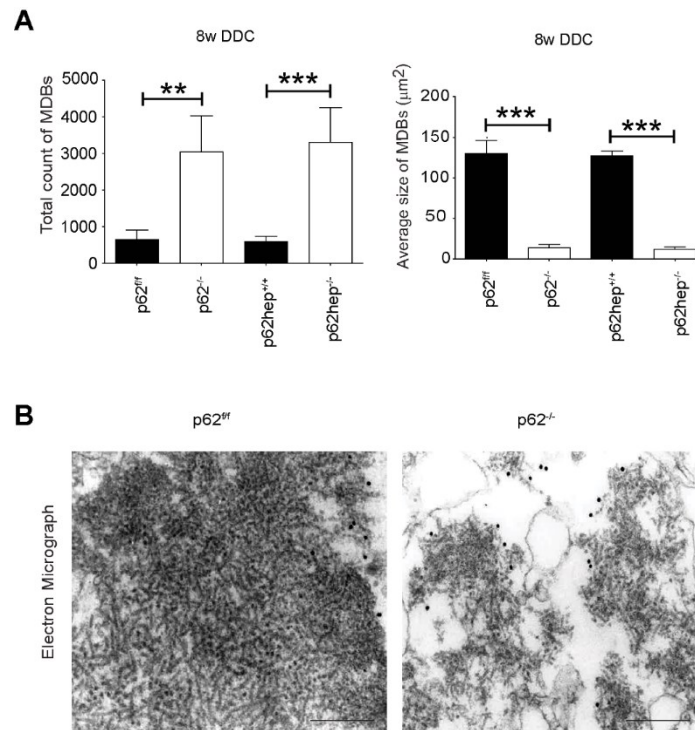


**Figure 16. Loss of p62 influences the size but not the filament ultrastructure of MDBs.**

(A) Morphometric scoring of the M<sub>M</sub>120-1 immunofluorescence confirmed the visual impression that p62-deficient mice contained more small MDBs but rarely developed larger

inclusions. ( $n \geq 10$ ) Values are expressed as mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . **(B)** Electron micrographs depict characteristic MDBs with densely but haphazardly arranged filaments in  $p62^{f/f}$  mice while only small filamentous MDBs are seen in  $p62^{-/-}$  mice. Note that the ultrastructure of the MDB filaments is identical in both genotypes. Scale bar = 500 nm.

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### **3.2.4. Loss of p62 does not affect keratin 8/18 modifications but alters the binding of other keratins to MDBs**

Keratins are the primary components of MDBs. To analyze molecular structural features of MDBs, we used the luminescent conjugated oligothiophene h-HTAA, which labels cross  $\beta$ -sheet structure of proteins (Mahajan et al., 2011). Double staining with h-HTAA and the M<sub>M</sub>120-1 antibody showed no positivity for cross  $\beta$ -sheet structures in hepatocytes of  $p62$ -deficient and corresponding control mice fed a normal diet (Figure 17A, panel 1). On the other hand, in DDC-fed mice both  $p62$ -deficient and “mature” MDBs were positive for cross  $\beta$ -sheet conformation indicating that DDC-induced structural changes of K8 to cross  $\beta$ -sheet conformation in MDBs were independent of  $p62$  (Fig 17A, panel 2-4). The formation of high molecular weight cross-linked K8 species, increased amount of keratins (Fig 17B), and K8>K18 mRNA ratio (Figure 18A) observed after DDC exposure also did not differ among the genotypes. However, the protein levels of transglutaminase 2 (Tg2), which was reported to form glutamine-lysine crosslinks of keratins in MDBs (Strnad et al., 2007), were decreased in DDC-intoxicated  $p62^{-/-}$  livers in comparison to  $p62^{f/f}$  livers (Figure 18B).

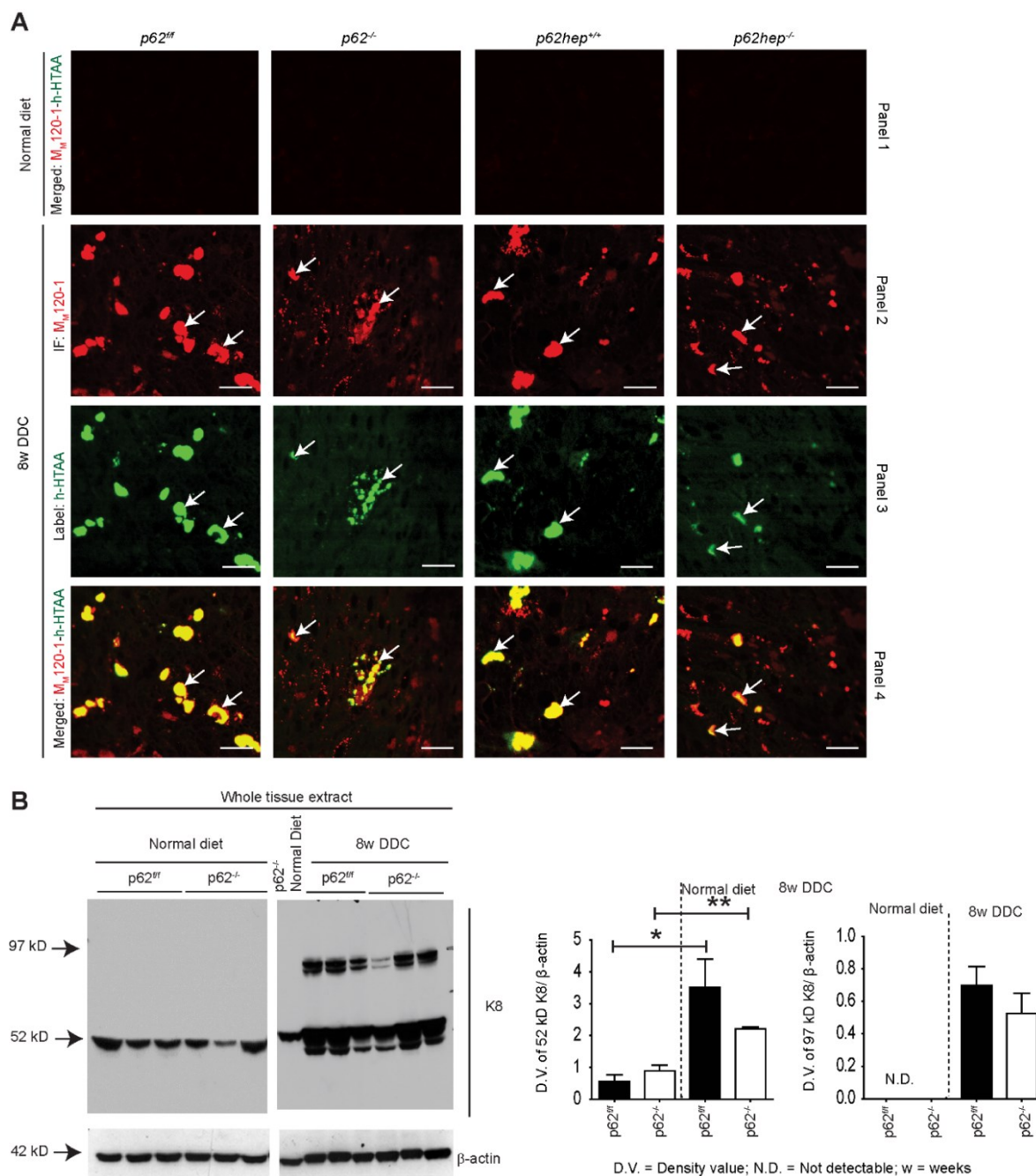


**Figure 17. p62 affects neither the extent of DDC-induced protein misfolding nor keratin cross-linking.**

(A) A combination of immunofluorescence staining with M<sub>M</sub>120-1 antibody (red) and labeling with the luminescent conjugated oligothiophene h-HTAA (green) was performed on liver sections of p62<sup>-/-</sup>, p62<sup>fl/fl</sup>, p62<sup>hep</sup><sup>-/-</sup> and p62<sup>hep</sup><sup>+/+</sup> mice fed a normal diet (panel 1) or DDC for 8 weeks (panels 2-4). Mice fed a normal diet did not show any positive labeling with h-HTAA or

M<sub>M</sub>120-1 (panel 1). DDC-intoxicated livers showed colocalization (yellow) of h-HTAA and M<sub>M</sub>120-1 indicating the presence of cross  $\beta$ -sheet conformation in MDBs (arrows) (panel 2;  $n \geq 10$ ) (scale bar = 20 $\mu$ m). **(B)** Whole liver tissue extracts of p62<sup>f/f</sup> and p62<sup>-/-</sup> mice fed a normal diet or 8weeks DDC were analyzed by western blotting with a K8-specific antibody and densitometric quantification was performed ( $n = 3$ ). DDC-treatment resulted in an increased amount of K8 (52 kD; bands with lower molecular mass resembled degradation products) and formation of crosslinked K8 dimers (97 kD). However, the extent of dimer formation did not differ significantly between p62<sup>-/-</sup> and p62<sup>f/f</sup> mice.  $\beta$ -actin was used as a loading control. \* $p < 0.05$ , \*\* $p < 0.01$ .

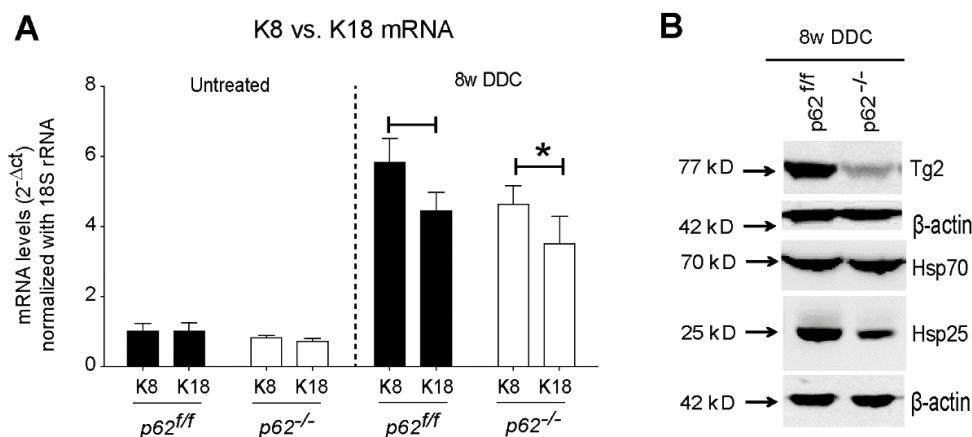


**Figure 18. Characterization of MDB components.**

**(A)** qPCR for K8 and K18 was performed on untreated and DDC-treated livers of p62<sup>f/f</sup> and p62<sup>-/-</sup> animals.  $\beta$ -actin was used as a normalization control. The transcript levels of both K8 and K18 were upregulated in DDC-intoxicated livers of both p62<sup>f/f</sup> and p62<sup>-/-</sup> mice when compared with untreated mice of both genotypes. Moreover, the increase in transcript expression ratio of K8/K18 was observed in both DDC-intoxicated p62-deficient mice and wildtypes when compared with untreated controls. **(B)** Whole tissue extract from 8 weeks DDC-treated livers of p62<sup>f/f</sup> and p62<sup>-/-</sup> mice were immunoblotted for MDB components Hsp70, Hsp25 and Tg2. The expression of Hsp70 did not differ between p62<sup>f/f</sup> and p62<sup>-/-</sup> livers. However, the expression of Hsp25 and Tg2 was markedly decreased in p62<sup>-/-</sup> livers when compared to wildtypes.

In chronically DDC-treated wildtype and p62-deficient mice, a pronounced ductular reaction was observed extending to some degree into the peripheral parenchyma. Double immunofluorescence staining with K8/18-K7 (Figure 19A) and K8/K18-K19 (Figure 20A) antibodies was used for detection and quantification of ductular cells. It was also noted that MDBs present in DDC-intoxicated p62<sup>fl/fl</sup> and p62<sup>-/-</sup> livers were also positive for K7 (Table 11, Figure 19B). Interestingly, double immunofluorescence staining with K17-K8/18 antibodies revealed K17 positivity of a subset of ductular cells (Figure 21A) but also for occasional polygonal hepatocytes (resembling intermediate cells) (Figure 21B). The significance of this K17 immunoreaction is unclear. It could indicate differentiation towards a glandular lineage (Trojanovsky et al., 1992). Moreover, K17 antibody showed positivity of approximately half of wildtype MDBs whereas only a small subset of MDBs was positive for K17 in p62-deficient livers (Figure 21B, Table 11).

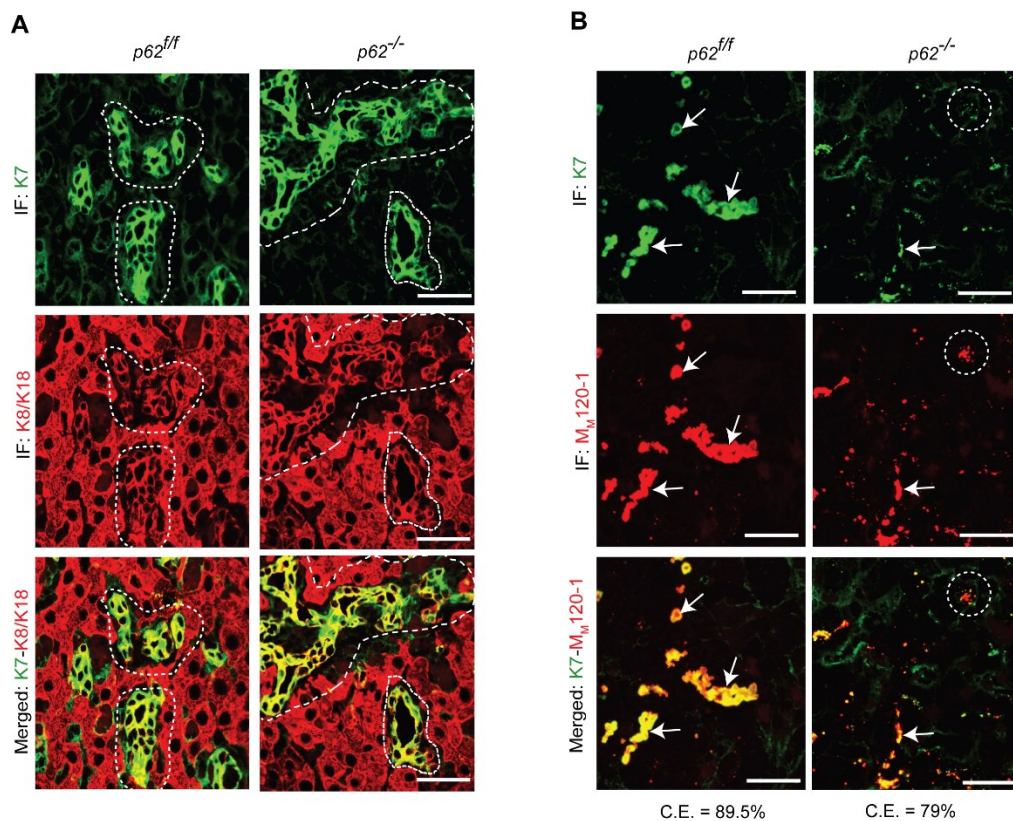


**Figure 19. Detection of K7 in DDC-intoxicated p62<sup>fl/fl</sup> and p62<sup>-/-</sup> mouse livers.**

(A) Double immunofluorescence staining with antibodies against K7 (green) and K8/K18 (red) shows the positivity of ductular epithelium K7 in (dotted line) (scale bar = 20µm). (B) Double

immunofluorescence staining with antibodies against MDB marker M<sub>M</sub>120-1 (red) and K7 (green) revealed a greater colocalization efficiency (C.E.) of the two antibodies in p62<sup>f/f</sup> livers than in p62<sup>-/-</sup> livers (p62<sup>f/f</sup>; C.E.=89.5%; p62<sup>-/-</sup>, C.E.=79%). MDBs positive and negative for K7 are indicated by arrows and dotted circles respectively (scale bar = 20μm).

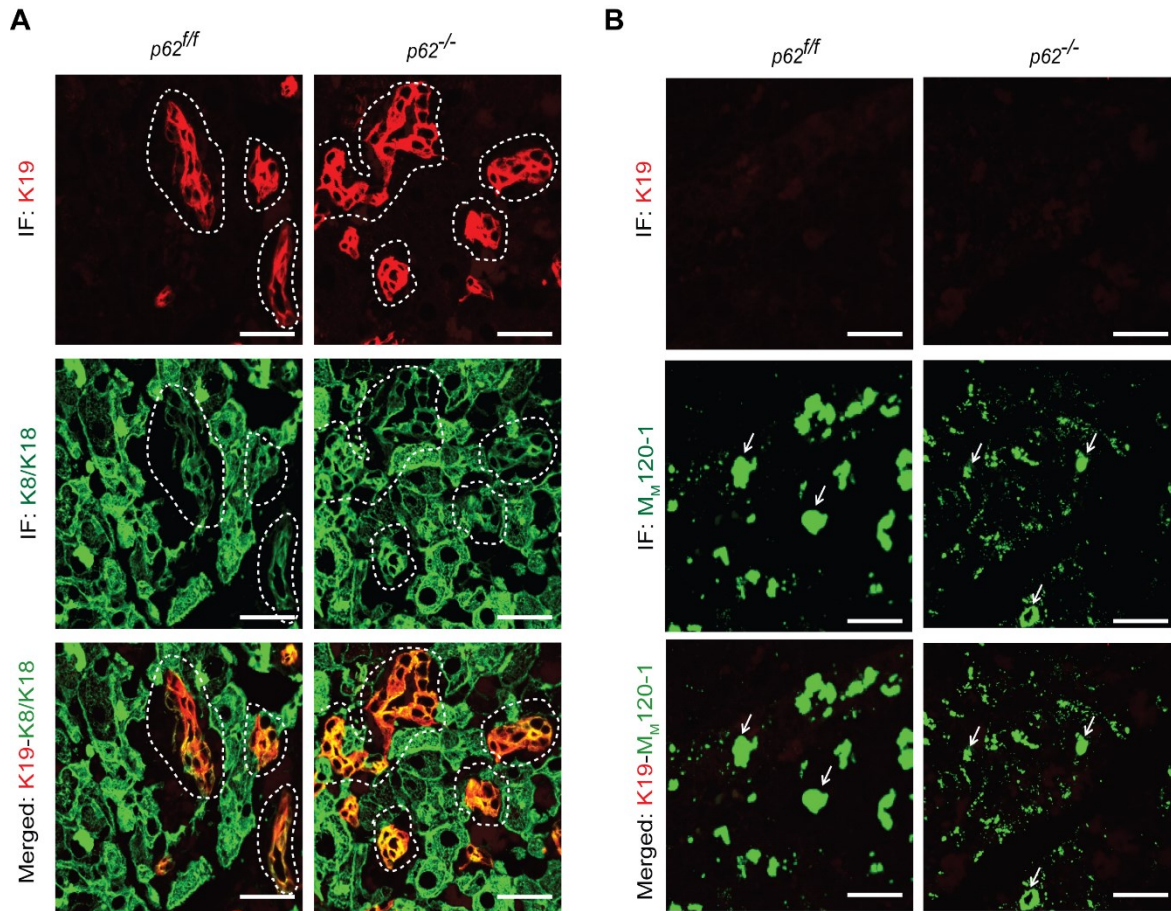


**Figure 20. Detection of K19 in DDC-intoxicated p62<sup>f/f</sup> and p62<sup>-/-</sup> mouse livers.**

(A) Double immunofluorescence staining with antibodies against K19 (red) and K8/K18 (green) shows the positivity of ductular epithelium (irregular dotted lines) (scale bar = 20μm). (B) Double immunofluorescence staining with antibodies against MDB marker M<sub>M</sub>120-1 and K19 showed no positivity of MDBs for K19 (arrows) (scale bar = 20μm). Note that only this area/image selected for the representation of M<sub>M</sub>120-1 positive MDBs excludes the marking of K19 positive ductules present in other areas of the same liver section.

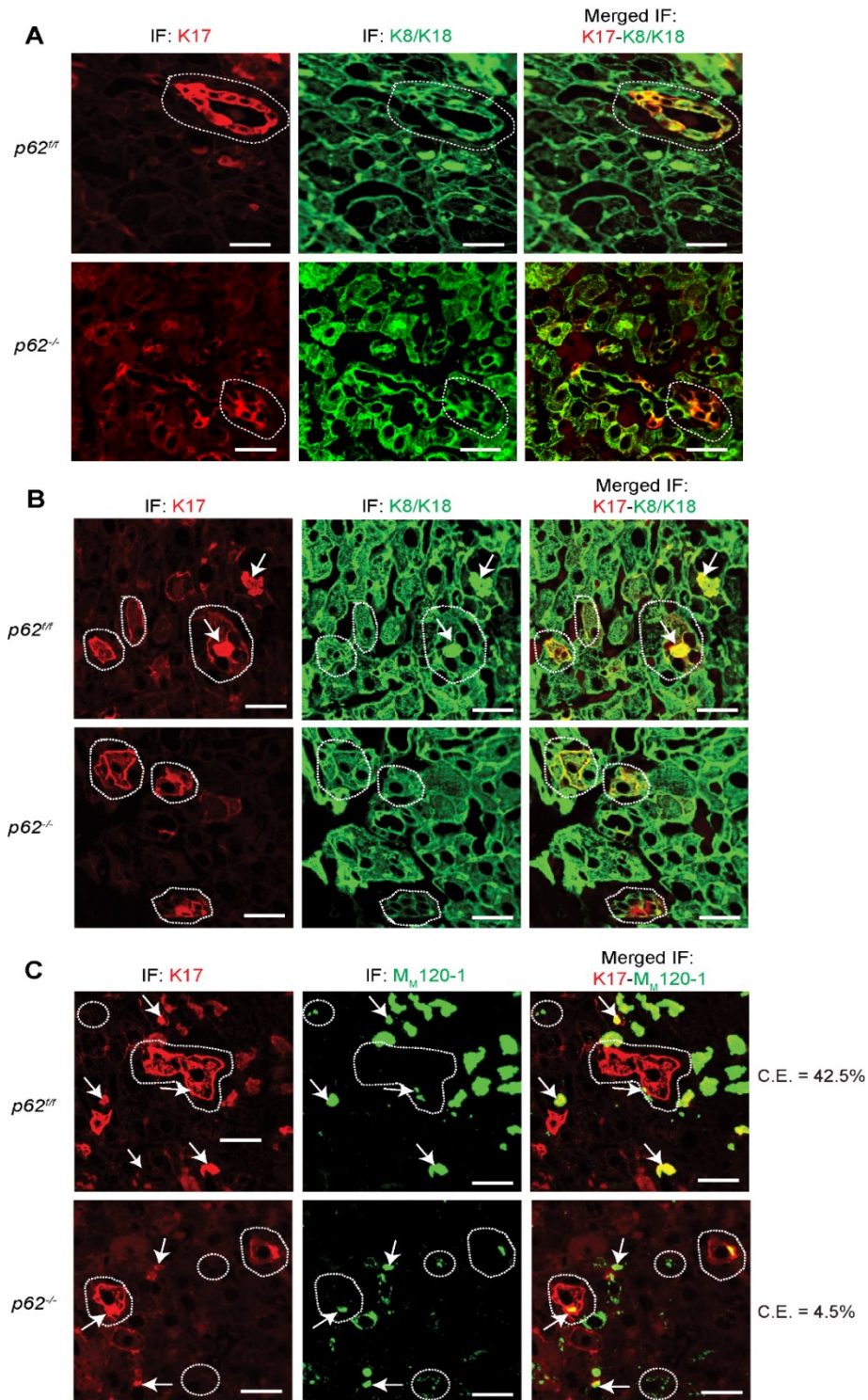


**Figure 21. Detection of K17 in DDC-intoxicated mouse livers.**

(A) Double immunofluorescence staining with antibodies against K19 (red) and K8/K18 (green) shows the positivity of ductular epithelium (irregular dotted lines) (scale bar = 20µm). (B)

Double immunofluorescence staining for K17 (red) and K8/K18 (green) showed MDBs positive for K17 (arrows) present in K17-positive hepatocytes (dotted lines) in both genotypes (Scale bar=20 $\mu$ m). (C) Double immunofluorescence staining with antibodies against K17 and M<sub>M</sub>120-1 antigen (MDB marker) showed a colocalization efficiency (C.E) of 42.5% in p62<sup>ff</sup> mice and only 4.5% in p62<sup>-/-</sup> mice. Colocalization efficiency was measured by counting  $\leq 200$  random MDBs from five different animals of each genotype. MDBs positive for K17 are indicated by arrows and MDBs negative for K17 are indicated by dotted circles (Scale bar = 20  $\mu$ m).

**Table 11. Quantitative assessment of MDB components in p62<sup>ff</sup> and p62<sup>-/-</sup> livers.**

| MDB components | Colocalization efficiency |                    |
|----------------|---------------------------|--------------------|
|                | p62 <sup>ff</sup>         | p62 <sup>-/-</sup> |
| MM120-1        | 100 %                     | 100 %              |
| Keratin 8/18   | 100 %                     | 100 %              |
| Keratin 17     | 42.5 %                    | 4.5 %              |
| Keratin 7      | 89.5 %                    | 79 %               |
| p62            | 100 %                     | 0 %                |
| NBR1           | 97 %                      | 21 %               |
| Ubiquitin      | 96.20 %                   | 19.80 %            |
| Hsp25          | 46.70%                    | 0 %                |
| SMI 31         | 100 %                     | 0 %                |

Double immunofluorescence staining with antibodies against known MDB components was correlated with the results of M<sub>M</sub>120-1 immunostaining, which served as common reference, and colocalization efficiency was quantified for various proteins present in MDBs.

### 3.2.5. Loss of p62 impairs the binding of NBR1 to MDBs.

Since NBR1 can also be detected in large “classical” MDBs and shares both structural and functional similarities with p62 (Figure 22A) (Kirkin et al., 2009), we investigated whether

NBR1 is able to compensate for the loss of p62 in MDB formation. To this aim, we compared the mRNA and protein levels of p62 and NBR1 in p62<sup>f/f</sup> and p62<sup>-/-</sup> livers. Mice fed a normal diet showed similar mRNA levels of NBR1 and p62 (Figure 22B). Of note, an increased expression of NBR1 protein was observed in western blots of total liver extracts of p62-deficient mouse livers when compared to p62<sup>f/f</sup> livers (Figure 22C, left panel). The minimal levels of p62 seen in the western blot are due to very low concentrations of p62 in mice fed a normal diet (non stressed situation).

DDC-intoxication led to a dramatic increase in both mRNA and protein levels of p62 (Figure 22B). In contrast to p62, there was only a slight increase of NBR1 in DDC-treated p62<sup>f/f</sup> livers (Figure 22B and 22C, right panel). Surprisingly, NBR1 mRNA and protein expression was markedly reduced in DDC-intoxicated p62-deficient livers as compared to p62-deficient mice fed a normal diet (Figure 22B-C). Moreover, double immunofluorescence staining for NBR1 and M<sub>M</sub>120-1 antigen demonstrated an almost perfect overlap of both antigens in DDC-treated p62<sup>f/f</sup> and p62<sup>hep+/+</sup> livers whereas a much weaker colocalization was seen in p62-deficient livers (Figure 22D, Table 11). Triple staining for M<sub>M</sub>120-1 antigen, NBR1 and ubiquitin revealed that only MDBs containing ubiquitin were also positive for NBR1 (Figure 23). This demonstrates that p62 enhances the binding of NBR1 to MDBs, but the latter does not compensate for p62's role in the maturation of MDBs.

Next, we examined the expression of the chaperones Hsp25 and Hsp70 in DDC-intoxicated p62<sup>f/f</sup> and p62<sup>-/-</sup> livers. Interestingly, double immunofluorescence staining with antibodies to Hsp25 and the M<sub>M</sub>120-1 antigen (Table 11) revealed that the absence of p62 impaired the binding of Hsp25 to MDBs. Moreover, the protein levels of Hsp25 were reduced in DDC-intoxicated p62<sup>-/-</sup> livers in comparison to p62<sup>f/f</sup> livers (Figure 18B). On the other hand, Hsp70 protein was not different between DDC-intoxicated p62<sup>f/f</sup> and p62<sup>-/-</sup> livers (Figure 18B).

In the context of characterizing possible impacts of the absence of p62 on the composition of MDBs we have also investigated the presence of the phosphoepitope SMI 31 in MDBs since it has been described as common feature of MDBs and neurofibrillary tangles (NFTs) (Preisegger et al., 1992). We observed that the SMI 31 epitope was not present in p62-deficient MDBs which demonstrates that the SMI 31 epitope in MDBs is located on p62 and not on tau protein as originally reported for NFTs (Table 11).

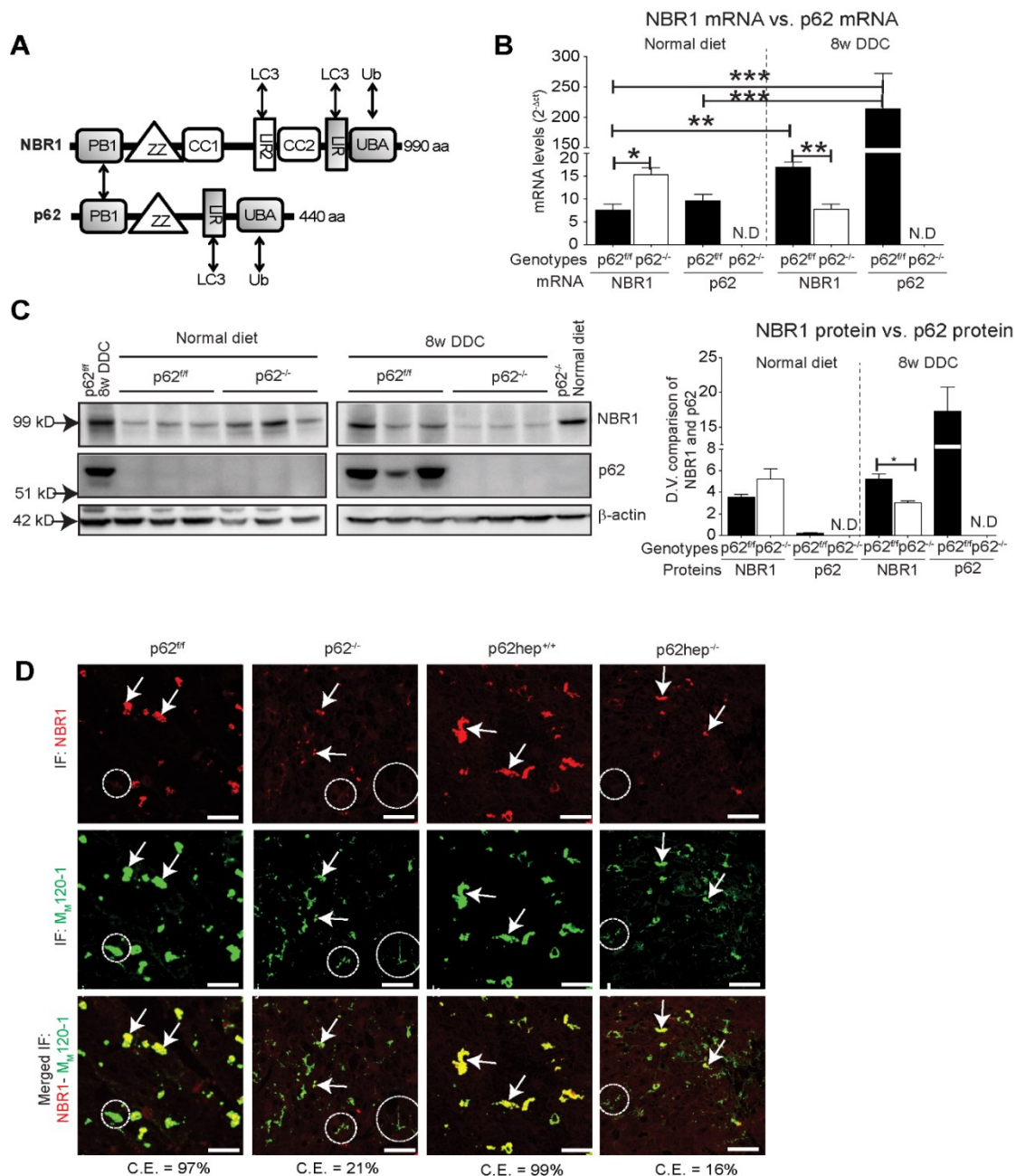


**Figure 22. Loss of p62 impairs recruitment of NBR1 to MDBs.**

(A). The scheme (modified from (Kirkin et al., 2009)) depicts the protein domain architecture of NBR1 and p62. The reported interaction partners and conserved domains are highlighted by double headed arrows and gray boxes, respectively. PB1, Phox and Bem1p domain; ZZ, zinc-binding domain; CC, coiled-coil domain; LIR, LC3-interacting region; UBA, ubiquitin-

associated domain; Ub, ubiquitin. NBR1 and p62 interact via their PB1 domains (Kirkin et al., 2009). **(B)** qPCR analyses of NBR1 and p62 mRNA expression in livers of p62<sup>f/f</sup> and p62<sup>-/-</sup> mice under normal diet or 8 weeks DDC. 18S rRNA was used as a normalization control (n = 5). **(C)** Whole liver tissue extracts of p62<sup>f/f</sup> and p62<sup>-/-</sup> mice fed a normal diet or 8 weeks DDC were immunoblotted for NBR1 (99 kD) and p62 (~55 kD). A sample from DDC-treated p62<sup>f/f</sup> liver was run on the same gel with liver samples from mice fed a normal diet (left panel) as common reference (right panel). Densitometric analyses of p62 and NBR1 are also shown (n = 3). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 **(D)** Double immunofluorescence staining with M<sub>M</sub>120-1 (green) and NBR1 antibodies (red) visualized the distribution of both proteins in livers of mice treated with DDC for 8 weeks. An extensive co-localization between NBR1 and M<sub>M</sub>120-1 (C.E = colocalization efficiency) was seen in animals with intact p62 production (p62<sup>f/f</sup> C.E = 97%, and p62<sup>hep+/+</sup> C.E = 99%) but not in the total (p62<sup>-/-</sup> C.E = 21%) and liver-specific (p62<sup>hep-/-</sup> C.E = 16%) p62-knockout animals (scale bar = 20µm). NBR1-positive and -negative MDBs are highlighted by arrows and circles, respectively.

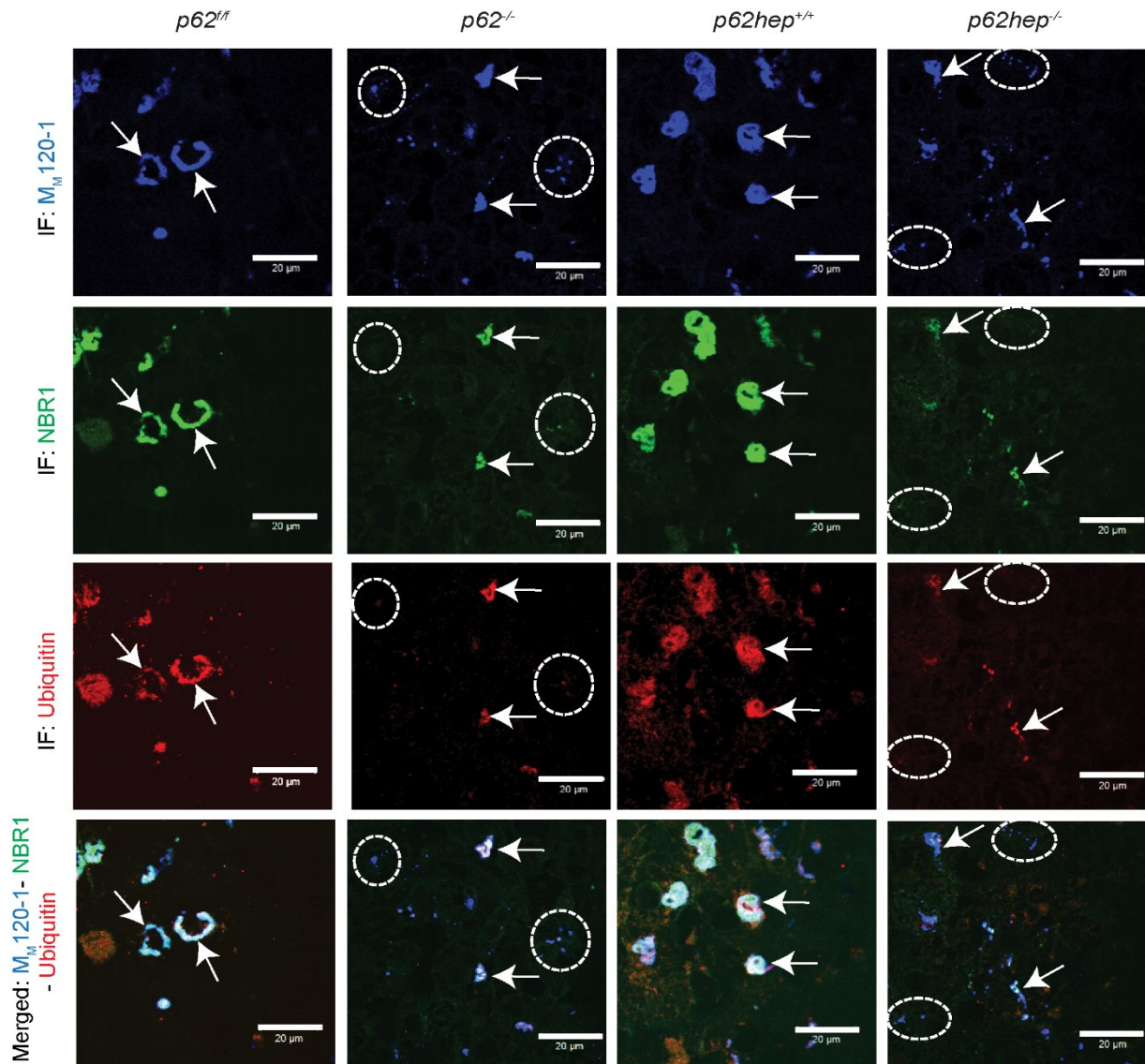


**Figure 23. Loss of p62 impairs recruitment of NBR1 and ubiquitin to MDBs.**

Triple immunofluorescence staining with antibodies against M<sub>M</sub>120-1 (MDB marker; blue), NBR1 (green) and ubiquitin (red) visualized the distribution of the respective antigens in *p62<sup>f/f</sup>* and *p62<sup>-/-</sup>* livers intoxicated with DDC for 8 weeks. NBR1-positive and -negative MDBs are highlighted by arrows and dotted circles, respectively. An extensive co-localization of NBR1 and the MDB markers M<sub>M</sub>120-1/ ubiquitin was seen in animals with intact p62 production (*p62<sup>f/f</sup>* and *p62<sup>hep+/+</sup>*) but less in the complete (*p62<sup>-/-</sup>*) and liver-specific (*p62<sup>hep-/-</sup>*) p62-knockouts. (Scale bar = 20μm).

### 3.2.6. p62 provides stability to MDBs

To determine whether p62 alters MDB dynamics, we analyzed the amount of inclusions in mice recovered from DDC intoxication on a standard diet for four weeks. As reported previously (Zatloukal et al., 1990b, Stumptner et al., 2001a), the recovery from DDC intoxication resulted in an almost complete disappearance of large MDBs (Figure 24A) while small aggregates occasionally persisted at the cell periphery in proximity to desmosomes (Figure 25). The numerical reduction of MDBs was much more pronounced in p62-deficient than in control livers as confirmed by morphometry (Figure 24B-C). In contrast, the reduction in size of MDBs was more prominent in controls (Figure 24C). In addition, insoluble ubiquitinated proteins and the high molecular weight insoluble K8 species were found at similar levels in DDC-fed p62<sup>f/f</sup> and p62<sup>-/-</sup> mice, but disappeared more quickly in p62-deficient animals upon recovery (Figure 24D, see stacking gels).

Moreover, no changes in the over-expression of K8 (52 kD and 97 kD) was observed in DDC-treated livers irrespective of the genotype (Figure 24D, see resolving gel). Ubiquitin levels were slightly decreased in DDC-treated livers of p62-deficient mice (Figure 24D, resolving gel). Further, we measured the levels of p62's binding partner microtubule-associated light chain 3-II to the light chain 3-I (LC3-II/LC3-I) ratio in the untreated control, DDC-treated and recovered mouse livers (Figure 26). In chronic DDC intoxication, we observed an increased LC3-II to LC3-I ratio in all genotypes when compared to untreated controls indicating autophagic activity. LC3-II to LC3-I ratios decreased in the recovery period to levels comparable to the untreated controls. There was no significant difference in LC3-II to LC3-I ratios between the genotypes (Figure 26).

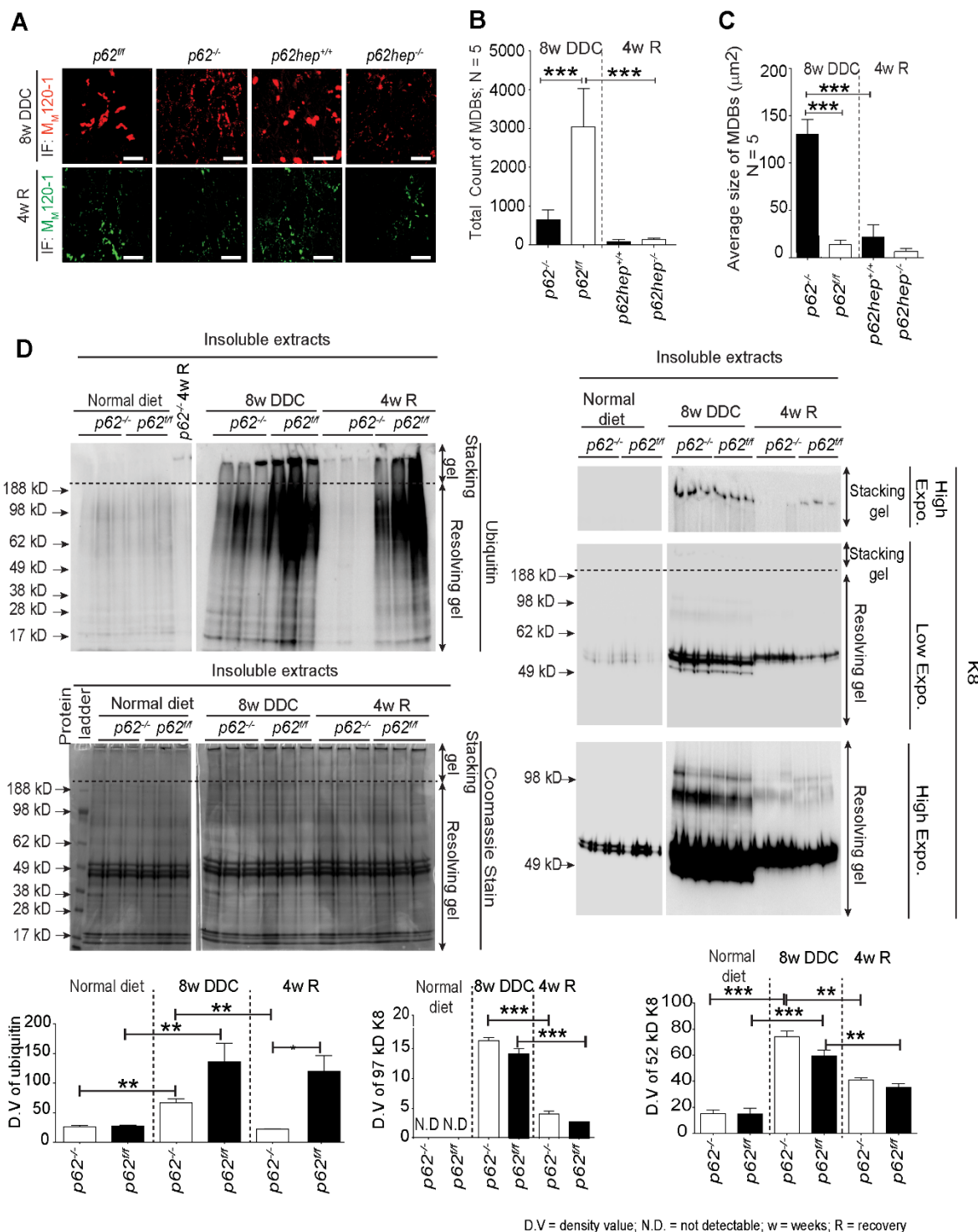


**Figure 24. Characterization of MDBs at 4 weeks of recovery after 8 weeks of DDC intoxication.**

(A) Double immunofluorescence staining of liver sections with M<sub>M</sub>120-1 and K8/K18 antibody visualized MDBs in livers of mice fed DDC for 8 weeks (8w DDC) as well as in livers of mice that were subsequently recovered on a standard diet for four weeks (4wR). Recovery led to a substantial reduction of MDBs both in mice with intact p62 production (p62<sup>f/f</sup> and p62<sup>hep+/+</sup>) and total (p62<sup>-/-</sup>)/liver-specific (p62<sup>hep-/-</sup>) p62 knockouts. (B) Morphometrical analysis showed that after recovery there was a significant reduction of the number of MDBs in the p62-deficient groups (n = 5). (C) No significant difference in MDB size was observed after recovery in the different genotypes, which confirmed the observations of immunofluorescence staining (n = 5). (D) Immunoblotting of insoluble liver protein extracts with antibodies against keratin 8 (K8) and ubiquitin and corresponding densitometric quantification (n = 5) showed that loss of p62 did not affect the accumulation of insoluble ubiquitinated proteins or formation of cross-linked high molecular mass K8 species during DDC intoxication. However, the disappearance of ubiquitinated proteins and K8 cross-linked proteins during the recovery period was accelerated in p62-deficient mice suggesting that the inclusions were less stable. A Coomassie-stained gel for indicated genotypes and treatment shows the protein loading used for western blotting. Values are expressed as mean ± SEM. (n = 5). \*p<0.05, \*\*\*p<0.001.

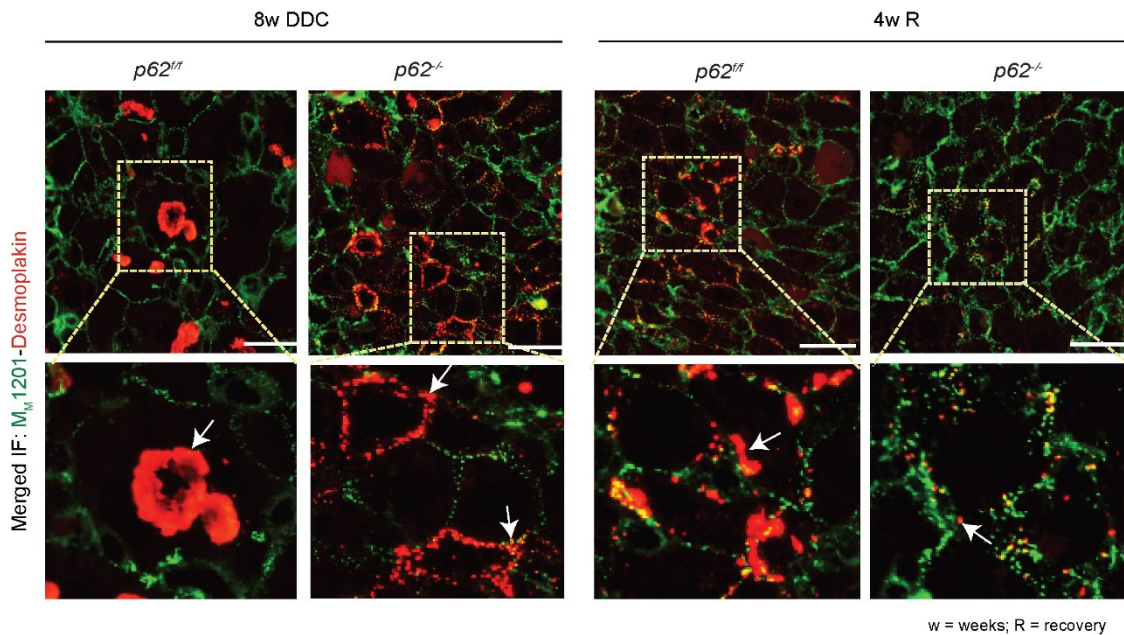


**Figure 25. Association of MDBs with desmosomes.**

Double immunofluorescence staining with antibodies against MM120-1 (MDB marker; red) and desmoplakin (desmosomal marker; green) visualized the distribution of the antigens in 8 weeks DDC-intoxicated (8w DDC) and 4 weeks recovered (4w R) p62<sup>f/f</sup> and p62<sup>-/-</sup> mouse livers. MDBs are indicated by arrows. (Scale bar = 20μm; inset showing higher magnification; scale bar = 10μm).

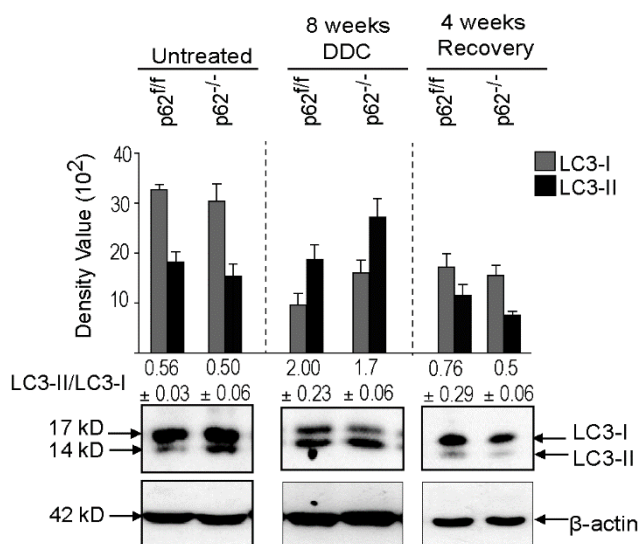


**Figure 26. Analysis of autophagy-related LC3-II/I ratio in DDC-intoxicated and -recovered livers.**

Whole tissue extracts from untreated mice, mice fed with DDC for 8 weeks (8w DDC) and from mice recovered on standard diet for four weeks (4wR) after DDC exposure were analyzed by western blotting with an antibody against LC3 as a marker of autophagy activation. Densitometric analysis depicted the intensity of both LC3-I and -II isoforms (dark/light bar) normalized to β-actin. DDC-intoxicated mice showed higher LC3-II/I ratio but recovered mice acquired attenuation of autophagy (i.e. lower LC3-II/LC3-I ratio). The extent of autophagy did not differ between total p62<sup>-/-</sup> and p62<sup>f/f</sup> mice of the same treatment regimen.

### 3.2.7. p62 alters the cellular defense mechanisms

Previous studies with the DDC model suggested that the increased accumulation of p62 and MDB formation is, at least in part, a response of the liver to oxidative stress (Singla et al., 2012). Nrf2 (Nuclear factor (erythroid-derived 2)-like 2)-antioxidant pathway is a key signalling pathway determining the cellular defense against oxidative stress (Kim and Keum, 2016). It regulates the expression of cytoprotective genes involved in the detoxication and elimination of reactive oxidants by enhancing cellular antioxidant capacity. Upon DDC intoxication, Nrf2 protein was increased in liver nuclear extracts of p62<sup>fl/fl</sup> and p62<sup>-/-</sup> (Fig 7A) mice suggesting dissociation of Nrf2 from Keap1 complex and nuclear translocation of Nrf2, followed by an increase in transcript levels of Nrf2 target genes, such as Nqo1 and Gst (Fig 7C). Interestingly, in addition to the translocation of Nrf2 into the nucleus there was also a marked overexpression of the Nrf2 mRNA in DDC-intoxicated livers (Fig 7A) suggesting additional transcriptional mechanisms regulating Nrf2 expression level, which to some extent were influenced by p62 since Nrf2 mRNA expression and the nuclear translocation were slightly reduced in p62-deficient mice when compared to the p62<sup>fl/fl</sup> livers. This reduced Nrf2 expression was accompanied by a reduction in Nqo1 and Gst mRNA levels in p62-deficient livers when compared to p62<sup>fl/fl</sup> livers (Fig 7C). No significant difference in the transcript and protein levels of Nrf2-interacting partners Keap1 (Kelch-like ECH-associated protein 1) and Nrf2 target gene Hmox-1 (hemeoxygenase 1) was observed between DDC-intoxicated p62<sup>fl/fl</sup> and p62<sup>-/-</sup> livers (Fig 7B). Taken together, these data indicate an impaired cellular defense mechanism in DDC-intoxicated p62-deficient liver leading to more pronounced injury. Although, this was not readily apparent at light microscopy (Fig 1A), the significant increase in the number of Ki-67-positive mitotic cells and the slightly enhanced ductular reaction in p62-deficient livers support this assumption (Fig 7D).

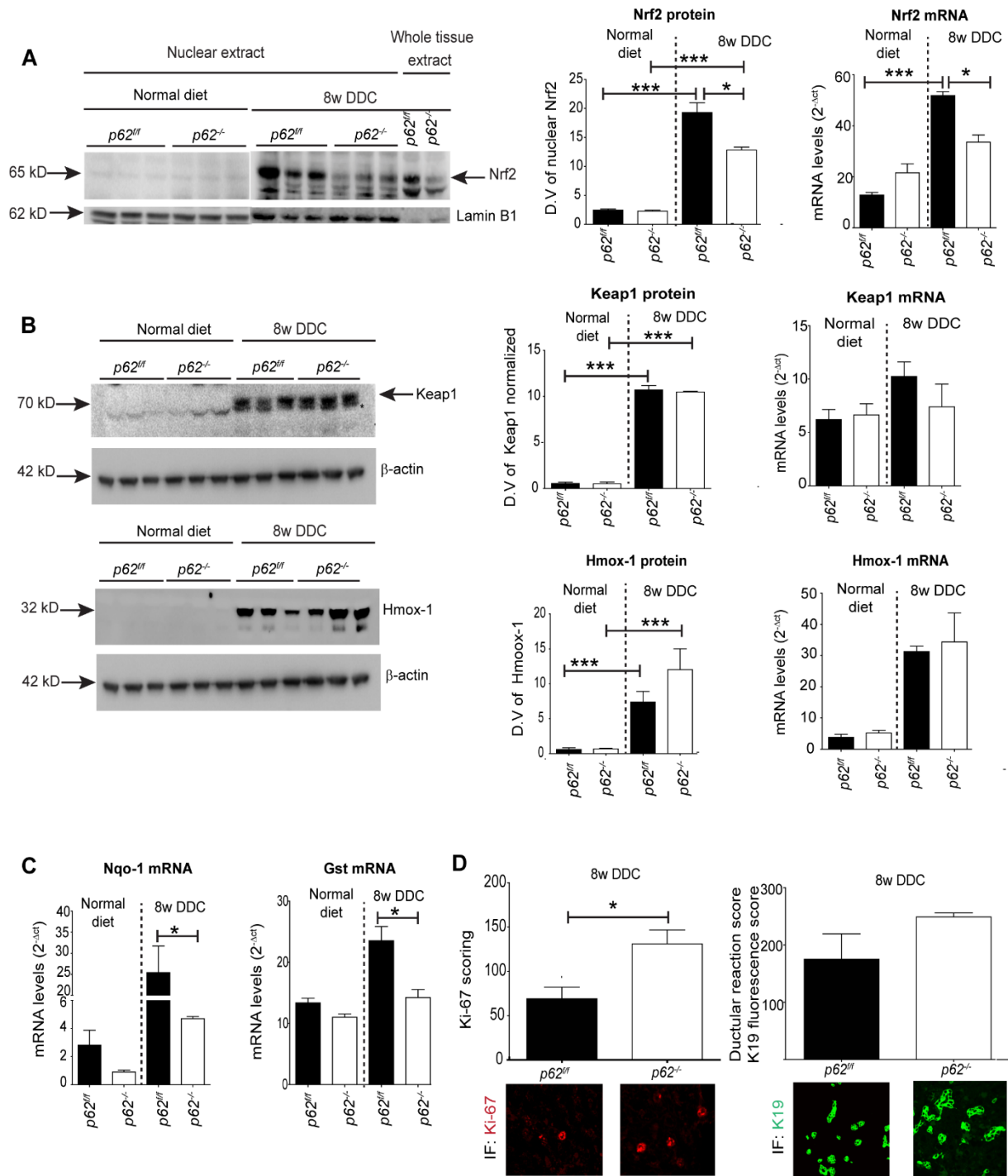


**Figure 27. Absence of p62 alters cellular defense mechanisms.**

(A) Liver nuclear extracts and whole tissue extracts of *p62<sup>f/f</sup>* and *p62<sup>-/-</sup>* mice fed a normal diet and of mice DDC-intoxicated for 8 weeks were analyzed by immunoblotting with antibody against Nrf2 (65 kD). Furthermore, quantitative qPCR analyses of Nrf2 mRNA (n = 5) was

performed from the same mice. Corresponding densitometric quantification is shown for nuclear extracts (n = 3). Lamin B1 (62 kD) was used as nuclear loading control. **(B)** Liver whole tissue extract from p62<sup>fl/fl</sup> and p62<sup>-/-</sup> mice fed a normal diet or 8 weeks DDC were immunoblotted for Keap1 (70 kD) and Hmox1 (32 kD) and qPCR analyses were performed for Keap1 and Hmox-1 mRNA (n = 5). Densitometric quantification for Keap1 and Hmox-1 are shown (n = 3).  $\beta$ -actin was used as a loading control. **(C)** qPCR analyses of Nqo1 and Gst mRNA levels were performed in livers of mice (n = 5) fed normal diet or after 8 weeks of DDC-intoxication. 18S rRNA was used as a normalization control. **(D)** Morphometric assessment of Ki-67-positive hepatocytes (Ki-67 score) and K19-positive bililary cells (ductular reaction score) was made by counting at least ten high power fields (400X) of five DDC-intoxicated livers of indicated genotypes stained with Ki-67 and K19 antibodies. At least five animals per genotype were used for qPCR, Ki67 and ductular reaction scoring. Values are expressed as mean  $\pm$  SEM. \*\*p< 0.01, \*p<0.05.

# Results: Part II

## Investigation of the role of p62-isoforms in $\alpha$ -synuclein mediated aggregation

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### 4.1. Aims of the study

Parkinson's disease (PD) is a neurodegenerative disorder caused by the loss of dopaminergic neurons of the substantia nigra pars compacta of the brain. The symptoms/pathology of PD is exemplified by the acquisition of motor deficits, including the bradykinesia, rigidity and resting tremor and the presence of cytoplasmic protein aggregates called "Lewy Bodies" in substantia nigra (Samii et al., 2004, Shulman et al., 2011). Currently, the number of people suffering from PD and related diseases in the world is estimated to be more than 24 million with 4.6 million new cases registered every year and not completely curable. Yet, the efficacy of the symptomatic treatment declines after five to ten years of treatment and the patient develops increasing cognitive dysfunction, motor impairment and postural difficulties. The primary aim is to understand the disease pathology by generating animal models mimicking human Parkinson's disease, in which to test and develop new therapeutics (Beal, 2010b).

The major drawback of the existing animal models of PD is the non-availability of "Lewy body" pathology and selective reproduction of cardinal symptoms of PD (Masliah et al., 2000). Mice overexpressing  $\alpha$ -synuclein develop accumulation of  $\alpha$ -synuclein, p62 and ubiquitin positive inclusions, but fail to reproduce fibrillar aggregates containing cross  $\beta$ -sheet conformation, typical of Lewy bodies (Masliah et al., 2000).

Our previous study on the role of p62 in MDB formation showed that the availability of p62 has a profound effect on the ultrastructure of MDBs [(Lahiri et al., 2016); Results Part I]. In the absence of p62, the MDB filaments seem to lose their compactness and aggregates appear as loosely bound and granular but, interestingly the molecular structural property of cross  $\beta$ -sheet formation (amyloid-like) remains unchanged. We conclude this study by proposing that p62

acts as “molecular glue” in the maturation and stabilization of Mallory-Denk bodies (Lahiri et al., 2016).

In this project, we want to investigate the role of p62 in Lewy-Body formation to understand whether the observations made in liver holds true to other cellular aggregates. p62 being the multifunctional stress adaptor protein and a constant component of Lewy bodies of Parkinson’s Disease and also other inclusions of neurodegenerative disorders such as neurofibrillary tangles of Alzheimer’s disease and TDP-43 aggregates of amyotrophic lateral sclerosis (Zatloukal et al., 2002, Odagiri et al., 2012) may provide us with clues to the unified molecular pathway of pathogenesis of aggregates.

Hence, if a more perfect model could be developed which more accurately stimulates the in-vivo pathological and phenotypic features of PD; it would be a promising tool for the successful future research. The specific aims of this study are:

1. Investigating the structural and molecular role p62 in the formation of  $\alpha$ -synuclein aggregates in a cell culture system.
2. The generation of a mouse model capable of reproducing the entire spectrum of Lewy body pathology.

**Note:** This is an ongoing project in the laboratory of Prof. Kurt Zatloukal at the Institute of Pathology. The experiments for investigating the specific aim 1 performed by Pooja Lahiri during the study period of 2013-2014 are shown as results.

## **4.2. Preliminary Results**

### **4.2.1. Identification of N-terminal variant of human p62/SQSTM1**

We started our experiment with an aim to clone commercially available cDNA from I.M.A.G.E database (Source Biosciences) into an expression vector to understand the role of p62 dynamics in protein misfolding. When investigating the nucleotide database (NCBI-nucleotide), we found a discrepancy between full-length cDNA’s in the NCBI database and commercially available I.M.A.G.E cDNA for p62. With the aid of sequence alignment tool NCBI-BLAST, we

identified a second transcript variant of human p62 differing in the 5' UTR and lacks 252 bp spanning the exon 1 and partly exon 2. Thus, p62-transcript variant 2 consists of 7 exons and start translation from an inframe downstream start codon when compared to variant 1 resulting in a short N-terminal region (Figure 28).

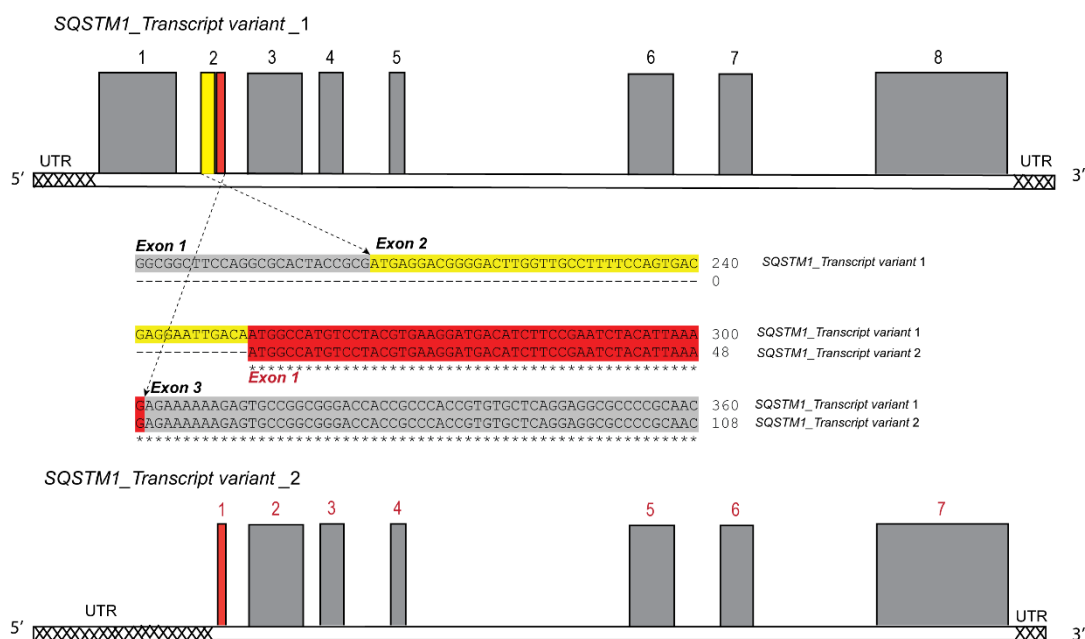


**Figure 28. Exonal view of transcript variants of human p62/SQSTM1.**

SQSTM1\_Transcript variant 1 (NM\_003900.4) consists of 8 exons (shown as grey boxes except exon 2 and labeled with black). SQSTM1\_Transcript variant 2 (NM\_001142299.1) lacks a part of N-terminal region (~ 252bp) and only consist of 7 exons (shown as grey boxed and labeled using red). The sequence of 5' UTR and 3' UTR are different for SQSTM1\_Transcript variant 1 and 2 (highlighted by cross markings). UTR, untranslated region.

Alignment of the corresponding protein sequences of p62/SQSTM1-transcript variants 1 (NP\_003891.1) and 2 (NP\_001135770.1) revealed that the shorter second isoform is devoid of the protein-interaction domain PB1. Hereafter, full-length p62 isoform-1 and -2 will be referred as p62\_H1 and p62\_H2 respectively.

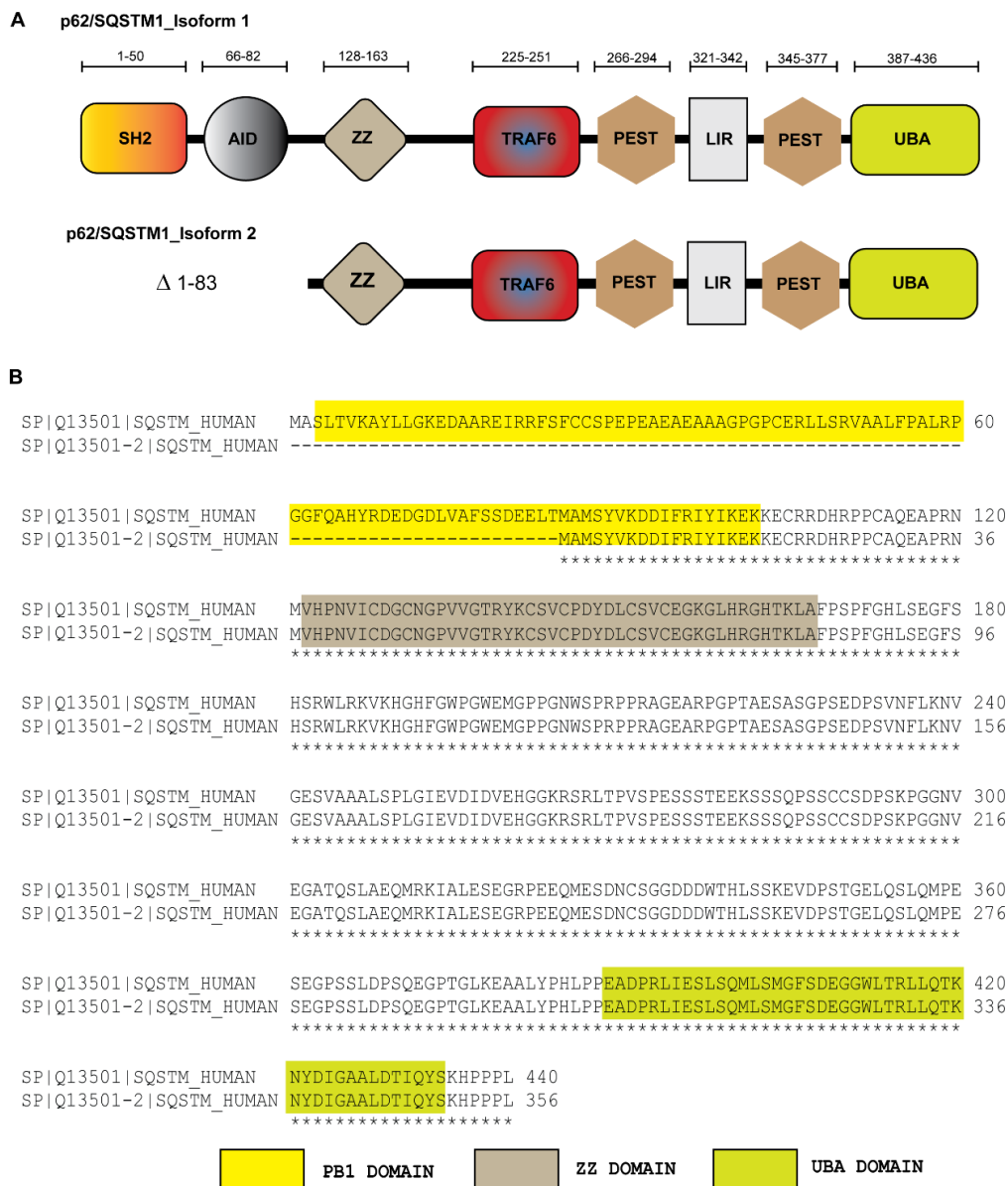


**Figure 29. Protein architecture of human p62 isoforms.**

(A). Scheme showing the various protein domains of p62/SQSTM1\_Isoform1 or p62\_H1 and p62/SQSTM1\_Isoform2 or p62\_H2. (B). Protein sequence alignment of p62\_H1 and p62\_H2. Highlighted areas depicts various domains. Note that p62\_H2 is devoid of PB1 domain.

Further, we analyzed the expression pattern of both p62\_H1 and p62\_H2 mRNA in human liver and brain. p62\_H1 and p62\_H2 mRNA was upregulated in both SH and HCC when compared

to normal liver. However, we observed a decrease in p62H1 and p62H2 in glioblastomas when compared to normal brain. The data suggest that p62\_H1 and p62\_H2 have similar mRNA expression pattern in different tissues but, varies among different disease states.

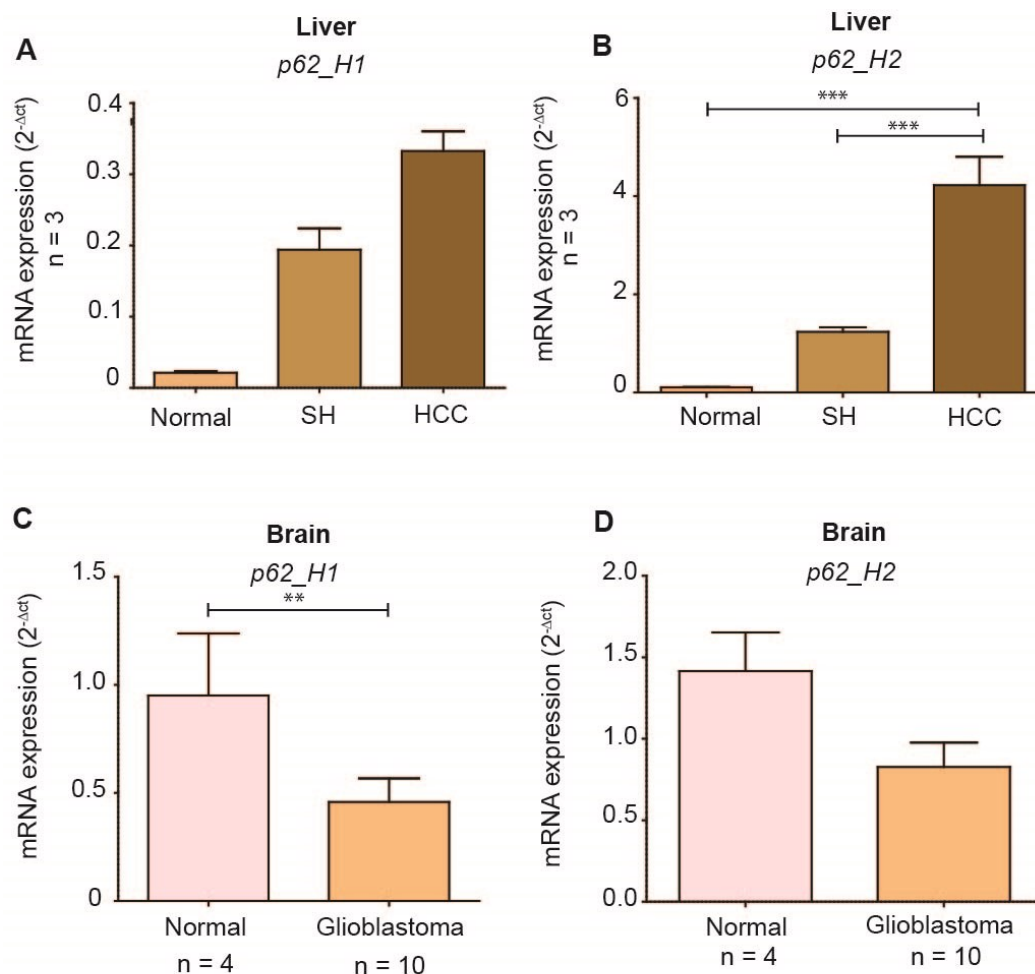


**Figure 30. mRNA expression of p62\_H1 and p62\_H2.**

qPCR analysis of p62\_H1 and p62\_H2 was performed on RNA samples from (A) normal, SH and HCC affected livers and (B) normal brain and glioblastoma affected brain. HCC showed maximum expression of p62\_H1 and p62\_H2, followed by SH and normal liver. On contrary, normal brain showed higher expression of p62\_H1 and p62\_H2 than glioblastomas. Data was analyzed using student's t-test. \*\*p<0.01, \*\*\*p<0.001

### 4.2.2. Influence of p62-isoforms on interaction with aggregation-prone proteins

Pathogenic protein aggregates of various diseases such as MDBs in SH, Lewy bodies in PD or DLB are known for its high cross  $\beta$ -sheet content, and the presence of p62 and ubiquitin. The stimuli required for the acquisition of cross  $\beta$ -sheet structure by primary proteins in protein aggregates differ from disease to disease. For example, K8 in MDBs undergoes a conformational change from  $\alpha$ -helix to cross  $\beta$ -sheet upon chronic oxidative stress whereas  $\alpha$ -synuclein in Lewy bodies adopts a  $\beta$ -sheet conformation in its mutated states of A53T, A30P, and E46K and are associated early-onset PD.

To determine the impact of p62-isoforms in protein aggregation, we have constructed a deletion mutant of p62 (partially mimicking p62\_H2) by deleting the SH2 domain of PB1 (p62<sup>ΔSH2</sup>). SH2 domain is responsible for the protein-protein interaction mediated by p62 and induces a cascade of downstream processes required for various cellular processes. Transfection of p62<sup>wt</sup> (mimicking p62-H1) and p62<sup>ΔSH2</sup> (partially mimicking p62\_H2) individually in CHO-K1 cells showed that p62<sup>wt</sup> aggregates displayed mixed morphology ranging from mostly smaller circular aggregates to globular aggregates however, deletion of SH2 led to globular aggregates (Figure 31). Interestingly, only p62<sup>ΔSH2</sup> aggregates were positive for h-HTAA labelling, indicating that lack of adaptor domain SH2 induces a conformational change in the p62 leading to the formation of cross  $\beta$ -sheet rich aggregates (Figure 31).

Now the question was how the deletion of SH2 domain influences the binding of p62 to other aggregation prone proteins. To this aim, we utilized the established in-vitro model of MDB formation to investigate and compare the aggregation pattern of p62<sup>wt</sup> or p62<sup>ΔSH2</sup> with K8 and K18. K8 aggregates are known for its susceptibility towards acquisition of cross  $\beta$ -sheet conformation and K18 forms aggregates only when associated with other aggregation prone proteins such as p62 or ub (Stumptner et al., 2007).

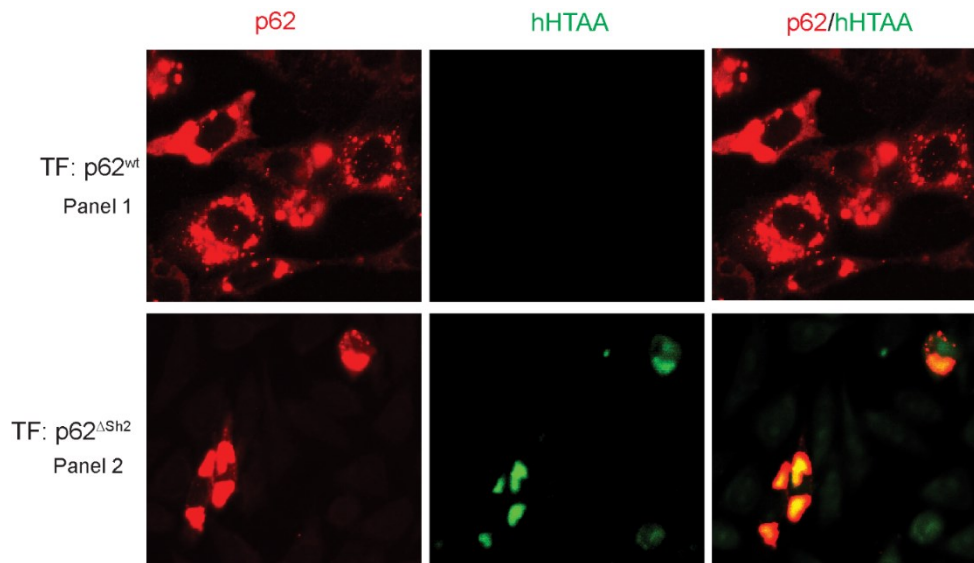


**Figure 31. Deletion of SH2 domain induces cross  $\beta$ -sheet conformation of p62.**

CHO-K1 cells were transfected with p62<sup>wt</sup> (panel 1) and p62<sup>ΔSH2</sup> (panel 2) alone and stained with antibody against p62 and labelled with hHTAA to detect cross  $\beta$ -sheet conformation of resulting aggregates. p62<sup>ΔSH2</sup> but not p62<sup>wt</sup> aggregates acquire cross  $\beta$ -sheet conformation. Scale bar = 20  $\mu$ m

Cotransfection of p62<sup>wt</sup>, K8 and Ub led to the formation of cross  $\beta$ -sheet rich in-vitro MDBs as shown previously (Stumptner et al., 2007, Mahajan et al., 2011) (Figure 32 A, panel 2) . The deletion of p62-SH2 domain did not seem to alter the binding of p62 to K8, suggesting that the binding of p62 to ubiquitinated K8 occurs via p62-UBA domain (Figure 32, panel 3) (Stumptner et al., 2007). The p62<sup>ΔSH2</sup>-K8 aggregates were more globular in appearance than p62-K8 aggregates and retained the cross  $\beta$ -sheet conformation (Figure 32, panel 3). Furthermore, cotransfection of K18, p62<sup>wt</sup> and ub resulted in the formation of aggregates similar to what we observed in K8, p62<sup>wt</sup> and ub transfection (Figure 32, compare panel 1-2 vs. panel 4-5). However, K18 aggregates alone or in combination with p62-ub fails to acquire cross  $\beta$ -sheet conformation. Interestingly, when K18 was transfected with p62<sup>ΔSH2</sup> and ub, the resulting aggregates acquire cross  $\beta$ -sheet conformation (Figure 32, panel 6) suggesting that the deletion of p62-SH2 alone is capable of conferring conformational changes in a protein aggregation system. The data further suggests that the inability of K18 to induce MDBs in K8 knockout

mice is not a direct result of lack of K8 but also depends on the stability of K18 and p62 *in-vivo*. These observations are of particular importance as the second isoform of human p62 (p62\_H2) is devoid of SH2 domain and may regulate the amyloid conformation of other pathogenic aggregates.

#### **4.2.3. Role of p62 in $\alpha$ -synuclein mediated aggregation in-vitro**

It was clear from the *in-vitro* model of MDB formation that deletion of p62-SH2 domain could confer amyloid characteristics to K18-p62 aggregates in the absence of a preferred substrate protein for cross  $\beta$ -sheet conformation, i.e., K8. To further investigate the role of p62 in Lewy bodies, we have performed a series of transfection studies on CHO-K1 cells with the core components of Lewy bodies i.e.,  $\alpha$ -synuclein ( $\alpha$ -syn), p62<sup>wt</sup>, p62 <sup>$\Delta$ SH2</sup> and ub. It was noted that  $\alpha$ -syn could not form aggregates with p62<sup>wt</sup> unless ubiquitin was added to the system (Figure 33, Panels 1 and 2). On contrary,  $\alpha$ -syn can progressively form aggregates with p62 <sup>$\Delta$ SH2</sup> with or without ubiquitin (Figure 33, Panels 3 and 4). Moreover, only p62 <sup>$\Delta$ SH2</sup>-  $\alpha$ -syn aggregates acquire cross  $\beta$ -sheet conformation (Figure 33, Panels 5-8). The data suggests that p62 is involved in  $\alpha$ -syn aggregation either via ubiquitin association or via  $\beta$ -sheet interaction. Hence, it can be assumed that p62\_H2 lacking SH2 domain may drive the conformational change in  $\alpha$ -syn aggregates (Lewy bodies) with age in Parkinson's patients. Also, the absence of p62\_H2 in mice might be one of the contributing factors why  $\alpha$ -syn could not acquire amyloid conformation in traditional mouse models (Masliah et al., 2000).

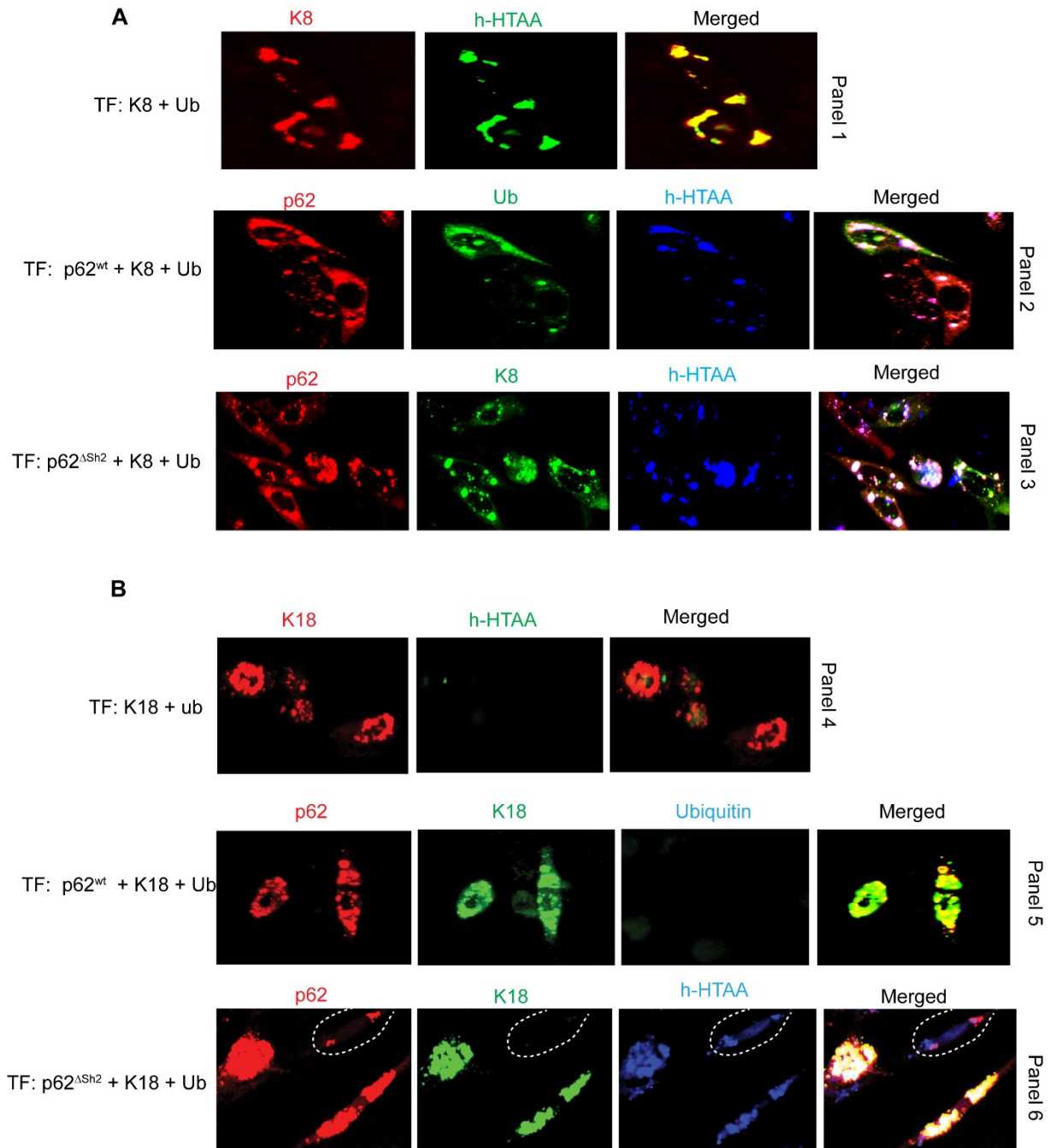
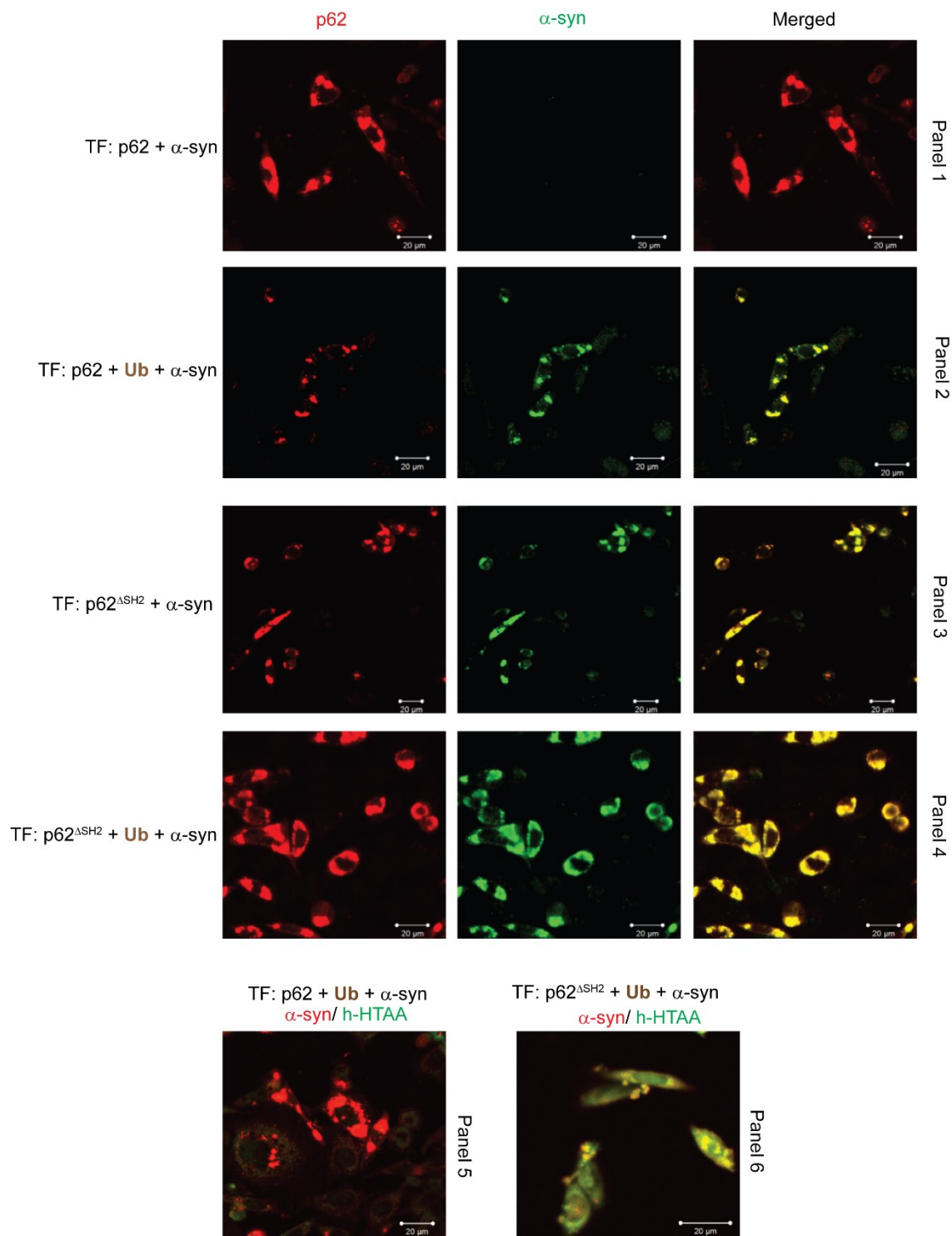


**Figure 32. Influence of p62-SH2 deletion on K8 and K18 aggregation.**

(A) CHO-K1 cells were transfected with K8+Ub (panel 1) and p62<sup>wt</sup>+K8+Ub (panel 2 and 3) and stained with antibodies against K8, p62, Ub and labeled with h-HTAA as indicated in the panels. K8 can form cross  $\beta$ -sheet rich aggregates alone or in combination with p62 and Ub. (B) Cells were transfected with K18+Ub (panel 4) and p62<sup>ΔSH2</sup>+K18+Ub (panel 5 and 6) and stained with antibodies against K18, p62, Ub and labeled with h-HTAA as indicated in the panels. K18

aggregates alone or in conjunction with p62<sup>wt</sup> cannot acquire cross  $\beta$ -sheet conformation. However, transfection of p62 <sup>$\Delta$ SH2</sup>, K18 and ub together results in cross  $\beta$ -sheet rich aggregates.



### Figure 33. Different modes of p62-mediated aggregation.

CHO-K1 cells were transfected in various combination with p62<sup>wt</sup>/p62<sup>ΔSH2</sup>, α-synuclein (α-syn) and with or without ubiquitin stained with indicated antibodies (see panels). α-syn cannot form aggregates with p62<sup>wt</sup> (Panel 1) unless ubiquitin is transfected in combination with α-syn+ p62<sup>wt</sup> (panel 2). The resulting ubiquitinated p62<sup>wt</sup>-α-syn aggregates does not acquire cross β-sheet conformation (panel 5). Moreover, α-syn forms aggregates with p62<sup>ΔSH2</sup> in the presence (panel 3) or absence of ubiquitin (panel 4). Ubiquitinated p62<sup>ΔSH2</sup>-α-syn aggregates showed cross β-sheet positivity. Scale bar = 20 μm

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# Discussion

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## 5.1. Physiological functions of p62 in MDB formation

The involvement of p62 in protein aggregation process is not straightforward. The presence of p62 as a general feature in protein aggregates of previously unrelated diseases of different etiologies indicates a functional role determined by the structural and functional properties of p62, possibly shaped by evolution. Studies over the last decade show p62's role in protein aggregation to be both neutral and antagonizing. The cellular starvation studies on genetic models of p62 promote the notion that p62 favors the formation of non-pathogenic aggregates with or without ubiquitin as a preparatory step towards degradation of the cargo by UPS or autophagy. However, no direct evidence on presumed enhancing effect of p62 on disease-related inclusions in-vivo is available. In this study, we have shown how p62 regulates the deposition of diseases-related inclusion, i.e., MDBs in stressed livers using an established model of DDC-induced MDB formation. DDC-mediated stress to an extent mimics steatohepatitis related liver injury and has greatly enhanced our understanding of MDB formation process over the years.

MDBs developed in DDC-intoxicated p62-deficient mice were smaller, granular and less distinct in their outlines when compared to that of the control mice (Figures 14-16). Thus, MDBs in p62-deficient livers resembled early stages of MDB development suggesting that the coalescence to larger aggregates is impaired in the absence of p62. However, these "early" p62-deficient MDBs already displayed the principal features of classical MDBs, i.e., cross  $\beta$ -sheet conformation, type II filamentous ultrastructure and M<sub>M</sub>120-1 positivity (Figures 14-17) (Yokoo et al., 1982, Zatloukal et al., 1990a, Mahajan et al., 2011). Considering p62's ubiquitin binding nature, the fact that ubiquitinated-keratin-rich "early" MDBs were formed in p62-deficient animals was surprising and suggestive of the presence of other ubiquitin adaptors such as NBR1. Recent evidence suggests that NBR1 and p62 oligomerize with each other via PB1 and shuttles the ubiquitinated cargo together towards degradation (Kirkin et al., 2009). NBR1 could also recruit ubiquitin-positive bodies to autophagosomes independently of p62 (Waters et

al., 2009). In our study, p62 deficiency led a slight increase in ubiquitinated proteins and NBR1 under basal conditions (Figure 22-24). However, upon DDC-treatment, p62 deficiency led to a decrease in NBR1 and ubiquitinated proteins and very few p62-deficient MDBs were positive for NBR1 and ubiquitin (Figure 22 and 23). The presence of ubiquitin negative MDBs suggests that incorporation of p62 to keratin-rich MDBs occurs before ubiquitin or other ubiquitin adaptors such as NBR1. Our data and previous immunohistological studies on inclusions of brain suggest that association of p62 to other primary components such as keratins in MDBs,  $\alpha$ -synuclein in LBs, tau in NFTs does not require ubiquitin and the binding of NBR1 to the central components is mostly weaker than p62 (Table 12) (Kuusisto et al., 2002, Kuusisto et al., 2003, Zatloukal et al., 2002, Stumptner et al., 2001a, Stumptner et al., 2002, Odagiri et al., 2012).

**Table 12. Correlation of p62 with central components, ubiquitin and ubiquitin adaptor NBR1 in various protein aggregates**

| Type of protein aggregates                | Central component     | p62 vs. central component                                                        | p62 vs. ub                                             | p62 vs. NBR1                                                            | Reference                                                                              |
|-------------------------------------------|-----------------------|----------------------------------------------------------------------------------|--------------------------------------------------------|-------------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| <b>Mallory-Denk bodies (SH, HCC)</b>      | Keratins (K8 and K18) | p62 present in "early MDBs" but not required for the initial keratin aggregation | p62 exceeds ub                                         | p62 exceeds NBR1; late component; weaker colocalization of NBR1         | Stumptner et. al., 2002<br>Zatloukal et. al., 2002<br>Lahiri et. al., 2016             |
| <b>Intracellular hyaline bodies (HCC)</b> | p62                   | p62 is the central component                                                     | p62 exceed ubiquitin; mostly Ub negative               | p62 exceeds NBR1; weaker colocalization of NBR1                         | Stumptner et. al., 2002<br>Zatloukal et. al., 2002<br>Lahiri et. al., unpublished data |
| <b>Lewy Bodies (brain-stem type; PD)</b>  | $\alpha$ -synuclein   | similar expression pattern of p62 + $\alpha$ -synuclein                          | similar expression pattern of p62 + ub                 | similar expression pattern of p62 + NBR1; late component ? not analyzed | Kusisto et. al., 2001<br>Kusisto et. al., 2002<br>Odagiri et. al., 2012                |
| <b>Glial cytoplasmic inclusion (MSA)</b>  | $\alpha$ -synuclein   | similar expression pattern of p62 + $\alpha$ -synuclein                          | similar expression pattern of p62 + ub                 | mostly NBR1 negative late component ? not analyzed                      | Kusisto et. al., 2001<br>Kusisto et. al., 2002<br>Odagiri et. al., 2012                |
| <b>Neurofibrillary tangles (AD)</b>       | tau                   | mostly p62 positive                                                              | p62 is present in early tau aggregates; p62 exceeds ub | Weaker colocalization of NBR1; possibly late component                  | Kusisto et. al., 2002<br>Zatloukal et. al., 2002                                       |

|  |  |  |  |  |                          |
|--|--|--|--|--|--------------------------|
|  |  |  |  |  | Odagiri et. al.,<br>2012 |
|--|--|--|--|--|--------------------------|

Now, the question arises that if p62 and ubiquitin association is a late event regulating the ubiquitination of primary components, what other factors determine the strong association of p62 with primary components of various inclusions. The answer may hold in the peculiar common structural features of aggregated proteins such as open hydrophobic residues, oxidized surfaces, phosphorylated SH2/SH3 (PXXP) motifs and cross  $\beta$ -sheet interaction through which p62 may bind directly or indirectly to primary components. Previous structural studies on p62 suggest that p62 binds to other PB1 domain containing proteins (such as NBR1) via heterodimerization of the domains in a front to back manner without ubiquitin interactions. However, the majority of the primary components of protein inclusions does not contain PB1 domains. Our studies on human p62 isoforms (Results part II) hinted towards cross  $\beta$ -sheet mediated interaction between p62 and central components. Previous in-vitro studies on MDB formation showed that p62\_H1 or p62<sup>wt</sup> can bind to cross  $\beta$ -sheet rich K8 aggregates to form type II MDBs with or without ubiquitin (Stumptner et al., 2007). Interestingly, the absence of SH2 domain in p62\_H2 led to its acquisition of cross  $\beta$ -sheet conformation and formation of more globular aggregates without ubiquitin (Figure 31). In this context, when K18 (which does not acquire cross  $\beta$ -sheet conformation) was transfected along with p62 <sup>$\Delta$ SH2</sup> or p62\_H2, it results in the formation of type II MDBs similar to that of p62+K8 transfections (Figure 32) (Stumptner et al., 2002, Stumptner et al., 2007). Further, we tested our hypothesis that the p62\_H2 may alone mediate aggregation by transfecting p62\_H2 in combination with primary constituent of Lewy-bodies i.e.,  $\alpha$ -synuclein. Studies till date has reported that wildtype  $\alpha$ -synuclein cannot progressively form aggregates in cell culture system whereas in animal models, aging leads to the formation of wildtype  $\alpha$ -synuclein aggregates in association with p62 and ubiquitin (Beal, 2010a, Tanik et al., 2013, Masliah et al., 2000). Genetic mutations such as A53T, A30P, and E46K in  $\alpha$ -synuclein are responsible for the formation of Lewy-body like inclusions both in-vitro and in-vivo with cross  $\beta$ -sheet conformation (Burre et al., 2012). Our experiments showed that p62\_H2 and not p62\_H1 can progressively form Lewy-body-like cross  $\beta$ -sheet rich aggregates with wildtype  $\alpha$ -synuclein. Hence, it can be assumed that p62\_H2 lacking SH2 domain may drive the conformational change in  $\alpha$ -synuclein aggregates (Lewy bodies) with age

in Parkinson's patients. Also, the absence of p62<sub>H2</sub> in mice might be one of the contributing factors why  $\alpha$ -syn could not acquire amyloid conformation in traditional mouse models.

As another possibility, p62 may binds to aggregates indirectly via chaperones as they are recruited to misfolded proteins as a first line of defense of the cellular system (Fink, 1999). Molecular chaperones such as Hsp25 and Hsp70 were previously described as a component of MDBs (Janig et al., 2005). In a DDC-primed mouse model, heat shock led to the expression of both Hsp25 and Hsp70 before MDB formation suggesting a functional role in MDB pathogenesis (Yuan et al., 1995). In our study, MDBs in p62-deficient livers were negative for Hsp25 (Table 11, Figure 18) suggesting a novel cooperative mechanism between p62 and Hsp25 in response to aggregates of misfolded proteins, which needs to be further characterized.

Furthermore, in contrast to the lack of involvement of p62 in the initiation of MDB formation, p62 stabilizes MDBs against degradation as indicated by the pronounced numerical reduction of MDBs and the more rapid disappearance of high molecular weight K8 and ubiquitinated proteins in p62<sup>-/-</sup> livers in the recovery phase after DDC intoxication (Figure 24). This marked decrease in high molecular weight crosslinked proteins could also be attributed to the observed decrease in Tg2 levels in p62-deficient livers (Figure 18). According to earlier reports, stimulated autophagy counteracts MDB formation (Harada et al., 2008). However, whether autophagy is involved in the clearance of MDBs after recovery from DDC intoxication has not been investigated. In our study, the presence or absence of p62 had no effect on the alterations of LC3-II/ LC3-I ratio in the different treatment groups (Figure 26). Thus, it is unlikely that p62-dependent impairment of autophagy is responsible for reduced stability of MDBs in p62-deficient mice. Although MDB-like inclusions have been detected in autophagosomes of K8-overexpressing mice treated with proteasomal inhibitor bortezomib (Harada et al., 2008), electron microscopic studies of mouse livers recovered from griseofulvin intoxication showed that MDB remnants were associated with desmosomes (Figure 25) but not autophagosomes (Denk and Franke, 1981). This finding corroborates our observations and suggests that autophagy does not play a major role in the elimination of MDBs during recovery. Moreover, we observed more pronounced reduction in the size than in the number of MDBs in both wildtype and p62-deficient recovered livers (Figure 24), which favors the assumption that the

elimination is caused by a cytosolic enzymatic degradation process, such as calcium ( $\text{Ca}^{2+}$ )-dependent proteinases (Nelson and Traub, 1982) or the UPS.

Characterization of the other facultative components of MDBs revealed K17 positivity for MDBs present in K17-positive hepatocytes (Figure 21). Furthermore, we observed the expression of K17 in a subset of ductular cells and in a few adjacent hepatocytes (Figure 21). Whereas the binding of K17 to MDBs was reduced in the absence of p62 (Table 11), the binding of K7 to MDBs was apparently independent of p62 (Figure 19, Table 11). The comparison of the composition of MDBs in  $\text{p62}^{\text{f/f}}$  and  $\text{p62}^{-/-}$  mice also showed that p62-deficient MDBs did not contain the SMI 31 phosphoepitope (Table 11). This observation provides further evidence that the SMI 31 epitope in MDBs is present on p62 and not on tau, where it has been originally described in neurofibrillary tangles (Bancher et al., 1989).

Taken together, On the basis of observations made in this study and previous reports on MDB formation, the major findings of our study in DDC-fed mice on the role of p62 in MDB pathogenesis can be summarized as follows: (i) Initially, K8 undergoes a conformational change to  $\beta$ -pleated sheet (Mahajan et al., 2011), gets transamidated and at least partly evades degradation and self-aggregates; (ii) subsequently, stress-induced over-expressed p62 binds to K8 possibly via cross  $\beta$ -sheet interactions and aids in ubiquitination of K8 via p62 UBA domains (Stumptner et al., 2007); (iii) p62 fosters the formation of larger “mature” MDBs by coalescence of early small aggregates; (iv) by a similar type of interaction, p62 enhances binding of other proteins to MDBs, such as NBR1, Hsp25 and K17; (v) p62 stabilizes the resulting MDB inclusions (Figure 34).

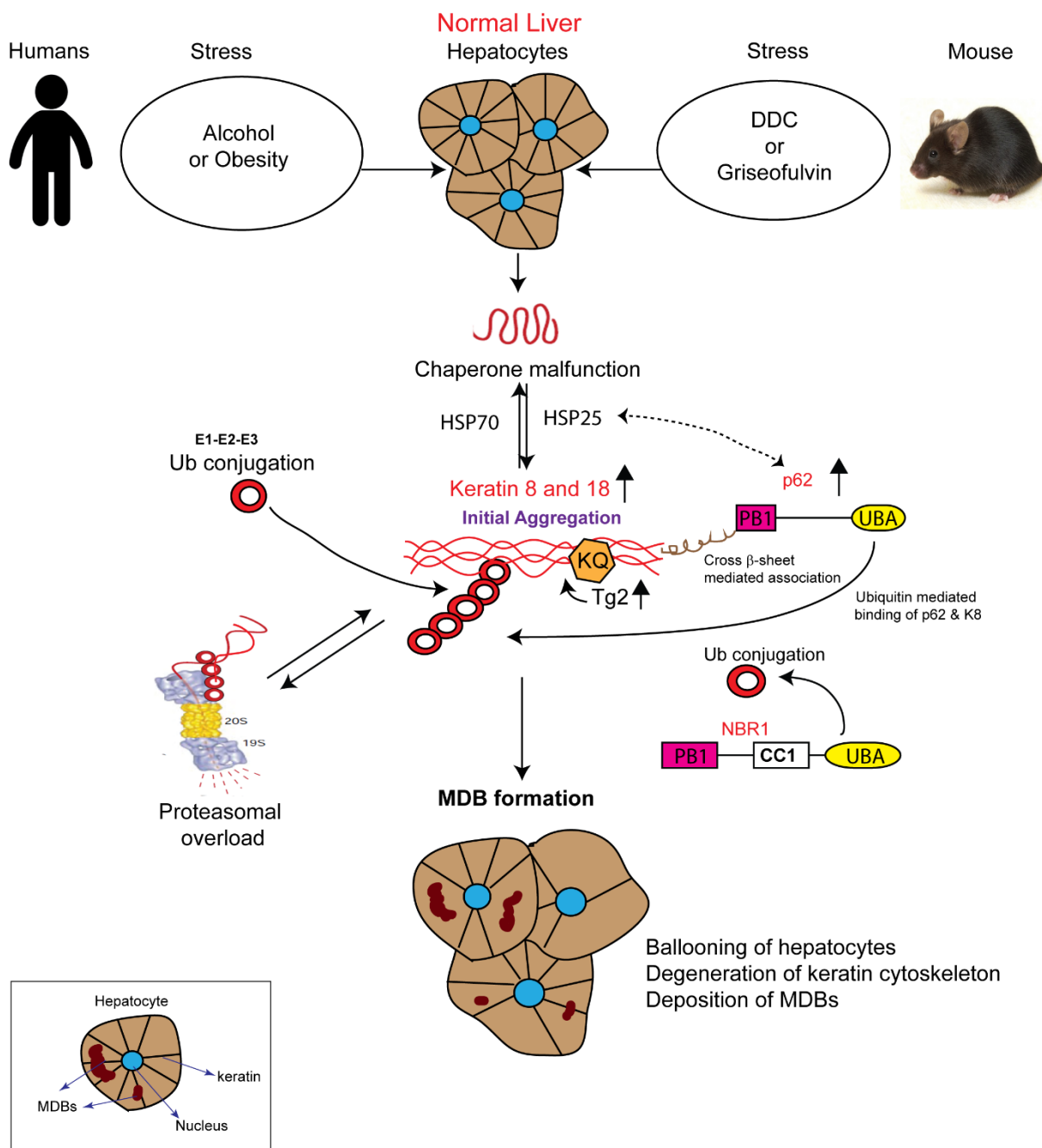


**Figure 34. Current understanding of the formation of MDBs.**

Excessive calorie intake/obesity or alcoholism in humans and treatment of hepatotoxins such as DDC or Griseofulvin in mice leads to the malfunction of chaperones (e.g., Hsp25 and Hsp70) and increase in the expression of K8 and K18. With increase in stress, K8 acquires cross  $\beta$ -sheet conformation, gets transamidated by TG2 at lysine (K) and glutamine (Q) residues and formation of insoluble cross-linked protein occurs. Oxidative and other types of stress also cause

the accumulation of misfolded keratins that can overwhelm the proteasome via accumulation of ubiquitinated or crosslinked proteins. In parallel, stress triggers the expression of p62. p62 can bind to K8 either via cross  $\beta$ -sheet association or via ubiquitin and incorporates NBR1 to the keratin-rich aggregates. The end result is the formation of MDBs.

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## 5.2. p62 and liver injury

In our model, we found no major difference with respect to liver morphology or liver damage-related serum enzyme levels between DDC-intoxicated wildtype and p62-deficient mice, despite differences in MDB formation (Figure 14). This was surprising since p62 exerts important functions in the cell by affecting NF $\kappa$ B signaling, regulating apoptosis through caspase 8-aggregation and modulating oxidative stress signaling and defense mechanisms (Komatsu et al., 2010, Jin et al., 2009). The latter are particularly pertinent in our model since oxidative stress is considered to play an important role in steatohepatitis of alcoholic and non-alcoholic etiology (Nikam et al., 2013, Nan et al., 2014). It was previously reported that p62 transcription is regulated by Nrf2 during chronic liver inflammation and oxidative stress (Jain et al., 2010). This is particularly pertinent in our model since oxidative stress is considered to play an important role in steatohepatitis of alcoholic and non-alcoholic etiology (Nikam et al., 2013). In the non-stressed situation, Nrf2 is sequestered in the cytoplasm by binding to its interaction partner Keap1, followed by ubiquitination and proteasomal degradation by E3 ubiquitin ligase cul3 (Cullinan et al., 2004). Oxidative stress leads to a conformation change in the keap1-Cul3 association, inhibiting the enzymatic activity of cul3, leading to Nrf2 dissociation. Free Nrf2 translocate to the nucleus and initiates the transcription of various antioxidant genes such as Nqo1 and Gst (Kobayashi et al., 2004, Osburn and Kensler, 2008). Alternately, p62 competitively inhibits the Keap1-Nrf2 interaction, leading to the stabilization of Nrf2 allowing nuclear translocation and expression of cytoprotective genes (Komatsu et al., 2010). DDC intoxication led to nuclear translocation of Nrf2 that was less pronounced in p62-deficient livers than in p62<sup>f/f</sup> controls, which, at least in part, can be attributed to the known function of p62 as competitive inhibitor of Keap1 (Taguchi et al., 2011, Inami et al., 2011, Copple et al., 2010). Interestingly, DDC intoxication led to marked upregulation of Nrf2 mRNA

level and translocation of Nrf2 into the nucleus. This effect was weaker in p62 deficiency when compared to p62<sup>f/f</sup> livers. The decrease in transcript and protein levels of Nrf2 in p62-deficient mice upon DDC-stress can be attributed to the role of p62 as an endogenous modulator of Nrf2 expression during chronic stress situations (Figure 27). Similar observations were recently made in a mouse model of hepatocellular carcinoma where chronic stress (DEN treatment) led to a reduction of Nrf2 target genes including Gst and Nqo1 mRNA in p62-deficient livers (Umemura et al., 2016). The impaired activation of proteins involved in cellular defense mechanisms in p62 deficiency could also explain the increased cell turnover as demonstrated by increased Ki-67 positive mitotic cells and slightly enhanced ductular reaction suggesting more pronounced liver injury (Figure 27). These data further strengthen the purported role of p62 in activation of Nrf2 and promoting cell survival by creating a positive feedback loop by inducing the expression of cytoprotective genes. The lack of correlation of serum parameters with occurrence of MDBs and related liver injury to some extent mirrors the human situation since in humans also serum transaminases are no appropriate indicators of the activity of steatohepatitis (Chalasani et al., 2012).

Of note, untreated p62<sup>-/-</sup>, but not p62<sup>hep</sup><sup>-/-</sup> mice developed obesity at the age of 4 months (Figure 11), which is consistent with previously published data (Rodriguez et al., 2006, Muller et al., 2013). In addition, macrovesicular hepatic steatosis was present in p62<sup>-/-</sup> and microvesicular steatosis in p62<sup>hep</sup><sup>-/-</sup> mice (Figure 12). This difference is as yet unexplained. It could result from specific roles of p62 in different cell types and tissues affecting glucose metabolism, leptin resistance and ERK (extracellular signal-regulated kinases) activity (Rodriguez et al., 2006, Harada et al., 2013, Komatsu et al., 2007). Interestingly, this difference was eliminated in the course of DDC intoxication (Figure 15-17).

### **5.3. Different pathways of MDB formation in steatohepatitis and hepatocellular carcinoma**

On the basis of morphology, ultrastructural and chemical composition, MDBs are related to but not identical with IHBs (Denk et al., 2006). IHBs can be found in a few cases of HCC (Denk et al., 2006) and in livers of infants with copper toxicosis (Muller et al., 2004) occasionally in

coexistence with MDBs (Denk et al., 2006, Mahajan et al., 2011). They differ from MDBs in that they contain p62 and (although not constantly) ubiquitin but lack keratins (Zatloukal et al., 2007). IHBs can thus be regarded as *in vivo* equivalents of “p62 bodies” found in stressed tissue culture cells caused by increased expression of p62 (Komatsu et al., 2007, Kirkin et al., 2009, Bjorkoy et al., 2005, Pankiv et al., 2007). It can be speculated that p62 bodies can serve distinct cellular functions (Moscat et al., 2007). For example, p62 may reversibly recruit specific target proteins from their functionally relevant site(s) in the cell. By doing that, the protein may be stored until the appropriate signal causes a conformational change leading to the release of the protein (Moscat and Diaz-Meco, 2009b, Moscat and Diaz-Meco, 2009a). Our data and previous reports (Denk et al., 2006) suggest that MDB formation may occur via two different pathways (Figure 35). In the first, which is typical for human steatohepatitis and related animal models (e.g., DDC intoxication), small keratin-containing “early” MDB granules are generated in response to stress-induced conformational changes and ubiquitination of keratin, and the subsequent incorporation of p62 and NBR1 results in the formation of large “mature” MDBs. In the second pathway, which can be proposed on the basis of previous histological and ultrastructural analysis of IHBs in HCCs and idiopathic copper toxicosis, overexpression and accumulation of p62 precede keratin aggregation. The later incorporation of keratins into a matrix of aggregated p62 likely cause the transformation of p62-containing IHBs/aggregates into classical MDBs (Stumptner et al., 1999, Denk et al., 2006, Zatloukal et al., 2007).

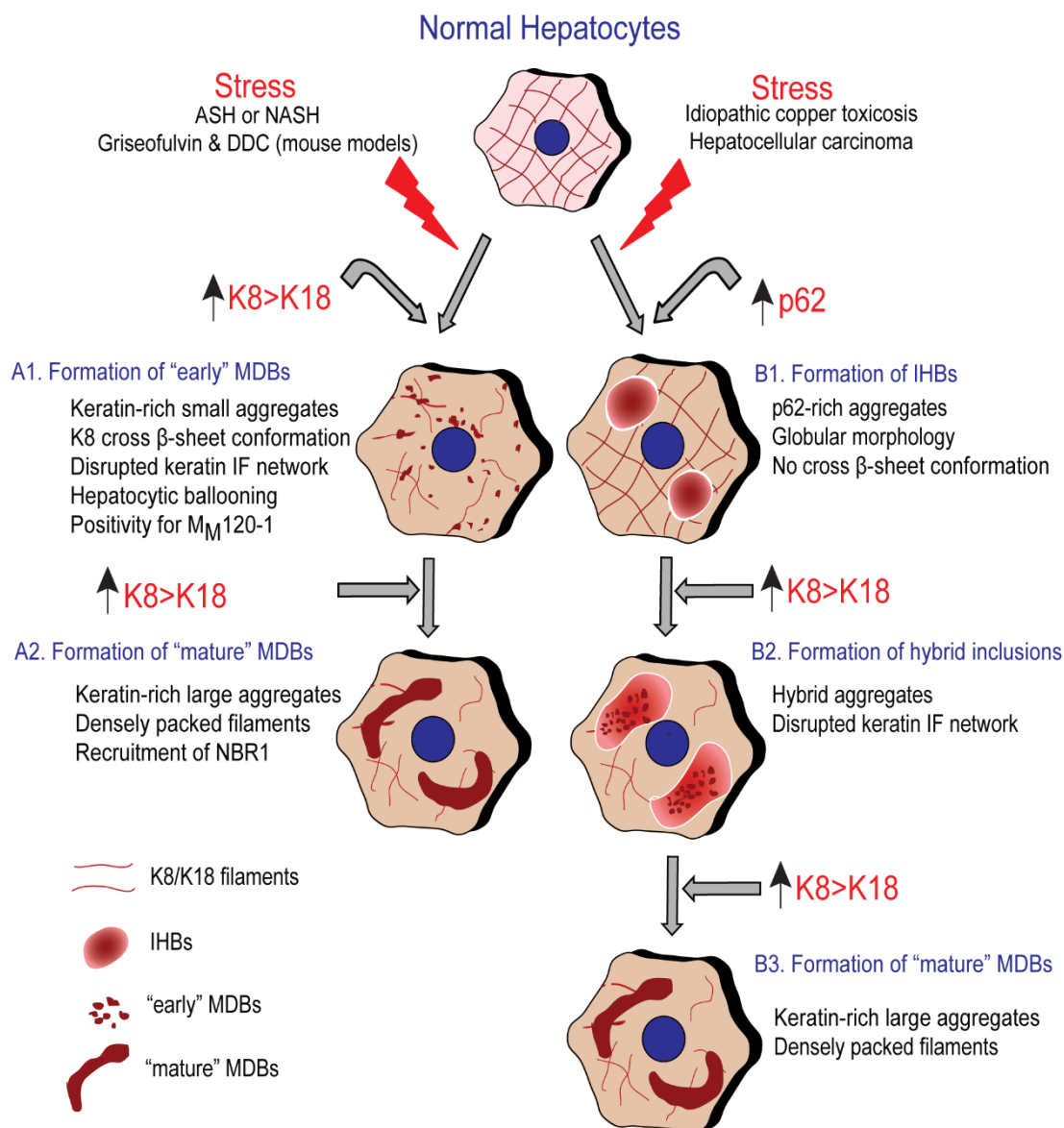


**Figure 35. Schematic drawing of the different role of p62 and keratin in two distinct hypothetical pathways of MDB formation.**

**(A1)** In the first pathway alcohol abuse or metabolic alterations associated with obesity in the context of human alcoholic or non-alcoholic steatohepatitis (ASH or NASH), and DDC or griseofulvin administration in mice trigger the upregulation of keratins (K8 and K18) with, increased K8:K18 ratio and cross  $\beta$ -sheet conformation of K8, which leads to the formation of small "early" MDBs with loosely arranged filamentous ultrastructure and M<sub>M</sub>120-1 antigen positivity. **(A2)** The coalescence of small MDBs to form large, compact "mature" MDBs occurs via incorporation of p62. Binding of p62 to MDBs also promotes the recruitment of NBR1 to

MDBs. **(B1)** In the second pathway (found in hepatocellular carcinoma and idiopathic copper toxicosis) p62-containing IHBs, which are negative for keratin and do not show cross  $\beta$ -sheet conformation, may progress to **(B2)** hybrid inclusions (showing mixed features of both IHBs and MDBs) by incorporation of keratins (K8 and K18). **(B3)** The hybrid inclusions may transform to “mature” MDBs upon further incorporation of K8 and K18. Adapted from (Lahiri et al., 2016)

## 5.4. Conclusions

Taken together, this study demonstrate new roles of p62 as adaptor for the recruitment of other proteins to MDBs, in the maturation of MDBs and as a stabilizer of MDBs, which might also be relevant to the pathogenesis of other protein aggregates containing p62 in various protein aggregation diseases. In addition, this study provides strong evidence that abnormal keratins behave as aggregation-prone proteins and are primarily responsible for the formation of MDBs.

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