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A study on the autophagy receptor p62/SQSTM1

Impact of ATF4 and 5´untranslated region of mRNA upon amino acid deprivation

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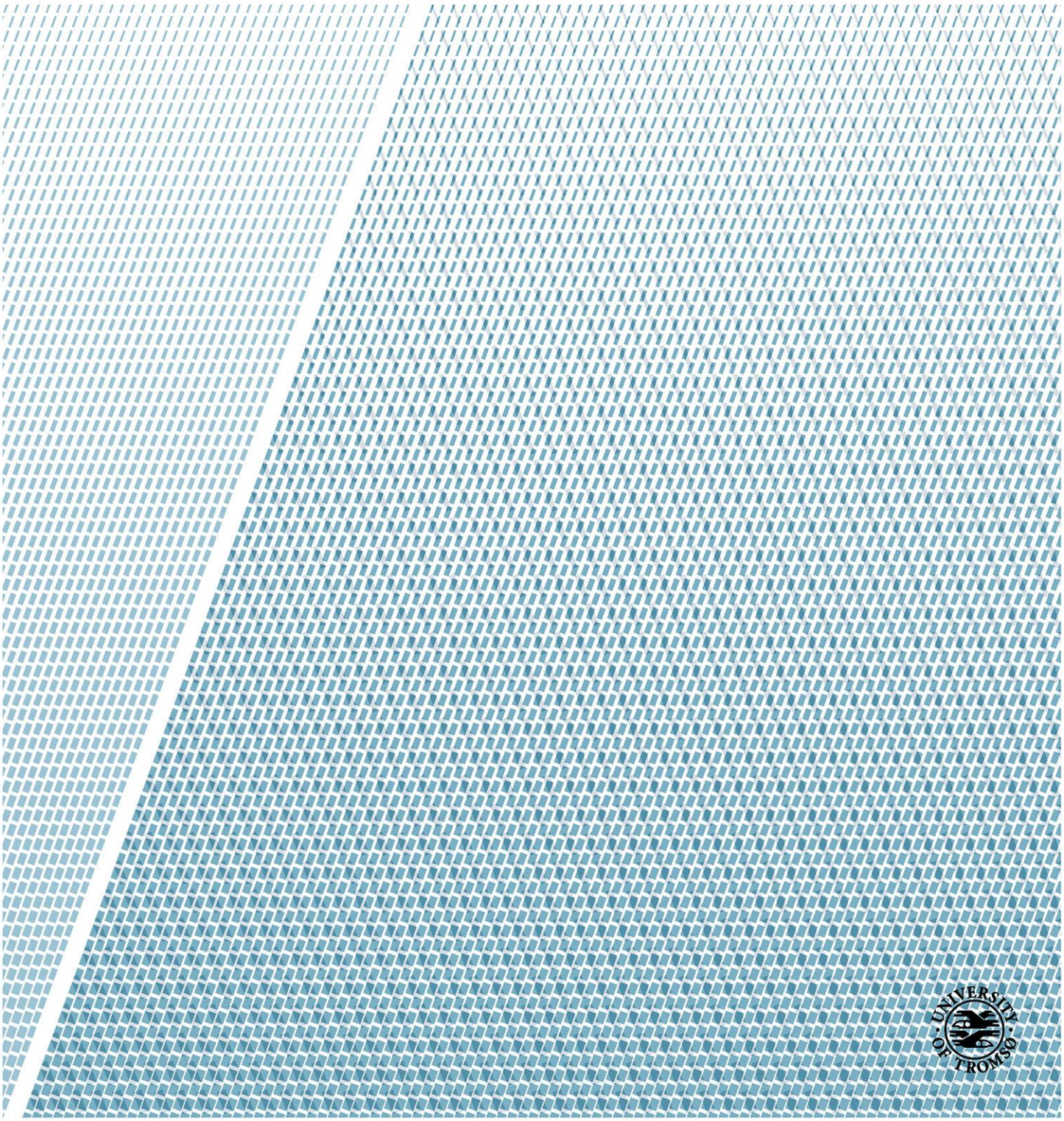

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Line Mari Fredriksen

Abstract

Autophagy is crucial for sustainment of cellular homeostasis, but there is limited knowledge of how autophagy is regulated. The present study examined characteristics of the first mammalian autophagy receptor described, the sequestosome-1 or p62/ SQSTM1, regarding transcriptional and translational expression under stress by amino acid starvation in the mammalian Hek293 cells.

Reviews report p62 to be restored during prolonged starvation in mouse embryonic fibroblasts and HepG2 cells. Initially p62 is also degraded by autophagy, but after 4-8 hours the level is restored. The transcription factor ATF4 is induced by starvation. Three aims were defined in the present study; first to test whether the p62 promoter is up-regulated by ATF4 compared to the inducing transcription factor NRF2, second to perform bioinformatics studies of the sequences in the p62 5´UTR and examine whether structures as "hairpins" related to the 5´UTR may explain levels of the p62 translation, and third to determine if different lengths of the 5´UTR have impact on the p62 translation under starvation.

Measures of expression of p62 promoter in Hek293 cells co-transfected with ATF4 expression plasmid compared to NRF2 as control, were assessed by reporter gene assay. To find whether ATF4 is involved in the upregulation of p62, and if translation of p62 is regulated by sequences in 5´UTR, bioinformatics and luciferase analyses were performed.

In the experimental set-up of Hek293 cells transfected with ATF4 and p62 promoter region, showed no enhancing effect on the p62 promoter to be detected. Bioinformatic analysis showed that the 5`UTR was unusually short and occurred in three main variants of 35, 62 and 100 bp. The 100 bp 5`UTR was predicted to form two hairpin structures.

The three variants of the 5`UTRs were cloned in front of the luciferase gene, and their effect on luciferase expression was measured under normal condition and upon prolonged starvation.

Interestingly, the 5`UTRs seemed to repress protein expression both under normal conditions and during starvation, possible due to formation of hairpin structures. In contrast the p62 5`UTRs co-transfected with an ATF4 expression plasmid did seem to reverse the repression of the p62 5`UTRs. Western blot of all extracts from starved Hek293 cells showed that the level of p62 decreased with 1- 4 hours of starvation, but after 6 hours an increase occurred. However, the LC3B lipidation level was modest. Taken together, the restoration of the p62 expression upon prolonged starvation may be due to ATF4 action on p62 5`UTR in a direct or indirect way.

Abbreviations and glossary

AARE	Amino Acid Response Element
Amp	Ampicillin
ATF4	Activation transcription factor 4
bp	Base par
BLAST	Basic Local Alignment Search Tool
BLAT	Blast Like Alignment Tool
CARE	C/EBP-ATF Response Element
C/EBP	CCAAT-enhancher-binding protein
CHOP	C/EBP homologous protein
DMEM	Dulbecco´s Modified Eagle´s medium
eIF2α	eukaryotic translation initiation factor 2 α
ER	Endoplasmatic reticulum
FCS	Fetal bovine serum
GAAC	General Amino Acid Control
Hek293 cells	Human embryonic kidney 293 cells
Kb	Kilobases
LB	Luria-Bertani-medium
LC3	Microtubule-associated protein 1 light chain 3
mTOR	mammalian Target Of Rapamycin
NEB	New England Biolabs
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PIC	Pre-Initiation Complex
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOC	Super Optimal broth with Catabolite repression medium
SQSTM1/ p62	Sequestosome 1
TFBS	Transcription Factor Binding Site
UBA	Ubiquitine associating
UPS	Ubiquitin-Proteasomal pathway
5`UTRs	5' untranslated regions

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Introduction

Cellular homeostasis and proteolysis

In all cells, to maintain homeostasis, there is interplay between composition and decomposition of various proteins used in cellular structures and processes. Protein degradation and synthesis are generally essential for this balance to occur (Ashford and Porter, 1962, Klionsky et al., 2007).

Proteolysis is the cleavage of proteins into polypeptides or amino acids, and in general this happens by hydrolysis of the peptide bond performed by enzymes termed proteases. Intracellular proteolysis may serve different functions as post-translational cutting of precursor peptides or pro-enzymes into functional proteins, or protein degradation in lysosomes or proteasomes. The degradation in lysosomal pathway is commonly considered as a non-selective process, but may become selective upon starvation and other cellular stress like oxidative stress. The lysosomes contain a wide variety of proteases such as cathepsins. Proteolysis in the proteasome pathway is known as a ubiquitin-dependent process targeting unwanted proteins into the proteasome.

Proteins have different size and structure, and they are degraded with a variety of rates. Abnormal proteins may be more rapidly degraded. The rate of degradation of intact proteins may vary depending on their functions, for example enzymes involved in some metabolic processes may be degraded much faster than enzymes whose activity is largely constant under most intracellular conditions.

Many of the rapidly degraded proteins may have regulatory functions, such as being transcription factors. The rapid degradation of these proteins is necessary for adaptive responses to outer stimulus, whereas other rapidly digested proteins are important for the mechanism behind the regulation of intracellular enzyme activity in response to specific signals. Degradation of proteins also plays a significant role for intracellular renovation by removal of incorrectly folded proteins or injured proteins, and such to avoid consequences of faulty or damaged proteins (Cooper and Hausman, 2000). In the sections below, the two main systems for protein degradation the ubiquitin-proteasomal pathway and the lysosomal pathway will be further described.

The ubiquitin-proteasomal pathway

The ubiquitin-proteasomal pathway (UPS) is considered as a selective pathway in which intracellular protein determined to be degraded is tagged with ubiquitin in covalent bond. The ubiquitin protein discovered in 1980 (Ciechanover et al., 1980) is containing 76-aminoacids, and is evaluated as a highly conserved protein in eukaryotic phylogenetic approaches (Hochstrasser, 1992, Ciechanover et al., 1980). Recent studies have showed that ubiquitin plays a major role in the selective autophagy pathway in which the mammalian autophagy receptor p62/SQSTM1 (sequestosome-1, also known as p62) recognizes their cytosolic cargo by binding to conjugated ubiquitin linked to the cargo (Bjorkoy et al., 2005).

The ubiquitin-proteasomal pathway consists of a complex array of enzymes which link the chains of conjugated ubiquitin. The 26S proteasome is found to be a large multicatalytic protease complex that degrades proteins marked with ubiquitin. Three enzymes are recognized to contribute. The first enzyme in the process is the ubiquitin-activating enzyme (E1), and ubiquitin unit with specificity destined for conjugation to a protein is activated. Further, the ubiquitin is transferred to an ubiquitin carrier protein (E2), and along with the ubiquitin binds to the protein to be ubiquitinated. In the last step ubiquitin is conjugated to the protein target by the ubiquitin ligase (E3). E3 has its specific protein substrate. Typically, it binds to the lysine 48, but can also bind the second lysine parts (Lecker et al., 2006).
A deficit of this UPS pathway for regulated proteolysis has been suggested to be involved in pathogenesis of several human diseases such as neurodegeneration and cancer (Hernandez et al., 2004, Dikic et al., 2010).

Lysosmal degradation

The lysosome organelle is central in intracellular degradation of extracellular material, and acidic hydrolases in the lysosome perform proteolytic decomposition of proteins and organelles. Extracellular materials and plasma membrane proteins are supplied to the lysosome through the endocytic pathway. On the other hand, materials originating from cytosol (e.g., proteins, lipids, glycogens) are provided to the lysosome via various forms of autophagy (Mizushima et al., 2011).

Autophagy

The term autophagy is from the Greek, meaning “auto” oneself, and “phagy”, to eat, and refers to any cellular degradative pathway making the delivery of cytoplasmic cargo to the lysosome (Levine and Kroemer, 2008). Lysosome-based degradation of cytoplasmatic materials can occur in various forms. In two recent reviews of autophagy (Rogov et al., 2014, Mizushima et al., 2011), it is argued that autophagy serves two major functions in cellular homeostasis. The authors claim evidence for autophagy having evolved both as a pathway for 1) restoring intracellular nutrient supply to counteract effects of cellular starvation, and 2) as a control process to prevent cellular damage caused by toxic macromolecules and deviant organelles.

A third role considered of autophagy, and derived from the former control mechanism, is the intracellular immune function by this system to protect against invading microbes (Levine and Kroemer, 2008, Mizushima et al., 2011). Three different types of autophagy leading to lysosomal degradation are commonly reported in mammalian cells, called microautophagy, macroautophagy and chaperone-mediated autophagy (Figure 1). Macroautophagy comprises the most common known mechanism often referred as “autophagy” (Levine and Kroemer, 2008).

Macroautophagy

Macroautophagy is a process characterized by formation of a distinctive organelle, the autophagosome. Autophagosomes are described as intracellular vesicles consisting of a double membrane that captures a portion of cytosol to be degraded. Further on, their outer membrane use with late endosomes and lysosomes, and thus merging their content in larger compartments. This is considered a well preserved phylogenetic pathway that may have evolved on the basis of periods lacking nourishment, and the cell had to forsake parts of cytosol to survive. However, it also may have evolved to specifically degrade exaggerated or toxicity proteins, organelles or intracellular pathogens. Macroautophagy comprises two major pathways; one is named bulk autophagy and is characterized by sequestration of random parts of the cytosol. The other more selective pathway has been uncovered more recently, and unlike the bulk autophagy, this occurs during nutrient rich conditions.

An increasing number of subcellular structures have been found to be removed from cytosol by selective autophagy. However, what molecular elements that contributes to this pathway is highly specific, and mostly unknown. Research on yeast cells have shed light on more details of these selective ways, and more specifically the discovery of the selective autophagy receptors in yeast (Rogov et al., 2014, Kirkin et al., 2009, Johansen and Lamark, 2011). Similar receptors have been recognized in mammalian cells. In mammalian cells the autophagy receptor p62 / SQSTM1 was the first selective autophagy receptor to be described. Corresponding to the yeast Atg19, both contribute to tag cargo to the mechanism of autophagy, and promote formation of autophagosome (Rogov et al., 2014, Kirkin et al., 2009, Johansen and Lamark, 2011). Now we also know other autophagy receptors in mammalian cellssuch as NBR1, NDP52, Optineurin and Tax1BP1.

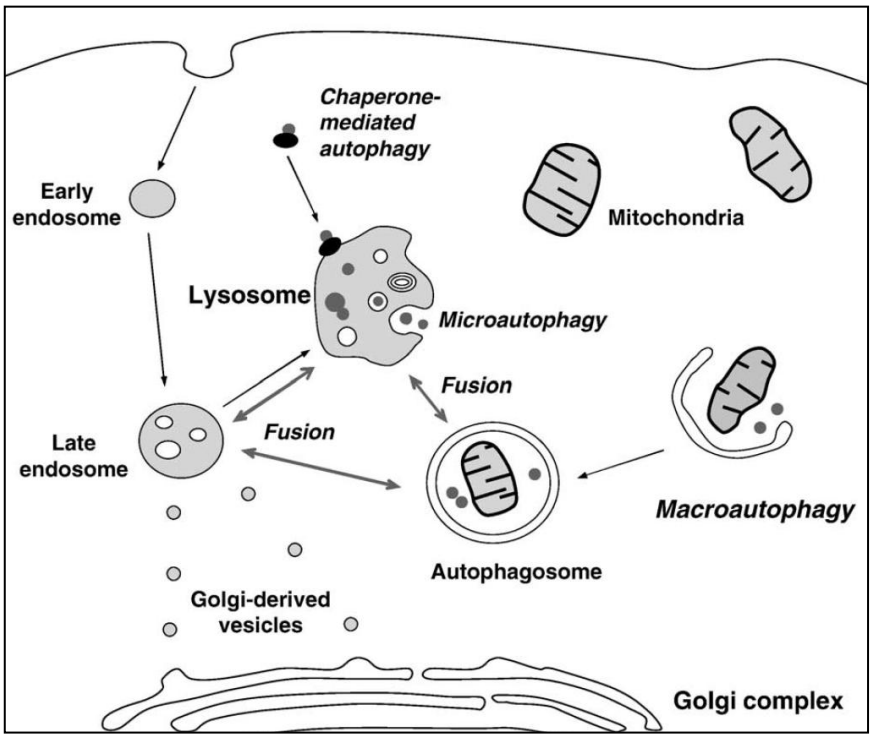

Figure 1. Display of different types of autophagy. Adapted from (Terman and Brunk, 2005).

Microautophagy

Microatuophagy involves direct acquisition of substrates without prior sequestration. Small components of cytosol enter lysosomes by invaginating of an endosomal or lysosomal

membrane, and this is also known as a selective pathway (Rogov et al., 2014). The cargo for microautophagy may include various macromolecules or organelles (Cuervo, 2004).

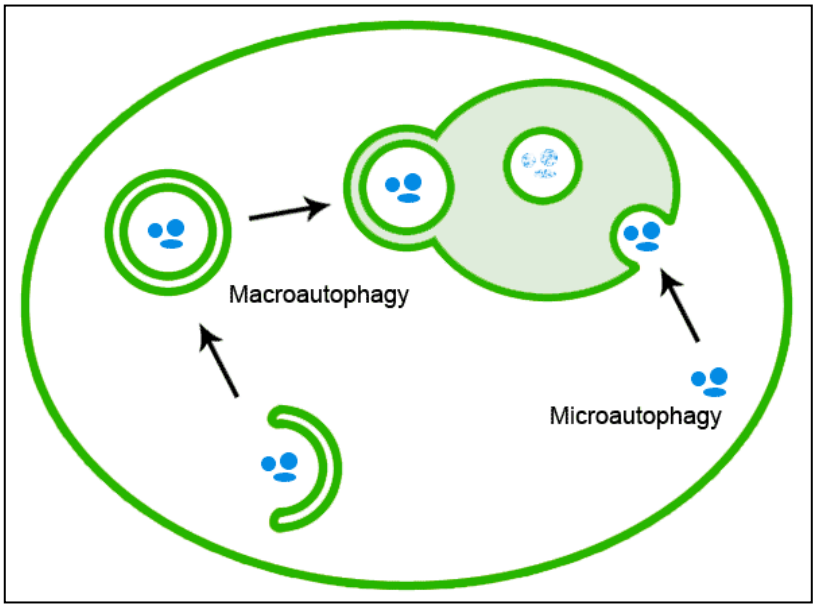

Figure 2. Adapted from Wikipedia. Comparison of the process of macroautophagy versus microautophagy.

Chaperone mediated autophagy

In molecular biology, chaperones are proteins that mediate structural changes of macromolecules by covalent folding or unfolding and assembly. Chaperones contribute to macromolecules forming functional units(Ellis, 2006). Chaperone-mediated autophagy (CMA) which is a selective protein degradation pathway, the Hsc70 chaperones recognize soluble proteins containing a KFERQ tag and translocate them from the cytoplasm to the lysosome without any vesicular trafficking. The protein chaperone complex will interact with another part of the CMA complex in the lysosomal membrane, and then further be transferred into the lysosomal lumen to be further degraded (Jaeger and Wyss-Coray, 2009, Kettern et al., 2010, Rogov et al., 2014).

Selective autophagy and autophagosome formation

A hallmark of autophagy is the formation of the autophagosome. This occurs over several steps, and which are respectively initiation, nucleation, elongation and later are the ligation of membrane to form the “preautophagosome” or phagophore. When it closes its double

membranes into a vesicle, it becomes an autophagosome. This will further be ready to fuse with the lysosome, see Figure 3.

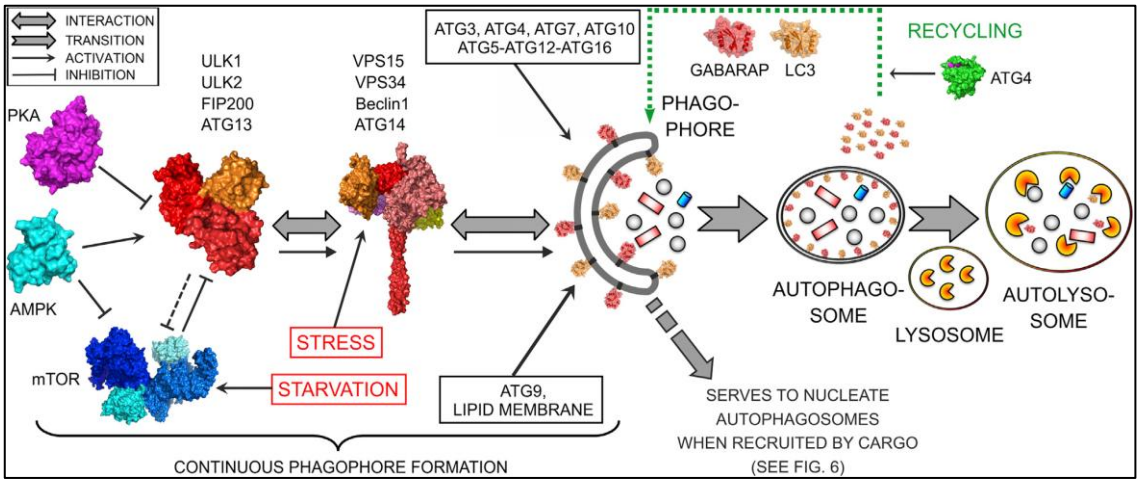

Figure 3. Schematic presentation of the autophagosome formation in the autophagy pathway adapted from (Rogov et al., 2014).

The autophag reseptores recognizes its cargo by binding to specific molecular elements such as selected unfolded regions of a protein or conjugated ubiquitin (Ub). Furthermore, propagation of self-oligomerization will create a platform for autophagosome formation. Ubiquitin-like modifiers (UBLs) -Atg8 / LC3 / GABARAP family, Atg5, Atg9 and Atg1-Atg13-Atg complex are known factors contributing to formation of the skeleton and initiating development of the lipid membrane to form the autopahgosome.

Research on yeast cells has revealed a total of 35 different autophagy-related genes that take share in regulation of different steps of autophagosome formation. The Atg proteins involved in this process consist of several functional units including two ubiquitin-like conjugation systems that play a centra role in the pathway (Mizushima et al., 2011, Johansen and Lamark, 2011, Rogov et al., 2014).

Initiation of the autophagosome and autophagy pathway

Different steps in the autophagy pathway are extensively accounted for in a recent review by Rogov and his collegues (Rogov et al., 2014). Some properties of the paths relevant to my project shall be referred. In yeast it is found that the initiation of autophagy was made by an

Atg protein complex Atg1-Atg13-Atg17, and a corresponding complex is identified in mammalian cells. This complex is evolutionarily conserved, and the ULK1 and ULK2, which are homologues of Ser / Thr kinase, corresponds to atg1 in yeast cells. ULK1/2 is phosporylated by mTORC1 (mammalin rampamycin) under cellular condition of nutrient deficiency. The mammalian ATG13 and FIP200 represents Atg13 and Atg17 in yeast. The whole complex is the mammalian initiation ULK1 / 2-ATG13-FIP200, however in contrast to the complex in yeast cells; it is independent of the cell nutrition status. Signaling kinases, like AMP activated protein kinase (AMPK) and protein kinase A (PKA) may influence phagophore initiation at the level of the ULK1/2 complex.

Nucleation

The next step in the autophagosom formation is nucleation. It is found to be controlled by the lipid hVps34 and its regulatory subunits ATG14L (Barkor) Beclin1 (ATG6) and hVPS15. Main results of hVps34 kinase activity is production of phosphotidylinositol 3-phosphate (PI3P) who recruits factors that promote phagophore nucleation. The elongating step in mammalian cells is regulated by the ATG9L1. This is a trans-membrane protein that supplements lipid membrane to the growing phagophore. The UBL complex Atg5-Atg12 and Atg8 / LC3 / GABARAP-phosphatidylthanolamine (PE) has been detected with impact on this process.

Elongation and closing phase

This step involves the two ubiquitin systems. Ubiquitin (Ub) is synthesized as a precursor protein that needs to proteolytic activated. Proteolytic maturation leads to exposure of its C-terminal Gly (Glycine) part which can bind a Lys (lysine) part belonging to another Ub or substrate protein. This contributes to initiate a cascade of enzymatic reactions involving activation (E1), conjugating (E2) and ligation (E3) enzymes, and different chains of Ub may have different functions, such as regulation of receptor internalization, attacking proteins degradation and assembling of signaling complex. Proteins containing Ub-biniding domains (UBDs) interact with Ub. Ub signals can be reversed by deubiquitin enzymes (Dubs), that are proteases that cleave the bond between Ub and its substrate.

The first small ubiquitin-like modifier (UBL) discovered was the Atg12 found as an autophagy protein. Atg12 is conjugated to lysine section of Atg5 containing two UBL

domains as Atg12-Atg5 complex. In mammalian cells, a corresponding complex is found along with the Atg16, and which has E3 similar activity for the second UBL specific system Atg8-PE (Fujita et al., 2013). Atg5 platforms contribute to build autophagosome platforms and to phagophore expansion. The Atg8-Atg4 complex receives a C-terminal with a Gly portion and is further activated by Atg7 (E1) , transferred to Atg3 (E2), and finally linked to the amino group of PE incorporated in the phagophore. When the autophagosom is completed, the Atg8 is de-conjugated by Atg4. In humans there are identified six Atg8 homologues; LC3A, LC3B and LC3C and various GABARAP, GABARAPL1 and GABARAPL2 / GATE-26 (Rogov et al., 2014).

The adapter protein p62/SQSTM1 in selective autophagy

The sequestosome 1 (SQSTM1) or p62 protein (p62/SQSTM1) serves as a scaffold or adapter protein (Geetha and Wooten, 2002). It plays an important role in the selective autophagy, but it also contributes to various cellular signaling pathways such as in NFKB/NFkB (nuclear factor of kappa light polypeptide gene enhacher in B-cells) activation, nerve growth factor signaling, and caspase activation. Therefore, p62/SQSTM1 has multiple functions in bone metabolism, obesity, inclusion body formation and tumor genesis (Wooten et al., 2006, Seibenhener et al., 2007, Moscat and Diaz-Meco, 2009, Komatsu et al., 2012).

The p62 is a small protein consisting of 440 amino acid (Joung et al., 1996) and contains an N-terminal with a PB1 domain followed by a ZZ-type zinc finger domain, nucleær localization signals (NLS), nuclear export signal (NES), LIR (LC3 intragerende region) and KIR (KEAP1 intragerende region) and a C-terminal UBA (Ub-associated) domain. See Figure 4.

Regarding autophagy, p62 interacts with the Atg8 proteins/LC3 and GABARAP family at the LC3-interacting region (LIR). This region is required for autophagy degradation of p62/SQSTM1 (Noda et al., 2010, Johansen and Lamark, 2011). The P62/SQSTM1 Phox and Bem1 (PB1) domain is a domain that allows p62 to integrate with protein kinases (ex. MEKK3, MEK5 and NBR1). It is also a domain for homopolymerization of p62. p62/SQSTM1 also acts as a cargo receptor for degradation of ubiquitin substrate. Because of

UBA (Ubiquitin binding domain), it makes SQSTM1 possible to operate as an adapter for the selective autophagy of ubiquitin substrates.

The p62/SQSTM1 also has the ability to move both to the phagophor membran and to the actual autophagosom-forming site (Itakura and Mizushima, 2011). Due to this, the p62 selectively incorporates into the autophagosome and then is degraded (Bjorkoy et al., 2005, Pankiv et al., 2007). The p62 is used as an indicator of autophagy flux (Klionsky et al., 2012, Mizushima et al., 2010).The expression of p62 is strictly regulated by a continuous degradation of basal autophagy. Failing or impairment of autophagy will lead to a massive accumulation of p62/SQSTM1 followed by aggregates which will contain p62/SQSTM1 and other ubiquitinated proteins. Accumulation of p62 is toxicity and neurodegenerative diseases and cancer are some examples (Komatsu et al., 2010). Under starvation, p62/SQSTM1is rapidly degraded and its total expression level is reduced. But how p62/SQSTM1 is regulated under starvation over time is unclear (Sahani et al., 2014).

One recent study (Sahani et al., 2014) has found that p62/SQSTM1 was upregulated under longer term starvation. Under conditions of nutritional deficiency, p62/SQSTM1 is first downregulated by autopagy, but then it is accumulated again after a few hours. This is independent of mTORC1 reactivation, but requires transcriptional upregulation of p62 and “de novo” protein synthesis. In this study the author also claimed evidence that amino acid derived for the autophagy-lysosome pathway are used for p62/SQSTM1 synthesis during prolonged starvation (Sahani et al., 2014).

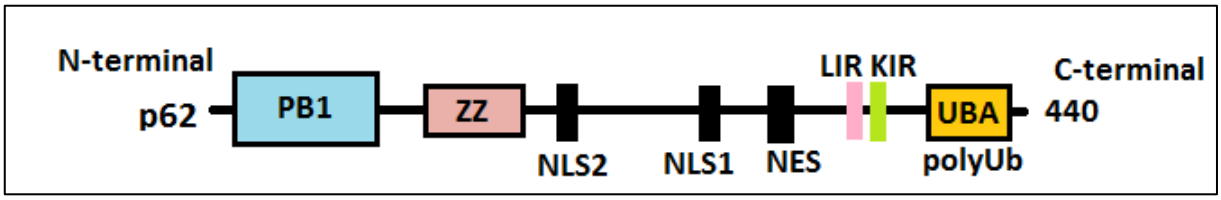

Figure 4. Map of p62 / SQSTM1 binding sites.

Transcription factors

In eukaryotic cells the expression of protein-coding genes is regulated during multiple steps. A crucial early step in this process from DNA to protein is the regulation of the initiation of transcription by the RNA polymerase II at a specific promoter site in the DNA. Further steps

are transcription, elongation, termination, and splicing out of these processes messenger RNA (mRNA) are synthesized. Further on, mRNA is moved out of the nucleus for translation in to polypeptide or protein at the ribosome. These steps are also regulated, but for gene expression the initiation transcription step is considered the major step (Casamassimi and Napoli, 2007, Barberis and Petrascheck, 2003).

Regulation of genes will turn on and off expression of specific protein coding genes at an appropriate level toned in with endogenous and exogenous signals in the cell environment. To monitor and control the transcription several regulatory proteins interplay in multi protein complexes.

Proteins that regulate transcription are defined as transcription factors, and can be categorized in three groups; general transcription factors (GTFs), activators and repressors, and co-regulators. The basal transcription machinery has GTFs together with RNA polymerase II as basic components. GTFs are received at position with RNA polymerase II at the promoter, and form the pre-initiation complex (PIC) (Roeder, 2005, Barberis and Petrascheck, 2003).

Proteins that act as activators and repressors are commonly called transcription factors. In the DNA, proximal and distal promoter regions or more distal regulatory regions like enhancers and silencers etc. are specific regions that transcription factors bind to promote or block the formation of the PIC. In a review Kardonaga (2004) has discussed recent knowledge of transcriptional factors as sequence-specific DNA binding regulatory factors that are modular in structure (Kadonaga, 2004), containing a DNA binding domain, one or more activation and/or repression domains and nuclear localization domain(s). Several well-defined DNA binding domains are detected. These are categorized according to their secondary structure.

Autophagy regulation under amino acid deprivation

Mammalian cells have evolved with several adaptive mechanisms to monitor and control homeostasis in nutritional deficiency (Kilberg et al., 2009). In this study amino acid starvation was imposed on cells, and is a main focus. Both the pathway of the mammalian target of rapamycin (mTOR) or FK506-binding protein 12-rapamycin-associated protein 1 (FRAP1)

(Brown et al., 1994, Moore et al., 1996) and the Amino Acid Response (AAR) signal transduction pathway are involved in regulation of the autophagy during detecting and adapting to amino acid availability and starvation (B'Chir et al., 2013, Chen et al., 2014). Yet there is much to explore about this.

Amino acid limitation induces autophagy in all organisms and cultured cells. Lack of amino acid and/or growth factors (ex. insulin) is a potent condition to induce autophagy. Rapamycin (mTOR) is a protein kinase involved in a nutrient-sensing pathway (Ma and Blenis, 2009), and contributes to inhibit the autophagy pathway, and inhibitors of mTOR hence promote autophagy. Unlike the mTOR pathway, in response to amino acid deprivation the amino acid response (AAR) [also called general amino acid control (GAAC) in yeast] signal transduction pathway is activated, which in turn regulates a number of steps in gene expression. This includes transcription starts site, transcription rate, mRNA splicing, RNA export, translation and several other processes (Kilberg et al., 2012, Kilberg et al., 2005).

The eIF2α - ATF4s pathway when lack of amino acids and autophagy

The p62 / SQSTM1 are one of the best known substrates in autophagy, and is therefore used as an indicator for degradation by autophagy. It has been shown that p62 expression may recur after hunger in cells. Initially p62 is degraded in autophagy; however, it appears that it is upregulated by longer time nutrient deprivation. Though, this is only shown in mouse embryotic fibroblasts and HepG2 cells (Sahani et al., 2014) not in HeLa and H293 (Sahani et al., 2014).

The General Control Nonderepressible 2 (GCN2)

The serine-threonine-protein kinase General control nonderepressible 2 (GCN2) is an amino acid sensor, and is found to play a key role in regulation of amino acid metabolism in response to amino acid deprivation (Hao et al 2005; Maurine et al 2005). Once bounded to uncharged tRNA, it completes the phosphorylation of the eukaryotic initiation factor 2α (eIF2α) at Serine 51, and hence eIF2α becomes inactivated. The phospholipid eIF2α acts as an inhibitor of eIF2B, which catalyzes transfer of GDP for GTP during the activation of the eIF2α complex. GCN2 is activated when it binds to any tRNA molecule, and this mediates

deficiency of any amino acid to trigger AAR. Phospho-eIF2α inhibits the general protein synthesis, however paradoxically it facilitates translation of some selected mRNA species, such as the transcription factor 4 (ATF4).

The activating transcription factor 4 (ATF4) in AAR

The GCN2 kinase is found to be an AAR pathway monitor for mediating the ATF4 pathway. ATF4 initiate increased transcription by binding to the CCAAT/enhancer-binding protein (C/EBP) to form at the C/EBP -ATF Response Element (CARE). Several genes contain a CARE this control region that modulate a wide specter of cellular actions to adapt nutritional stress.

Two open reading frames of ATF4 mRNA are the first upstream ORF (uORF1) and uORF2. They are located at 5` to the ATF4 coding sequence, and both are translated in the non-stressed condition. The sequence of uORF2 overlaps slightly with the ATF4 ORF. During amino acid deficiency resulting in decrease in the amount of eIF2 complex, then ribosome scanning ignores uORF2 and translation is re-initiated at the ATF4 coding region. Thus, the synthesis of ATF4 protein becomes selectively elevated in response to amino acid starvation. ATF4 activates transcription of different genes by binding to CARE sequences. Due to this pathway, ATF4 is considered to be a primary activating factor in the AAR.

The expression of P62 / SQSTM1 is elevated in response to amino acid deprivation in mouse fibroblasts embryos (MEFs)(B'Chir et al., 2014). Until now knowledge of the molecular mechanism behind transcription activation through eIF2α / ATF4 way of autophagy genes as p62 has been limited (B'Chir et al., 2013). Recent studies show that eIF2α / ATF4 path direct an autophagy gene transcriptional program in response to amino acid starvation or endoplasmic reticulum (ER) stress. It is described that the eIF2α kinases GCN2 and PERK, and ATF4 and the transcription factor CHOP are needed for transcription of several genes involved in the formation of autophagosomet (B'Chir et al., 2014).
A recent study reported an association relation between ATF4 and mTOR in the regulation of autophagy (Chen et al., 2014). The author found a feedback mechanism that controls the strength of autophagy by controlling amino acid uptake. Starvation leads to deactivation of mTOR, but in the absence of the amino acid glutamine the general amino acid control (GAAC) pathway (corresponding to AAR) is triggered via ATF4, which upregulates amino

acid uptake by synthesis of increased amount of transporter SLC7A5, a leucine transporter. This in turn will reactivate mTOR and suppress further autophagy (Chen et al., 2014). By knockdown of ATF4, the major transcription factor in the GAAC pathway, or of SLC7A5, reduced mTOR reactivation and hence significant higher levels of autophagy.

To sum up, in the above sections I have reviewed findings of a correlation between ATF4 and mTOR in the regulation of autophagy (Chen et al., 2014), and discovery of correlation between eIF2 / ATG4 way and upregulation of p62/SQSTM1 under amino acid starvation (B'Chir et al., 2014), but it is still unresolved how p62 / SQSTM1is upregulated during amino acid starvation.

The 5`UTR role in translation

The translation process can be characterized in four stages; in initiation, elongation, termination and ribosome recycling. The main part of regulation occurs in the initiation step by the start codon AUG identified and decoded by methionyl tRNA, which is specialized for initiating. In eukaryotes a scanning mechanism performed by the 40s ribosomal subunit detects the start codon. This ribosomal subunit becomes loaded with Met-tRNA to a preinitiation complex (PIC), and binds to mRNA near the 5`end, then it scans the 5`untranslated region (5`UTR) for an AUG codon. Reduced efficiency occurs if the RNA has structures that prevent the ribosomes to interact with the 5`UTR in a single-stranded form, or later to scan the 5`UTR. In addition decoy AUG codons in the 5`UTR may hinder scanning ribosomes to recognize a correct start codon (Sonenberg and Hinnebusch, 2009).

The 5' untranslated regions (5`UTRs) or leader RNA is the sequences of an mRNA located upstream to the initiation site, and have an average a length of 100-200 nucleotides (Pesole et al., 2001). The 5'UTRs have a significant role in regulating the initiating of translation. Genes show differences in their 5'UTRs and these seem to determine regulation of their translation. Initiating translation in eukaryotes needs the recruitment of ribosomal subunits at either the 5`m7G cap structure or at an internal ribosome entry site (IRES). The initiation codon is typically placed downstream, that it presupposes ribosomal motion to this site. However ribosomal subunits may bypass (shunt) sequences of the mRNAs in a non-linear way during initiation of translation. Shunting allow mRNAs containing uAUGs or a "hairpin structure" to

be translated specifically (Araujo et al., 2012). The RNA that has the property to form hairpin structures will ease the initiation of translation. Although transcription is a significant factor to regulate the gene expression in a cell, the amount of mRNA does not alone determine the amount of protein synthesized (Vogel et al., 2010). Translational modulation of the present mRNA allows for rapid shift in amount of the encoded protein by translation. In this way homeostasis may be maintained without more permanent changes in cell physiology that changing transcription would represent.

Summing up of the background for this study

Several studies have shown that the level of p62 / SQSTM1 is up-regulated in cells exposed to oxidative stress, various drugs such as deprenyl and valproate, and several different signaling molecules as PDGF (platelet derived growth factor), IFN-g (interferon gamma) and TLR (toll-like receptor) signaling. In contrast the p62 / SQSTM1 level is down-regulated when the cell is exposed to amino acid starvation and hypoxia-activated autophagy, but recent studies show that p62 / SQSTM1 is upregulated after a prolonged period of starvation(Sahani et al., 2014). However, less is known about regulatory elements in the p62 / SQSTM1 promoter and 5'UTR, and how the signal paths are converging on these regulatory elements.

The p62 / SQSTM1 promoter is rich of CpG nucleotides, and contains two serum responsive regions. The PDEF-responsive site is discovered and an Sp1 binding site and an AP1 binding site also are identified. The transcription factor ATF4 is in the AP1 family, and binds potentially to the AP1 binding site. Recently it was shown that the NRF2 binding site at position-1320 on p62 / SQSTM1 leads to significantly increased p62 expression under oxidative stress, and that p62 / SQSTM1 resist the oxidative stress response in a positive "feedback" inhibition of NRF2 expression(Jain et al., 2010) Transcription factor NRF2 is a redox-sensitive transcription factor that plays an important role in redox homeostasis under oxidative stress.

Aims and hypotheses

The p62/SQSTM1 has two well-studied of functions:
I) It works as specific cargo receptor in autophagy, where it has the ability to detect proteins in the cell to be degraded, and to concentrate them in specific regions called "p62 bodies".
II) It acts as "scaffold protein" in various signaling pathways. When a cell is exposed to oxidative stress or starvation, the p62 / SQSTM1 accumulates in round aggregates which are subsequently degraded by autophagy. This indicates that in order to maintain a certain level of p62 / SQSTM1 under stress, the cell must initiate novo synthesis.

The purpose of this project is therefore to look at how the expression of the transcript of p62 / SQSTM1 in mammalian cells (Hek293 cells) can be regulated under stress.
Based on literature accounted for in the sections above the following hypotheses can be set up in the study:

1. The p62 promotor may be regulated by ATF4. In mouse embryonic fibroblasts and HepG2 cells the p62 is restored during prolonged starvation. Initially p62 is degraded by autophagy, but after 3-6 hours the level is restored. In part this process is regulated by transcription. It is also shown that the transcription factor ATF4 is induced by starvation.

2. Different lengths of the untranslated 5´UTR may have impact on the p62 translation under starvation (stress) . Sequences in the p62 5´UTR could form structures like “hair-pins” that moderate the p62 translation.

Research questions

The objectives are to test these hypotheses by the following research questions examined in the study:

1) To test whether p62 promotor is up-regulated by ATF4 compared to NRF2, by which it is known to be upregulated.

2) To perform biometrics of the sequences in the p62 5´UTR in front of the luciferase gene and examine whether structural aspects as “hairpins” related to the 5´UTR may explain regulation of the p62 translation.

3) To determine whether different lengths of untranslated the 5´UTR have impact on the p62 translation under starvation (stress) compared to a control system .

4) To examine if ATF4 modifies effects of the 5´UTRs under starvation compared to a control system

Materials and Methods

Materials

General materials used in this study are listed in tables. The methods are described below.

Growth medium for bacterial cultures and mammalian cell lines

Table 1 displays the composition of the growth medium for bacterial cultures and mammalian cell lines used in this study.

Table 1. Growth medium for bacterial cultures and mammalian cell lines

Growth media for bacterial cultures	LB(Luria-Bertani)-medium	10g Bacto Trypton 5g Bacto yeast extract 10g NaCl dH_2O to1L pH adjusted to 7.5 with NaOH Antibiotic: Ampicillin
	LB agar plate	10g Bacto Trypton 5g Bacto yeast extract 10g NaCl 15g agar dH_2O to 1L pH adjusted to 7.5 with NaOH
	Super optimal broth with Catabolite repression (SOC) medium	20 g Bacto Trypton 5g Bacto yeast extract 10ml 250mM KCl 5g $MgCl_2$ 20mM glucose dH_2O to 1L pH adjusted to 7.5 with NaOH
Growth media for mammalian cell culture (Hek293)	Dulbecco´s Modified Eagle´s medium 10 % Fetal Calf Serum 100 U/ml Penicillin 100 g/ml Streptomycin	

Different general buffers used

Some of the most general buffers used in the various methods in this study are listed in Table 2.

Table 2. General buffers

Method	Buffer	Content
General buffers	TE buffer	10mM Tris/HCl, pH 8.0 (adjusted with HCl) 1mM EDTA
	1xPhosphat buffered saline (PBS)	0,1 mM Na-phosphat buffer, pH 7.2 0.7 %NaCl
Buffers used in restriction enzyme digestion	NEB 1.1 buffer	10mM Bis-Tris-Propane-HCl 10mM $MgCl_2$ 100µg/ml BSA pH 7.0, [4]
	NEB 2.1 buffer	50mM NaCl 10mM Tris-HCl 10mM $MgCl_2$ 100µg/ml BSA pH 7.9 [4]
Gel-electroforesis	20xMinigel buffer	193,75 g Tris-Cl 27,22 g NaOAc 14,9 g EDTA dH_2O to 2L
	6xT gel loading buffer	0,25 % Bromophenol blue [1] 60 mM EDTA pH 8.0 0.6 % SDS [1] 40 %(W/v) sucrose sterile filtered
	1-kb ladder	1.0 µl 1kb-ladder stock(1.03 µg/µl) 24.0 µl TE buffer pH 8.0 5.0 µl 6xT gel loading buffer
	100-bp ladder	1,0 µl 100 bp-ladder sock 24.0 µl TE buffer pH 8,0 5,0 µl 6xT gel loading buffer
	1 % agarose gel	1 g agarose [1] 5 ml of 20xminigelbuffer 95 ml H_2O
	1.5 %	1.5 % agarose [1] 5 ml of 20xminigelbuffer 95 ml H_2O
SDS-PAGE	4 x Separating gel buffer	181.65 g Trizma-base 4 g SDS dH_2O to 1 L pH 6.8, adjusted with HCl
	4 x Concentrating gel buffer	60.55 g Trizma-base 4 g SDS dH_2O to 1 L pH 6.8, adjusted with HCl
	10 % separating gel	2.5 ml acrylamide mix 40 % [2] 2.5 ml separating gel buffer 4.9 ml H_2O 100 µl 10 % APS(Ammonium persuphate) 10 µl TEMED (Tetramethylethylenediamine)[2]
	15 % separating gel	3,75 ml acrylamide mix 40 % [2] 2.5 ml separating gel buffer

		3.64 ml H_2O 100 µl 10 % APS(Ammonium persuphate) 10 µl TEMED (Tetramethylethylenediamine) [2]
	4 % concentrating gel	1ml acrylamide mix 2.5 ml concentration gel buffer pH 6.8 6.4 ml dH_2O 100 µl 10 % APS 10 µl TEMED
Western blot	1xTBS-T buffer	75 ml 2M NaCl 10 ml 1M Tris-HCl, pH 8.0 1 ml Tween20 914 ml dH_2O
	5 % dried milk solution	2.5 g dried milk (Magermilch powder) [3] in 50 ml TBS-T
	Transfer buffer	1.88 g Tris 28.55 g Glycine 150 ml methanol dH_2O to 1 L

Figures 5 and 6 display the different ladders used in agarose gel electrophoresis by the 1-kb DNA ladder and the 100-bp ladder respectively [4]. The figures 7 and 8 show Biotinylated protein ladder used in Western Blot [4], and prestained protein marker [5] respectively.

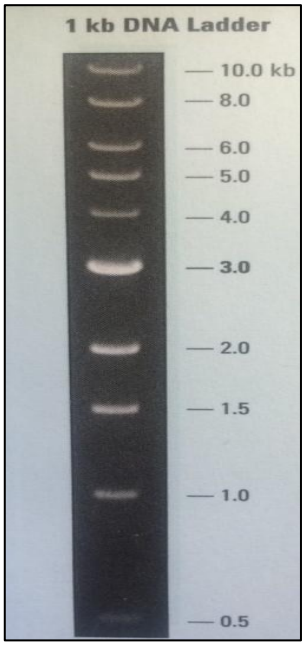

Figure 5. 1-kb DNA ladder [4]

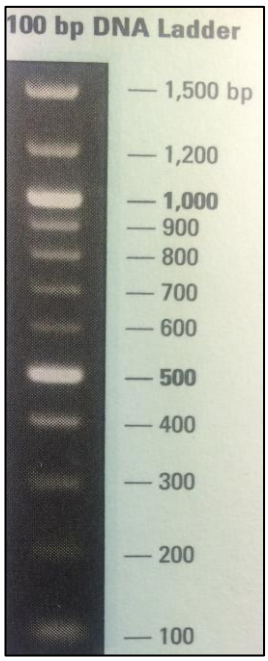

Figure 6. 100-bp DNA ladder [4]

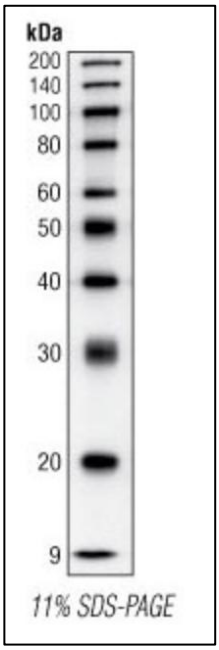

Figure 7. Biotinylated protein ladder [4]

Figure 8. Prestained protein marker [5]

Antibodies

Both the primary and secondary antibodies used in western blotting in this study are listed in Table 3.

Table 3. Antibodies used for western blot

	Antibody	Supplier	Dilution
Primary Antibodies:	Rabbit anti-LC3B	Sigma	1:1000
	Mous-anti-p62/SQSTM1 (LCK ligand)	BD Boscionse	1:1000
	Anti-biotin mat ladder		
	Rabbit anti-Actin	Sigma	1:1000
Secondary antibodies:	Anti-mouse HRP	Sigma	1:1000
	Anti-rabbit HRP	Sigma	1:1000

Restriction enzymes

The restriction enzymes used in this study are listed in Table 4.

Table 4. Restriction enzymes

Enzyme name	Recognition sequence	Concentration	Supplier
Hind III	AAGCTT	20.000 U/ml	New England Biolabs
Nco1	CCATGG	1.000 U/ml	New Enland Biolabs
KpnI	GGTACC	4.000 U/ml	New England Biolabs
Nar1	GGCGCC	5.000 U/ml	New England Biolabs

Primer sequences used for cloning

Table 5 shows the p62 5´UTRs primer sequences (produced by Life Technologies) used in cloning in front of the luciferase gene in the reporter gene vectors pGL3-Basic and pGL3-Control. The primers are made for inserting into the HindIII – NcoI sites of the luciferase vectors.

Table 5. The primer sequences used for cloning of p62 5'UTRs in front of Luciferase gene.

P62 5FW-100 nt AGCTTCTCGAGGCGGGGCGGGGCCTCCGCGTTCGCTACAAAAGCCGCGCGGCGG CTGCGACCGGGACGGCCCGTTTTCCGCCAGCTCGCCGCTCGCTATG P62 5REV-100 nt CATGCATAGCGAGCGGCGAGCTGGCGGAAAACGGGCCGTCCCGGTCGCAGCCGC CGCGCGGCTTTTGTAGCGAACGCGGAGGCCCCGCCCCGCCTCGAGA P62 5FW-62 nt AGCTAAAAGCCGCGCGGCGGCTGCGACCGGGACGGCCCGTTTTCCGCCAGCTCG CCGCTCGCTATG P62 5REV-62 nt CATGCATAGCGAGCGGCGAGCTGGCGGAAAACGGGCCGTCCCGGTCGCAGCCGC CGCGCGGCTTTT P62 5FW-35 nt AGCTACGGCCCGTTTTCCGCCAGCTCGCCGCTCGCTATG P62 5REV-35 nt CATGCATAGCGAGCGGCGAGCTGGCGGAAAACGGGCCGT

Sequencing primer

The primer used for sequencing is shown in Table 6.

Table 6. Primers used for sequencing

Primers	Primer sequence	Supplier
3´Gbasic (RVprimer3)	CTAGCAAAATAGGCTGTCCC	Promega

Enzymes

The enzymes and their reaction buffers used for cloning in this study for general methods are listed in Table 7.

Table 7. Enzymes and reaction buffer needed together

Enzyme	Consentration	Reaction buffer	Content Reaction buffer	Supplier
T4 DNA ligase	400.000 units/ml	10 X T4 DNA Ligase Reaction buffer	50 mM Tris-HCl 10 mM $MgCl_2$ 1 mM ATP 10 mM DTT pH 7.5, 25°C [5]	New England Biolabs(NEB)
T4 polynucleotide kinase	10.000 Units/ml	T4 Polynucleotide Kinase Reaction Buffer	70 mM Tris-HCl 10 mM $MgCl_2$ 5 mM DTT pH 7.6, 25°C [4]	New England Biolabs(NEB)

The cDNA constructs

The different plasmids constructs used in this study are listed in Table 8, and schematic of the pGL3-Basic and Control reporter gene vector are shown in Figure 9.

Table 8. Plasmides

cDNA construkt	References
pGL3-p62 promoter (-2305/+46)	Hanne Britt Brenne
pcDNA3-HA	-
pCH110	-
PCI ATF4	-
pDEST-myc-hNRF2	-
pGL3-Basic reporter gene vector	Promega
pGL3-Control reporter gene vector	Promega
pGL3- Basic + 100 nt of p62 5´UTR	This thesis
pGL3- Basic + 62 nt of p62 5´UTR	This thesis
pGL3-Basic + 35 nt of p62 5´UTR	This thesis
pGL3-Control + 100 nt of p62 5´UTR	This thesis
pGL3-Control + 62 nt of p62 5´UTR	This thesis
pGL3-Control + 35 nt of p62 5´UTR	This thesis

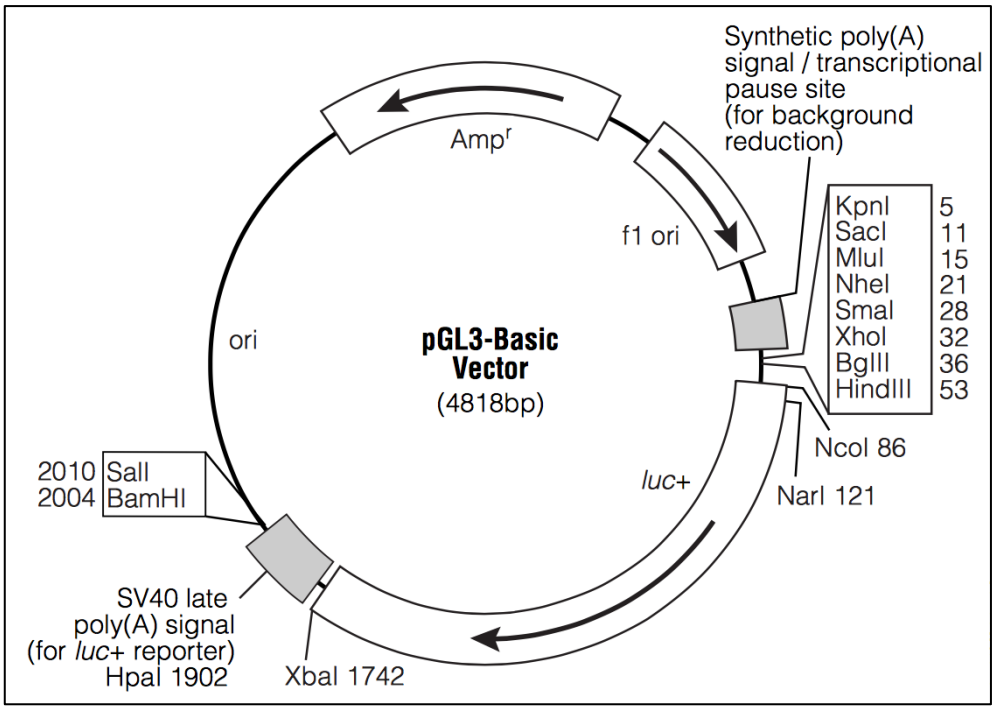

Figure 9. The pGL3-Basic vector where the p62 5´UTRs are cloned in front of the luciferase gene [6]

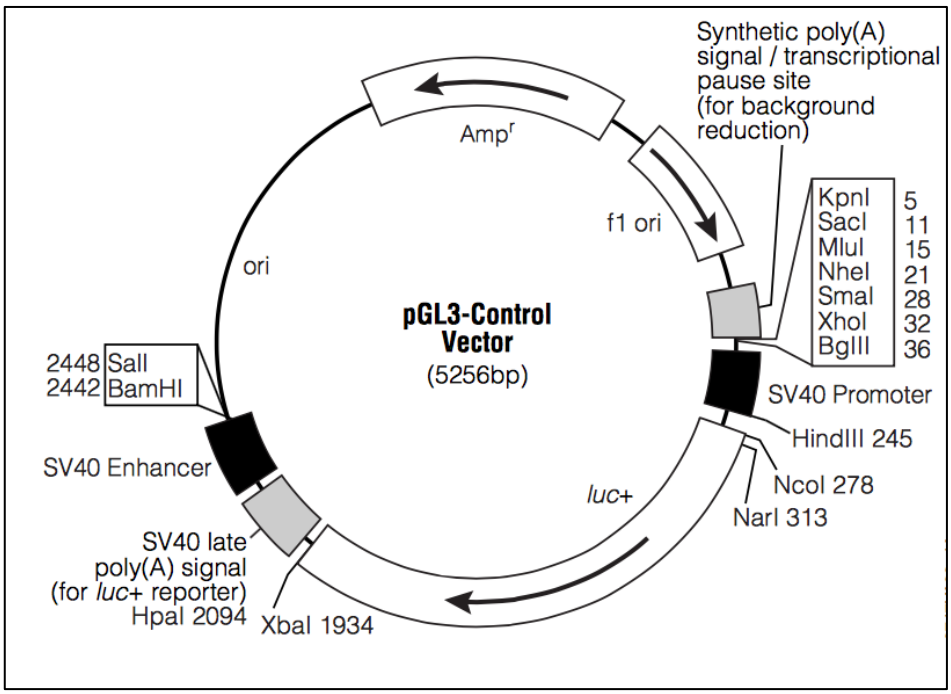

Figure 10. The pGL3-Control vector where the p62 5´UTRs are cloned in front of the luciferase gene [6]

Methods

Cloning of p62 5´UTRs in front of the Luciferase gene

The first step in this study was the cloning of p62 5´UTRs in front of the Luciferase gene. The 5´UTRs were cloned into the luciferase reporter plasmids of pGL3-Basic and pGL3-contol as showed in Figure 6 and 7. The Primers were phosphorylated, annealed and ligated into the HindIII-NcoI of the vectors.

The pGL3 luciferase reporters are specified to analyze factors affecting the mammalian gene expression. The backbone of the pGL3 luciferase vectors is created for increased expression, and contains a coding region for firefly (Photinus pyralis) luciferase that has been optmimazed to adjust transcriptional activity in transfected eukaryotic cells. Additionaly the Luciferase reporter gene vectores contain different features aiding in the structural characterization of the regulatory sequences that is studied.

Step 1: Streak from freezer stock and over night culture

The first step in this procedure was to streak the plasmids from the freezer stock on LB (luriabertani) + AMP (ampicillin) plates. They were incubated over night in 37 °C. The day after, three colonies from each plate were picked and cultured over night in 4 ml LB + Amp medium with shaking at 37 °C.

Step 2: Preparing the plasmids

From E.coli the Luciferase reporter plasmids pGL3-Basic and pGL3-Control where purified from the O.N cultures as described below

Plasmid purification

Plasmid purification is applied to purify plasmid DNA from a bacterial culture, and is referred to as the alkaline/SDS method (Birnboim and Doly, 1979). By this method cells are lysed using SDS, and under alkaline condition the chromosomal DNA denatures. Then, neutralization with acidic sodium acetate aggregates the chromosomal DNA and high weight RNA, while SDS-protein complexes precipitates under the high concentrations of sodium acetate. Centrifugation then pellets chromosomal DNA, most proteins and large weight RNA,

while covalently closed circular (CCC) DNA will stay in the supernatant. The CCC DNA is then purified from the supernatant by binding to a column (Birnboim and Doly, 1979).

Miniprep

Miniprep was used to purify the plasmid DNA. We applied the "GenElute ™ Plasmid miniprep Kit (Sigma) ®". Miniprep method is based on the alkaline/SDS method, and is quickly performed with a relatively small amount of impure DNA. In the procedure by GenElute MiniprepKit a binding column binds the plasmid DNA and low ionic strength conditions elutes it

Procedure:

All steps where performed at room temperature, and all centrifugation was done at 17 000 x g. 1,5 ml ON culture from each over night culture where centrifuged for 1 min and then the supernatant was removed. Further the cells were resuspended in 200 µl of Resuspension solution and the pellets were dissolved by vortexing. Then 200 µl of Lysis solution was added. The mix was gently mixed and put on hold for 5 minutes. Further, 350 µl of neutralization solution was added, and the mix was gently inverted 4-6 times. The solution then was centrifuged for 10 min. In the meantime the binding column was prepared. The binding column was placed in a collection tube, and 500 ml Column preparation solution added to it. Further the supernatant was transferred to the binding column (hereafter called column) and centrifuged in 1 min further, and flow discarded. Then 750 µl Wash solution was added to the column and centrifuged for 1 min. The flow was discarded. The column was further centrifuged one more time to dry the column. Thereafter the column was transferred to a new collection tube and 100 µl Elution Solution was added.

Step 3: Determination of DNA concentration

The DNA concentration of the plasmid mini-prep was measured by using a NanoDrop ND-100 Spectrophotometer (Available from Saveen-Werner).

Step 4: Phosphorylation and Annealing of oligoes

Because chemically synthesized oligonucleotides [7], which came as single DNA strands, are not phosphorylated at their either end, the oligoes needed to be phosphorylated before they should be annealed together for further proper ligation. The DNA fragments used without 5´phosphate group is phosphorylated by kinases. In the same process the annealing occurred. The procedure is to heat the oligonucleotides so that they can denature, and then introduce a

cooling period allowing the two oligoes to form base pair together. All this occurred in the same process.

Procedure:
1 µl oligo FW (100 µM)
1 µl Oligo REV (100 µM)
1 µl 10xT4 Ligation buffer (NEB)
6 µl dH_2O
1 µl T4 Ligation buffer (NEB)

This was mixed in a 0,5 ml microsentrifuge tube and incubated in a PCR machine with this program: 37°C for 30 min, 95 °C for 5 min and then ramping down the temperature to 25°C at 5°C per min.

Step 5: Linearisation procedures

The luciferase reporter vectors needed to be linearized. The vectors where cut with HindIII and Ncol by using the general method Restricion enzyme digestion. And then further purified using QIAquick PCR purification kit [8]. Further the DNA concentration was measured by nano-drop as described above and then runned on a 1 % agarose gel to verify linearization.

Restriction enzyme digestion

In the late 1960 Arber and Linn were the first to describe the restriction enzymes (Arber and Linn, 1969). The restriction enzyme cleaves blunt or staggered ends leaving a 3´ or 5´overhang. The restriction enzyme typically recognizes sites from 4 to 8 nucleotides.

Procedure:
Linearisation:
2 µl 10xNEB2.1 buffer
1 µg pGL3 luciferase reporter plasmid
0.5 µl HindIII
0.5 µl Ncol
dH_2O to 20 µl
This reaction mix where incubated at 37°C in 45 min, further 0.5 µl SAP was added and the incubation went for 15 more min.

Purification of digested vector

To exclude uncutted plasmids purification was performed. The procedure to purify the DNA used silica base membranes in a spin column binding PCR products lager than 100 bp.

QIAquick PCR purification kit from Qiagen procedure:
All steps of this procedure were performed at room temperature and centrifugation was performed at 13000 rpm in a table top centrifuge.

Five volumes of cold Buffer PB1 was added to one volume of the reaction mix in a 1,5 ml Eppendorf tube. Then a QIAquick column was placed in an empty 2 ml collection tube. The reaction mix was transferred to the column and centrifuged for 30-60 sec, and the flow-through was discarded. Thereafter 0.75 ml of Buffer PE was added to the column before centrifugation for 30-60 sec, and flow-through was discarded. The column and empty collection tube was centrifuged for 1 min. Then content of the column was transferred to a 1.5 ml Eppendorftube and 50 µl of buffer EB was added (to the center of column). At last the DNA was eluted by centrifugation for 1 min.

Agarose gel electrophoresis

In general this method separates DNA fragments based on their size. Agarose, which is a linear polysaccharide, obtained from agar, forms a rigid gel with pores when it is polymerized. The size of these pores is influenced by the concentration of the agar. For separation, when sufficient amount of DNA fragments is applied to the agarose gel, the negative charged DNA will move towards the positively charged electrode through the pores, and such be separated since smaller fragments will move faster towards the positively charged electrode. The separated DNA may be viewed with staining as described below in the specific procedure.

Procedure:
1.0 g agarose was mixed with 5 ml of 20xminigel buffer and 95 ml H_2O to a 1 % agarose gel. This solution was boiled in a microwave, and poured into a casting frame with comb inserted for formation of wells. The gel was sat to polymerize at room temperature in 45 min. Thereafter, the comb was removed before placing the gel in a tray, and Minigel buffer was poured over to cover the gel. DNA samples were mixed with 6xT gel loading buffer before applying it to the wells, together with a 1kb ladder. In the electrophoresis a 90V current was applied for 45 min followed by staining in Gelred-bath for 15 min. Further on, the gel was

exposed to UV light at 302 nm using a UV trans illuminator resulting in the DNA-bands visualized on the gel. For pictures a Multi-Cod IY Digital system was used.

Step 6: Ligation of oligoes into Luciferase vectors

Each annealed oligo (100, 62 and 35) was then ready to be ligated into each of the three luciferase reporter genes (pGL3-Basic and pGL3-Control).

Ligation of DNA fragments

This method is based on the discovery of DNA ligase by Weiss and his colleague who first reported on the reaction of the enzyme (Bernard Weiss, 1968). They found that DNA ligation covalently seals one 3´hydroksyl and 5´phosphate nick in a DNA strand, and DNA ligase catalyzes this reaction.

Procedure:

1 µl 10xligation buffer (NEB)

50 ng linearized Luciferase reporter plasmid

1 µl of diluted, phosphorylated and annealed oligo from step 2

1 µl T4 DNA ligase (NEB)

dH_2O to 10 µl

This was incubated under 25°C for 2 hours

Step 8: Transformation of E.coli DH5α

Transformation of bacteria is a process where DNA is taken up by the bacterial cell and can be integrated into the genome or maintained in the cell as a plasmid. Not all bacterial cell can be transformed, but a few strains have the natural ability to take up DNA, others can be made competent by different methods. *E.choli* DH5α used in this study is chemically competent.

Procedure:

E.choli DH5α was thawed on ice. 5 µl of the ligation product was added to 50 µl competent cells, and incubated on ice for 20 min. Thereafter the transformation mixture was heat-shocked at 37°C for 2 min, and then placed back on ice for 2 min. Then 500 µl SOC was added, and the mixture was placed in 37°C with 150 rpm shaking for 1 hour. Further, 200 µl of the sample was added to prewarmed LB plates with ampicillin and incubated at 37°C over night. Then 4 colonies from each plate was picked and transferred to 3 ml LB+amp medium

for overnight (O.N) incubation, and plasmid purification was performed using miniprep as described earlier.

Step 9: Validation of correct insert

After isolating the plasmid DNA from cultures using Miniprep the plasmids where validated with restriction enzyme digestion as described above using the restriction enzymes KpnI and NarI. Then the digested plasmids were run on a 1.5 % Agarose gel to see if the oligo is inserted. After the insert validating was done the sequence was verified by the general method Sanger sequencing which is used to verify correct cloning.

Restricting enzyme digestion procedure:
1 µl NEB1.1 buffer
3 µl purified luciferase plasmid
0.5 µl KpnI
0.5 µl NarI
5 µl dH_2O
This was incubated for 1 hour in 37°C

Gel electrophoresis process:
2 µl 6XT loading buffer was added to the digested plasmids. Then 1.5 g Agarose was added to 5 ml of 20xminigel buffer and 95 ml H_2O to make 1.5 % agarose gel. Then the further steps were the same as described above.

Sanger sequencing

The Sanger sequencing technique was developed by Fredrick Sanger and his colleagues (Sanger et al., 1977), and is based on dideoxynucleotide chain termination. Heating denatures DNA. Running the cycle many times will terminate the synthesis at different times resulting in fragments of different length. Separation the fragment by size and analysis of the emitted light for each band will refine the DNA sequence. Here Big Dye 3.1 DNA sequencing kit [9] was used. For analyzing the generated DNA fragments, the PCR reactive mix was sent to a in house sequencing lab, and an ABI PRISM 3130x1 Genetic analyzer was used to determine the DNA sequence.

Procedure:

1 µl BigDye

1 µl Seqencing buffer

150 ng plasmid

1 µl 3´GBasic primer (10 µM)

dH_2O to 10 µl

PCR program for sequencing

1. 96°C in 1 min
2. 96°C in 30 sec
3. 50 °C in 15 sec
4. 60°C in 4 min
5. Back to step 2, 25 times
6. 64°C ∞

Further analysis of the sequences was done using Chromas software [12] and Nation Center for Biotechnology Information´s search-tool BLAST(Gertz et al., 2006).

Freeze stock of E. coli DH5 α

Freezer stocks of the correct verified plasmids where created for later use by procedure described below.

Procedure:

From the over night culture a sample of 1.2 ml was mixed with 300 µl of 50 % glycerol in a cryotube. Then the tube was marked and frozen at -70°C

Mammalian cell cultures

In this study the Human embryonic kidney cell (Hek293) was used for luciferase assays and Western Blotting. The Hek293 cells were grown in a 5 % humidified CO_2 incubator at 37°C. All solutions used for cell cultures were preheated to 37°C. The Hek293 cells where grown in DMEM (100 U/ml penicillin and 100 g/ml streptomycin) with 10 % FCS.

Splitting and counting of cells

When cell cultures grow, the cells by dividing tends to grow too close together. Contact inhibition may cause them to inhibit or stop growing, detach and even die. By splitting procedures, the cells are detached from the surface, diluted and grown in a fresh medium. Hek293 cells need to be split every 2-4 days.

Procedure:

The growth medium was aspirated and 1xPBS was added to wash the cells. Thereafter the 1xPBS was removed and 1 ml Trypsin was added. The sample was incubated for 1 min so every cell could be loosened. Then 5 ml of DMEM with 10 % FCS was added to inactivate the trypsin.

The numbers of cells were counted by a Büchner chamber in a microscope. The counted number was multiplied with factor 10^4 to reflect the number of cells per ml solution.
A small volume of the cell-suspension was transferred to a new bottle, and fresh DMEM was added with 10 FCS, the volume depending on size of the bottle used.

Transfection of mammalian cells

Transfection is a process of uptake of genetic material as foreign DNA into eukaryotic cells by a non-viral method. The transfection can be either stabile or transient, which respectively indicate if the cells have incorporated the DNA into its genome or if it is only transiently within the cells and lost later on. There are several methods of transfection, and in this study the procedure Metafecten ®Pro (available from Biontex) was used. This method involves a lipid membrane that is formed around the DNA, and is further fused with the cell membrane introducing the DNA into the cells. During this procedure DMEM without antibiotic was used.

Transfection procedure for Luciferase assay

24 well plates were seeded by 20 000 Hek293 cells with 500 µl DMEM containing 10 % FCS and incubated for 48 hours. The cells had an equal amount of DNA transfected so there was no difference in the amount of DNA. The empty vector (pCDNA-HA) was used to equalize the amount of DNA in each well.

Further the appropriate amount of the expression vectors of interest (see table 9, 10, 11 and 12) where added together with the empty vector, and β-gal expression plasmid to 75 µl DMEM into 1.5 ml Eppendorftube. Every Eppendorftube filled with DNA and DMEM was represented by three parallels of the same mix of DNA of interest. Then carefully, 75 µl DMEM was added to 3 µl of Metafecten®Pro, and thereafter the DNA-DMEM mix was added to the Metafecten®Pro and incubated in room temperature for 20 min. Further on, this mixture was added to the Hek293 cells and incubated for 24 hours. Depending on the test performed, the cells were either exposed for starvation and then harvested, or no-starvation and harvested after 24 hours of incubation.

Table 9. Presentation of the amount of transfected PGL3-p62 5´UTR-luciferase vectors into the Hek293 cells for further use in the Luciferase assay

Expression vector	Amount of the vectors transfected	Amount empty vector: pCDNA3-HA	Amount of β-gal PCH1100 plasmid
pGL3-Basic 100 nt	50 ng	50 ng	10 ng
	75 ng	25 ng	10 ng
	100 ng	-	10 ng
pGL3-Basic 62 nt	50 ng	50 ng	10 ng
	75 ng	25 ng	10 ng
	100 ng	-	10 ng
pGL3-Basic 35 nt	50 ng	50 ng	10 ng
	75 ng	25 ng	10 ng
	100 ng	-	10 ng
pGL3-Control 100 nt	50 ng	50 ng	10 ng
	75 ng	25 ng	10 ng
	100 ng	-	10 ng
pGL3-Control 62 nt	50 ng	50 ng	10 ng
	75 ng	25 ng	10 ng
	100 ng	-	10 ng
pGL3-Control 35 nt	50 ng	50 ng	10 ng
	75 ng	25 ng	10 ng
	100 ng	-	10 ng

Table 10. Presentation of the amount of transfected pDEST-myc-hNRF2 with pGL3 p62 promoter (-2503) into the Hek293 cells for further use in the Luciferase assay

Expression vector and p62 promoter (-2503)	Amount of the vectors transfected	Amount empty vector: pCDNA3-HA	Amount of β-gal pch1100 plasmid
pGL3-p62/SQSTM1 promoter (-2503) pDEST-myc-hNRF2	50 ng 50 ng	50 ng	10 ng
pGL3-p62/SQSTM1 promoter (-2503) pDEST-myc-hNRF2	50 ng 100 ng	-	10 ng

Table 11. A presentation of the amount of transfected ATF4 with p62/SQSTM1 promoter (-2503) into the Hek293 cells for further use in the Luciferase assay

Expression vector and p62 promoter (-2503)	Amount of the vectors transfected	Amount empty vector: pCDNA3-HA	Amount of β-gal pch1100 plasmid
pGL3 p62/SQSTM1 promoter (-2503) pCI-ATF4	75 ng 50 ng	50 ng	10 ng
pGL3 p62/SQSTM1 promoter (-2503) pCI-ATF4	75 ng 100 ng	-	10 ng

Table 12. Presentation of the amount of transfected pGL3 p62 5´UTRs luciferase and the vector for expressing transcriptional factor ATF4 into the Hek293 cells for further use in the Luciferase assay

Expression vector	Amount of the vectors transfected	Amount empty vector: pCDNA3-HA	Amount of β-gal pch1100 plasmid
pGL3-Basic 100 nt and pCI-ATF4	50 ng 50 ng	50 ng	10 ng
	50 ng 100 ng	-	10 ng
pGL3-Basic 62 nt and pCI-ATF4	50 ng 50 ng	50 ng	10 ng
	50 ng 100 ng	-	10 ng
pGL3-Basic 35 nt pCI-ATF	50 ng 50 ng	50 ng	10 ng
	50 ng 100 ng	-	
pGL3-Control 100 nt pCI- ATF	50 ng 50 ng	50 ng	10 ng
	50 ng 100 ng	-	10 ng
pGL3-Control 62 nt pCI- ATF	50 ng 50 ng	50 ng	10 ng
	50 ng 100 ng	-	10 ng
pGL3-Control 35 nt pCI-ATF	50 ng 100 ng	50 ng	10 ng
	50 ng 100 ng	-	10 ng

Aminoacid and serum starvation

Further, the 5´UTR of SQSTM1/p62- transcript where also tested under stress condition by starvation to find out if stress has any impact of the amount of SQSTM1/p62 translated. The cells where starved using Hank´s Balanced Salt Solution (HBSS). This solution has no FCS, amino acids and low glucose. The transfected cells where starved for 3 hours and 6 hours, while Hek293 cells (without any transfected DNA) were starved for 1, 2, 4 and 6 hours.

Luciferase assay to determine expression of the gene SQSTM1 / p62

One of the aims of this study was to examine how the expression of SQSTM1 / p62 in mammalian cells can be regulated under stress, by studying the SQSTM1 / p62 promoter response to stress related transcription factors NRF2, NRF1 and ATF4, and if the 5'UTR of SQSTM1 / p62 transcript has impact on the amount of SQSTM1 / p62 which is known to be translated both under starvation and in normal conditions. The Luciferase reporter assay method was used to determine this.

The Luciferase assay can be categorized as a reporter gene assay to detect levels of a reporter gene controlled by front-lying regulatory sequence of interest. In this study the regulatory sequences of interest were the p62 5´UTRs which were cloned in front of the Luciferase gene in the reporter gene vectors (pGL3-Basic and Control) to see if any of these affected the amount of luciferase expression. The p62 promoter (-2503) and both the NRF2 and ATF4 transcriptional factors were also measured in different combinations with and without the p62 5´UTRs to see if there were any changes in the expression of luciferase.

Procedure:

Hek293 cells were transfected 24 hours prior to the harvest. The growth media was removed and the cells were washed with 1xPBS. Then, 60 µl of Tropix®lysis solution with 1 mM DTT was added to each well, and the cell lysates were transferred to an 1.5 ml Eppendorf tube. The tubes where centrifuged at 13.000 rpm using a table-top centrifuge for 3 min. Further, 20 µl of the supernatant was transferred to a 96-well titrer plate. Then, the Luminescence and β-galactiosidase activity using the Labsystemes Luminoscan (CLARIOstar, BMG LABTECH) and Tropix®lysis solution with 1mM DTT as blank was measured. Table 11 below indicates the reagents used for the measurement. The β-galactiosidase activity was assessed 45 min after luciferase measurement.

Table 13. Presentation of reagents for measurements of Luciferace activity and β-galactiosidase activity

Measurement	**Reagent**
Luciferase activity	15 µl Dual light®Reagent buffer A 60 µl Dual light®Reagent buffer B with 1 % Tropix Galacron-Plus®
β-galactiosidase activity	60 µl Tropix Accelerator II

Western blot preparations

This method was used to investigate whether p62 / SQSTM1 is regulated similarly in Hek293 cells as in HepG2 and MEF cells. The first preparation step to western blot is SDS-PAGE.

SDS-page

Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis (SDS-PAGE) is a method developed in the 1960s, by Shapiro and his team (Shapiro et al., 1967). This method is based on the observation that proteins, which have lost their secondary and tertiary structures and made negatively charged by SDS, are migrating towards a positive electrode in the gel plate. Exposed to current, the proteins will separate on the basis of size since smaller proteins migrate faster.

Procedure:

Preparation of the cell lysate

The cells were seeded out in a 6 well plate, and the cells in some wells were starved with Hank´s serum for 6 hours, 4 hours, 2 hours and 1 hour respectively, and as controls some were not starved at al. Then, the cells were harvested; growth medium removed, and cells were washed with 1xPBS. Further, 60 µl of 1xSDS loading buffer with 200mM DTT was added to each well, and the cell-lysates were transferred to 1.5 ml Eppendorf tubes. The lysated cells were sonicated for 2-3 sec, and then boiled for 5 min and spun down. Now, the cells lysated were ready for further examinations.

SDS-page preparation

Two plates, on glass and on plastic was separated by spacers and mounted in a gel caster. Components of 10 % separating gel was mixed and poured between the plates until 2/3 filled. Water was then added on the top of the separating gel to keep it straight and to avoid drying. The gel was left to polymerize at a minimum of 20 min. Then a 4 % concentrating gel was mixed and put on top of the separating gel after discarding the water. A comb was removed and electrophoresis buffer was added to the vertical and bottom reservoirs. 5 µl biotinylated protein ladder, 5µl pre-stained ladder was then loaded, and the samples also were loaded to the wells and the gel was run at 25mA for 80-100 min with water cooling. The protein-gel was now ready for further use.

Western blotting

George Stark and his team developed this western blot method detecting specific proteins in the late 1970´s (Renart et al., 1979). A mixture of protein is run on a SDS-PAGE gel. The proteins are then transferred from the SDS-PAGE gel to a membrane, and the membrane is treated with a primary anti-body for binding to proteins of interest. Further, the membrane is treated with Horseradish Peroxidase (HRP)-conjugated secondary antibody that will bind to the primary antibody. By treating the membrane with western blot reagents, the HRP-conjugated antibodies will emit light.

Procedure:

First, the proteins were run on a SDS-PAGE gel (with protein ladder). A membrane, two filter papers and two sponges were soaked in transfer buffer Size of the membrane and filter papers are the same as the separating gel. Then a sponge was placed in the bottom, followed by filter paper, the separating gel and then the last sponge. This was locked in a holder and placed in a blotting chamber, Trans-Blot®Turbo™ (bio-rad), the membrane-side facing the positive electrode. A 100 volt current was applied for 30 min, blotting the proteins onto the membrane. The membrane was then removed and blocked by soaking in 5 % dried milk solution for 1 hour. Then, 1.5 ml of TBS-T buffer was mixed with 1.5 µl of the primary antibodies in a 50 ml tube. The membrane was transferred to the tube and incubated on a rotator over the night at 4°C.

The next day the mix of TBS-T buffer and primary antibodies was drained, and the membrane was washed with TBS-T buffer 3 times (5 min between every round). Then 2 µl of the secondary antibody was added to the tube together with 2 ml of TBS-T buffer and put on the rotator for 1 hour in room temperature. Further, the mix of secondary antibodies and TBS-T buffer was drained and washed 3 times as described. Then 1 ml of Solution A and Solution B (western blotting luminol reagent) was mixed together in a tray, and the membrane was soaked for 5 min in the dark. The sample was then wrapped in plastic, and the signals were detected by a Lumi-Imager.

Statistical methods

The data were initially analyzed by descriptive methods. Testing differences in the continuous outcomes was done with t-tests when the assumption of normal distribution was met. Statistical analyses by use of student t-test were carried out by t-test calculator (http://www.quantitativeskills.com/sisa/statistics/t-test.htm) comparing the two means and with assumptions of independent samples (test group versus control). Mean, standard deviation and number of cases (parallels) were plotted in the calculator. The level of significance was set at $p < 0.05$. All tests were two sided.

References to methods applied

1. Merck, C.; available from; http://www.merckmillipore.com/NO/en?bd=1
2. Sigma-Aldrich, Norway; available from; http://www.sigmaaldrich.com/norway.html
3. GABRIEL SALITER; available from; http://www.organic-bio.com
4. New England Biolabs; available from; https://www.neb.com
5. Cell Signaling; available from; http://www.cellsignal.com
6. Promega; available from; https://no.promega.com
7. Invitrogen; available from; https://www.lifetechnologies.com/no/en/home.html
8. Qiagen; available from; https://www.qiagen.com/no/
9. Technelysium-pty; available from; http://technelysium.com.au

Results

ATF4 does not induce p62 / SQSTM1 promoter in Hek293 cells

In mouse embryonic fibroblasts and HepG2 cells it is found that level of p62 is restored during prolonged starvation (Sahani et al., 2014). Initially p62 is degraded by autophagy, but after 3-6 hours the level is restored again. In part this process is regulated by transcription. It is well known that the expression of transcription factor ATF4 is induced by cellular stress like starvation (Kilberg et al., 2009, B'Chir et al., 2013).

The purpose of this experiment was to test whether p62 is upregulated by ATF4 in the mammalian Hek293 cells. It is shown that the p62 promoter contains an ATF/CHOP sensitive site at position -1360/-1345. This site is important for upregulation of p62 under Leucine deprivation (B'chir et al., 2013). In this study they used a 1935 nucleotide construct of the p62 promoter upstream of the Luciferase gene. Here a construct containing 2305 nucleotides of the p62 promoter upstream of the luciferase gene in a pGL3 reporter plasmid was used (Brenne, 2010).

First, a bioinformatic analysis of the p62 promoter was conducted using the UCSC genome browser (http://mfold.rna.albany.edu), results are displayed in Figure 12A. It appears that there are several conserved Ap1 sites in the p62 promoter. ATF4 is in family with Ap1 transcription factors and may bind to similar sites in the DNA. This indicated a possible impact of ATF4 on the p62 promoter.

Next, the Luciferase assay was used as monitoring method to test whether ATF4 had an influence on the p62 promoter in Hek293 cells. ATF4 expression plasmid and p62 promoter sequences were co-transfected, and the luciferase expression was measured. Figure 11B displays our finding that ATF4 does not induces expression of p62. Instead there seems to be a slight reduction in promoter activity. The level of expression was increased around 2,5 fold when a plasmid expressing the transcription factor NRF2 was cotransfected. NRF2 was used as positive control since it is previously shown that p62 is up regulated by this transcription factor (Jain et al., 2010).

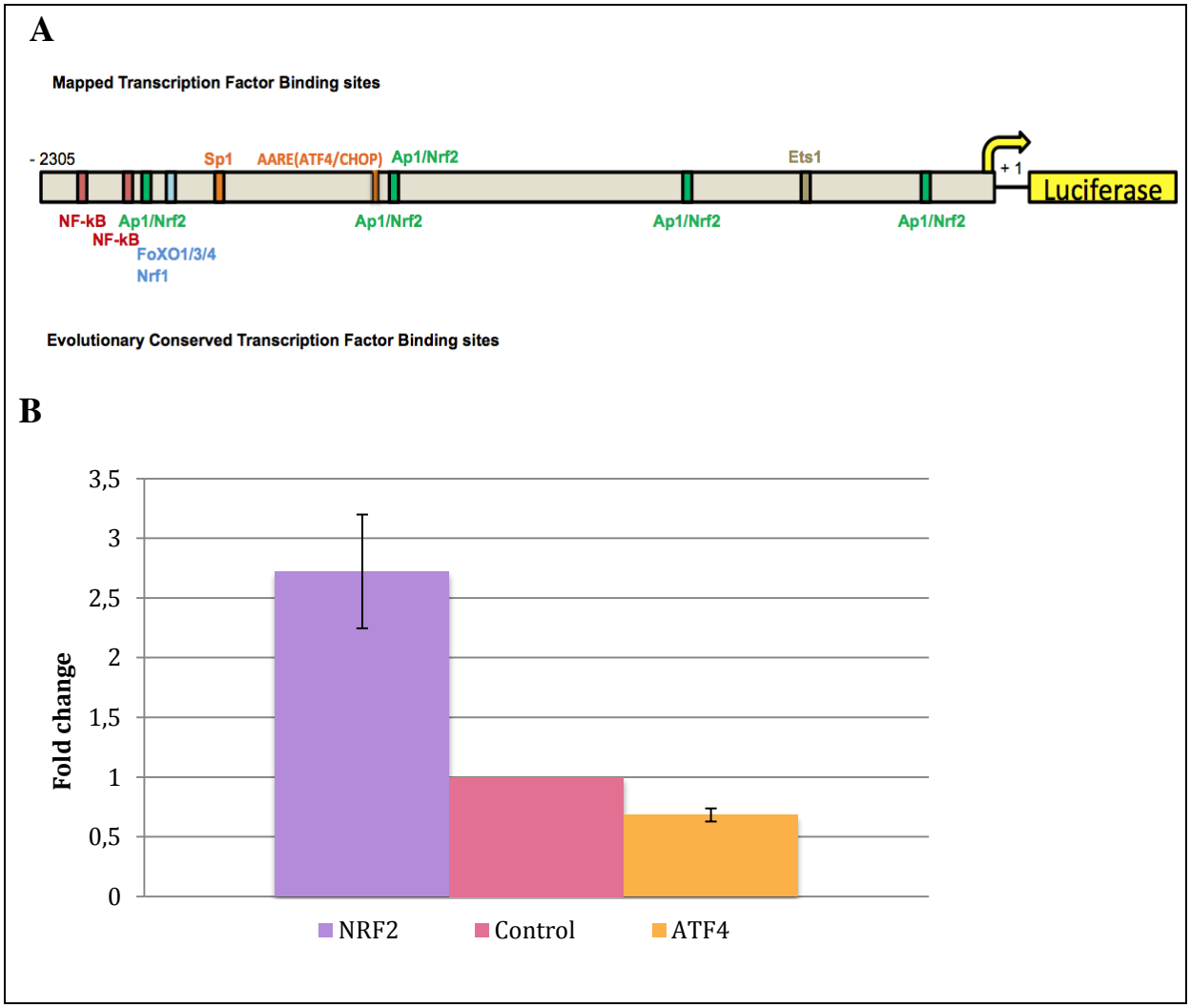

Figure 11. ATF4 does not induce transcription from the p62 promoter
A) Schematic display of SQSTM1/p62 promoter by its several evolutionary conserved transcription factor-binding sites. Experimentally mapped TF´s (transcription factors) indicated above and bioinformatically identified conserved sites indicated below. **B)** ATF4 compared with NRF2 as trancription factors regulating the expression of p62. Control groups each of 6 parallels, total n=18. Hek293 cells where transfected with 50ng of p62 (-2305/+46) promoter constructs, 50ng pcDNA3-HA (empty vector), 10 ng of pcH110 β-gal using Metafecten®Pro, and 50 ng ATF4 and 50 ng NRF2 expression vectors. Luciferase and β-galactosidase values were measured using the Topix®Luciferase assay kit. The results show luciferase/β-galactosidase ratio for ATF4 and NRF2 compared to the control (error bars: SD).

Cloning of p62 5´UTRs in front of the luciferase gene

Cellular stress like starvation also alters the factors involved in regulation of translation, and several stress induced proteins are regulated by sequences in their 5´UTR. To find whether expression of p62 may be regulated by sequences in 5´UTR a bioinformatic analysis was performed by using *the UCSC Genome Browser (https://genome.ucsc.edu/)*. Intrestingly it

was observed that p62 contains unusually short 5´UTR, and that most transcripts have 5´UTR of 35, 62 or 100 nucleotides (Figure 12A).

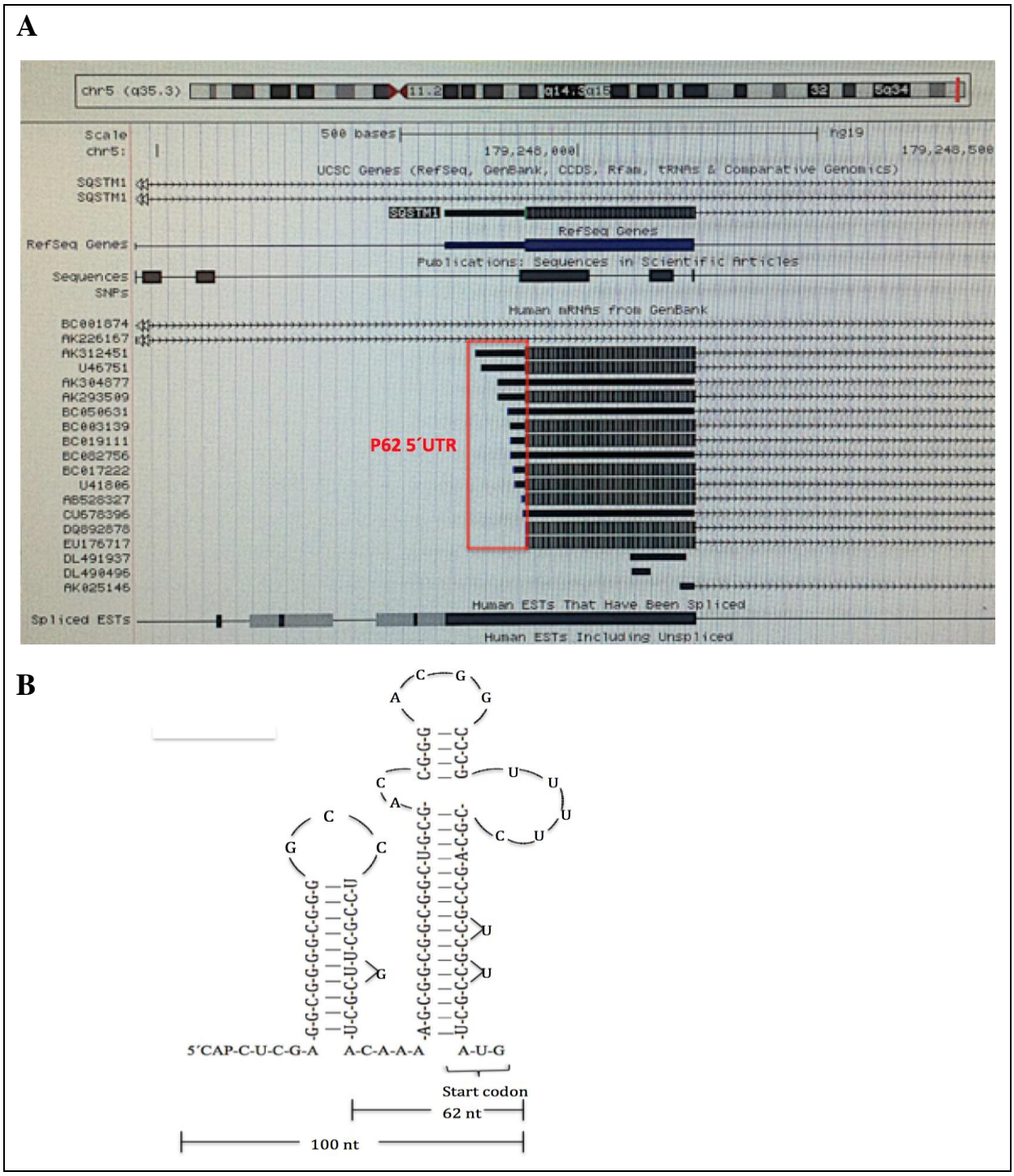

Figure 12. Analysis of the p62 5`UTR

A)Presentation of the different 5'UTR regions found in the UCSC Genome Browser on human p62. It appears that the 5'UTR regions found in different human cells are unusually short. **B)** Presentation of the two hairpins structures of the p62 5´UTR of 62 bp and 100 bp using the mfold program. The 5´UTR conting 100 bp is predicted to form two hairpin structures and the 5´UTR contsing of 62 bp is predicted to form one.

Further a structural analysis of the p62 5´UTR was performed using *the mfold program UNAfold web server* (http://mfold.rna.albany.edu). This computational analysis predicted that the p62 5´UTR can form two hairpins when it is 100 bp long, and one hairpin when it is 62 bp long (Figure 12B).

Cloning of p62 5`UTRs in front of the Luciferase gene

To measure the impact on gene expression of p62 / SQSTM1 with the different lengths of the p62 5'UTRs, the luciferase assay method was used. The three 5'UTRs were cloned into two different reporter gene vectors, the pGL3-Basic and pGL3-Control (Promega) (Figure 13A).

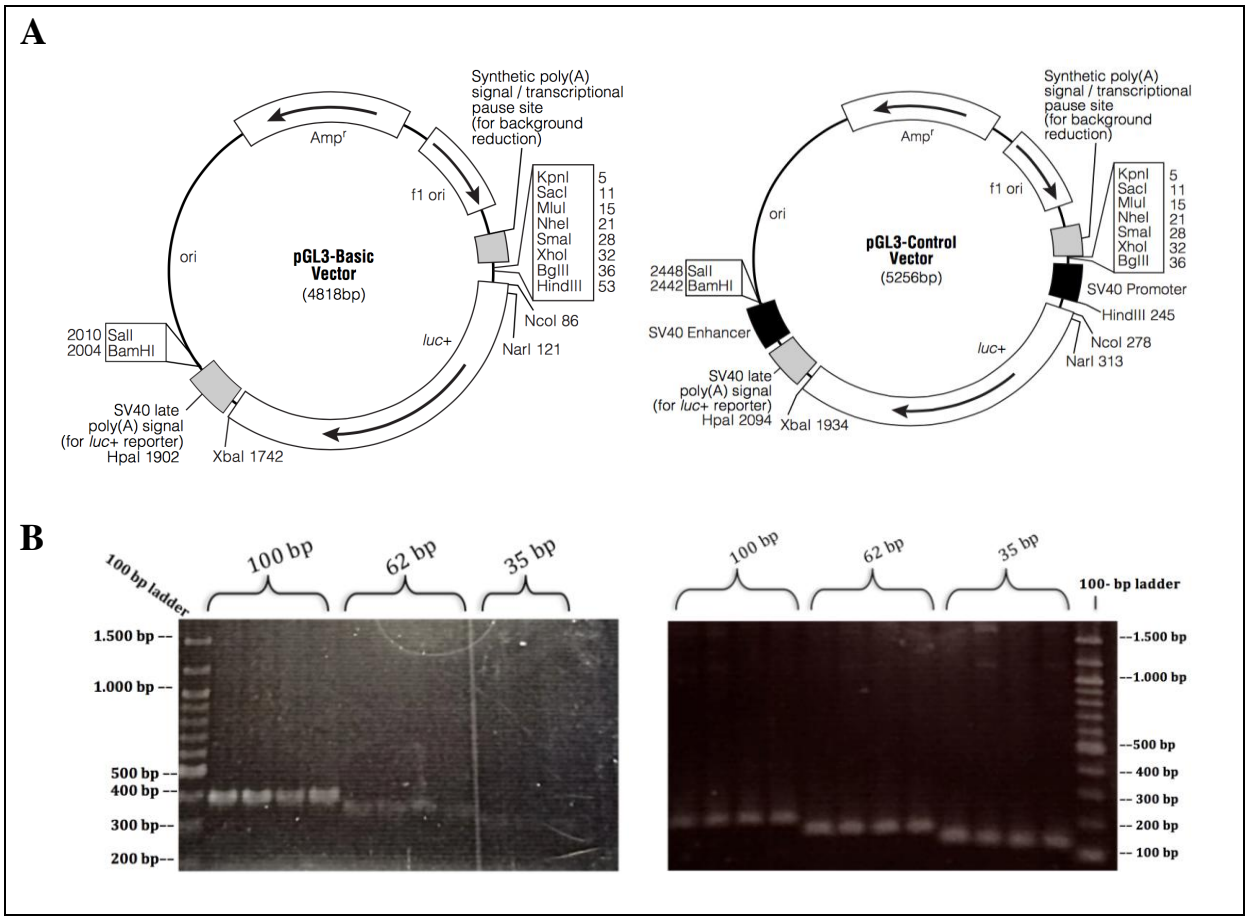

Figure 13. Presentation of the cloning of the p62 5´UTRs
A) Map of the pGL3-Basic vector and pGL3-Control used to clonie the different p62 5´UTRs (adaptet from Promega). **B)** The three different p62 5´UTRs where cloned successfully in to the pGL3-Basic ans pGL3-Control vectores. Oligoes were inserted into the HindIII - NcoI sites of the luciferase vectors. The gel represent 4 different clones of each of the 5´UTRs after restriction enzyme digestion using KpnI and NarI, to verify insertion of the oligoes representing the 5´UTRs of 35, 62 and 100 bp.

The pGL3-Basic vector does not contain eukaryotic promoter and enhancer sequence while the pGL3-Contol vector contains a SV40 promoter and enhancher sequence, which allows luciferase expression in many cells to become strong (Figure 13A). The pGL3-Basic vector was used as a control.

The three different 5´UTRs of 35, 62 and 100 bp where cloned into the pre-linearisated luciferase reporter plasmids in front of the luciferase gen using the HindIII - NcoI site. To validate the plasmids for correct inserts restriction enzyme digestion (Figure 3B), followed by DNA sequening was performed. Analyzes of the sequences by using *the Chromas BLAST* (http://technelysium.com.au/?page_id=27) confirmed that the inserts were correct (Figure 13B).

p62 5'UTR represses protein expression during normal conditions

The 5'UTR part of mRNA is known to contribute in translational regulation but little is known about regulatory elements in the p62/SQSTM1 5´UTR. In this part of the study the aim was to explore whether the up-regulation of p62 / SQSTM1 during prolonged starvation could be regulated by its 5´UTR.

The method used to determine the impact of the different lenghts of the 5´UTR in the expression of p62/SQSTM1 with and without starvation was luciferase assay. 50 ng and 100 ng of the three 5´UTRs in front of the luciferase gene in the pGL3-Basic and pGL3-Control vector were first tested in full medium in Hek293 cells. 50 ng and 100 ng of pGL3-Basic and pGL3-Control without insert of the p62 5´UTRs were transfected and worked as controls.
Figure 14A shows the impact of the 5´UTRs on luciferase expression upon normal conditions using the pGL3-Basic reporter vector. The results show luciferase/β-galactosidase ratio relative to the basal expression of pGL3-Basic without 5´UTR. The 5'UTR of 100 bp significantly enhanced expression of the luciferase gene, 62 and 35 bp 5´UTRs rather inhibited luciferase expression (Figure 14A).

Figure 14B presents the effect of the p62 5´UTRs on expression of luciferase in the pGL3-Control plasmid. The pGL3-Control has a strong SV40 promoter and an enhancer. Interestingly, all the p62 5´UTRs strongly repressed expression of the luciferase protein under normal conditions.

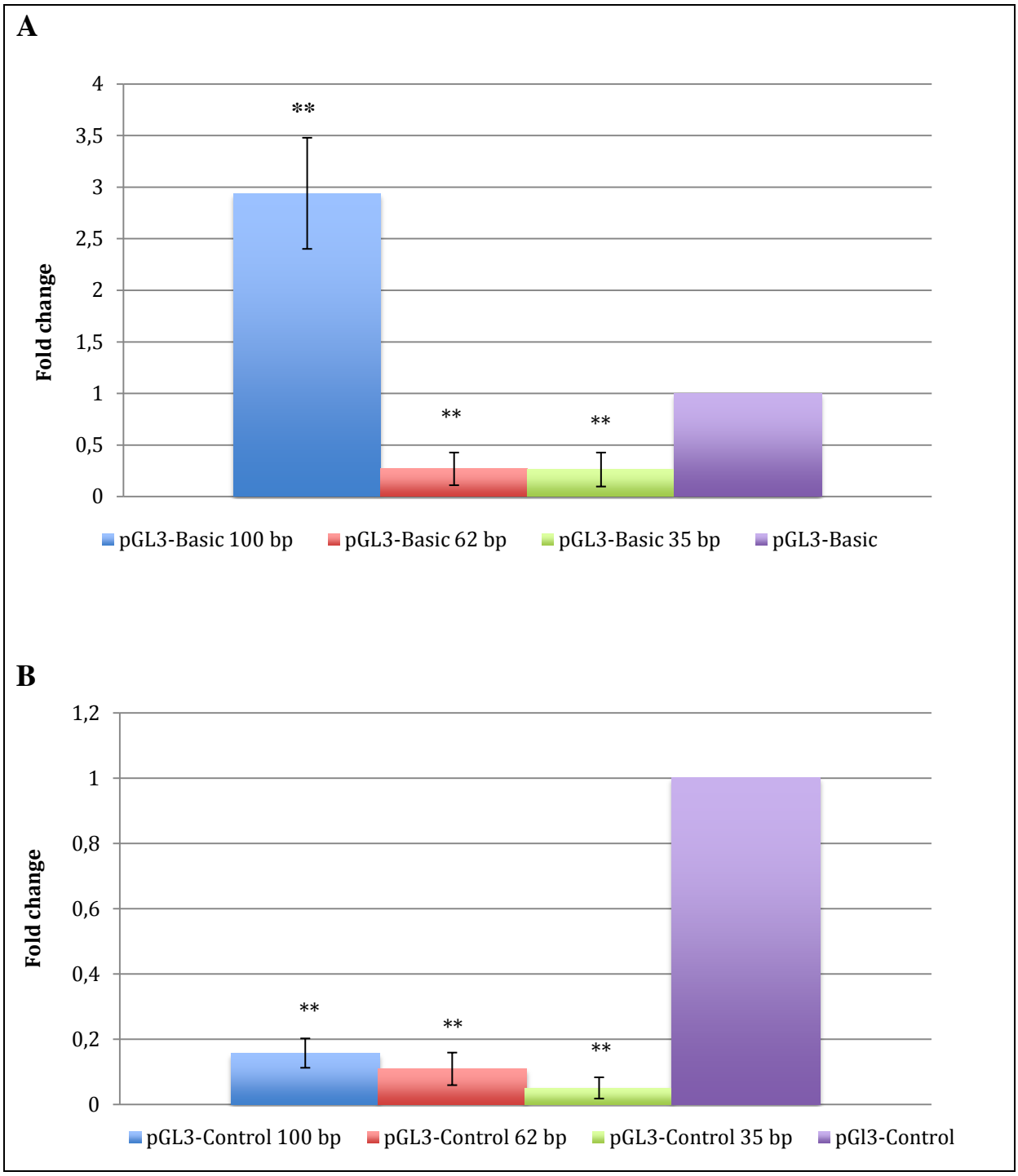

Figure 14. p62 5´UTRs repress expression of the luciferase protein

A) Impact of the various p62 5'UTRs on luciferase expression using pGL3-Basic reporter gene vector. Hek293 cells where transfected with 50ng pGL3-Basic 100 bp, 62 bp and 35 bp, 50 ng pcDNA3-HA (empty vector) and 10 ng of pcH110 β-gal using Metafecten®Pro. Luciferase and β-galactosidase values were measured using the Topix®Luciferase assay kit. The results show luciferase/β-galactosidase ratio. The results are relative to the basal expression in pGL3-Basic without 5´UTR. (** $p<0.01$ each group with 5 parallels, total n=20). **B)** The impact of the p62 5´UTRs on luciferase expression using pGL3-Control plasmid. Hek293 cells were transfected with 50 ng pGL3-Control 100 bp, 62 bp and 35 bp 50 ng pcDNA3-HA(empty vector) and10 ng of pcH110 β-gal Metafecten®Pro. Luciferase and β-galactosidase values were measured using the Topix®Luciferase assay kit. The

results show luciferase/β-galactosidase ratio. The results are relative to basal expression. The basal expression is pGL3-Control without the inserted p62 5´UTRs. (** $p<0.01$, each group with 5 parallels, total n=20).

p62 5´UTRs repress protein expression during starvation

In order to see whether the p62 5´UTRs have impact on p62 expressing during starvation, the luciferase reporter assay was performed in Hek293 cells using the pGL3-Basic, pGL3-Control and reporter vectors as described above. The Hek293 cells where starved for 3 or 6 h in Hanks medium the day after transfection. Both Figure 15A and 15B shows that starvation results in less expression of the luciferase protein. The repression is highest 3 hours after starvation. The p62 5`UTRs seems to repress the luciferase expression both under normal condition and up on starvation.

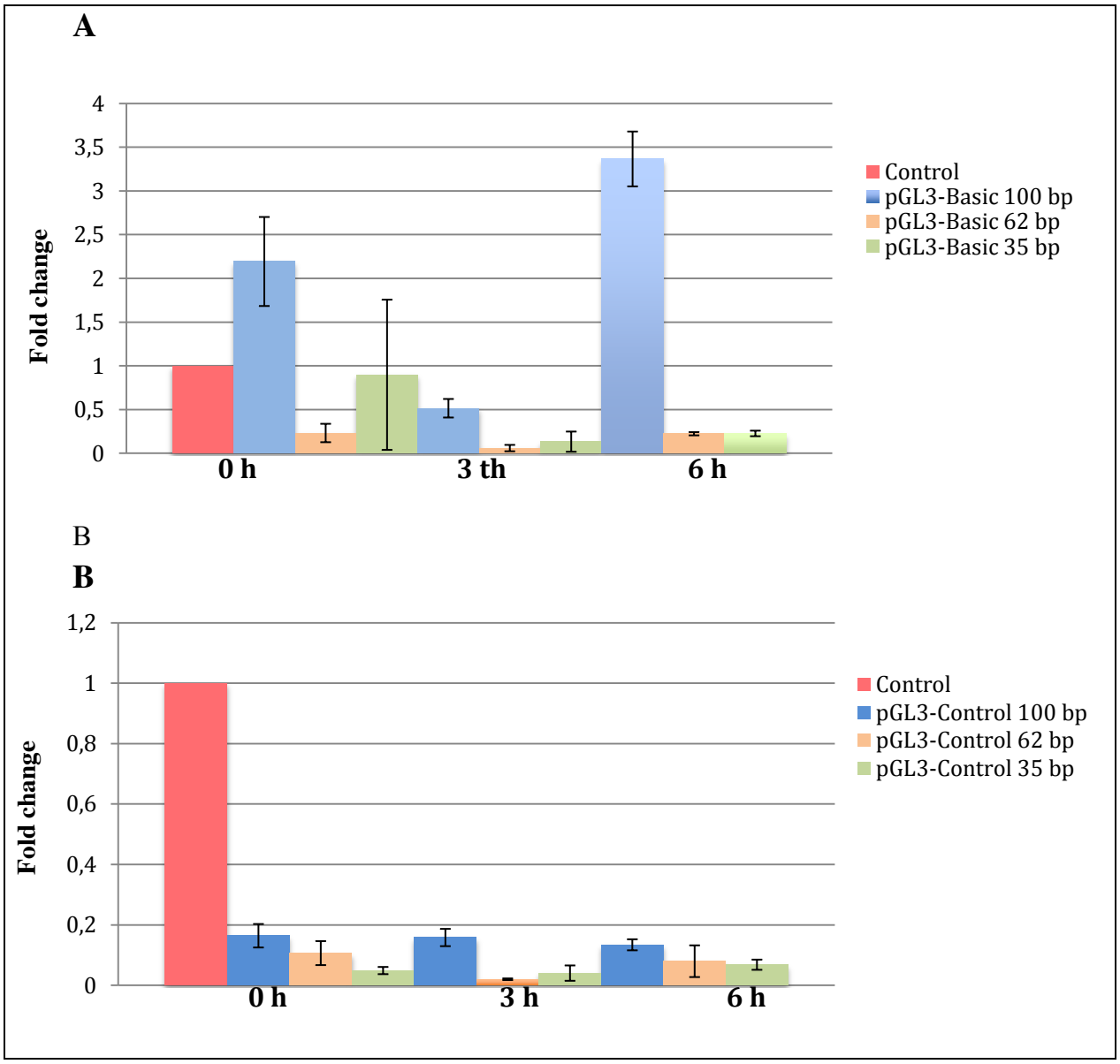

Figure 15. The p62 5´UTRs repress protein expression during starvation
A) Impact on protein expression using the pGL3-Basic vector. Hek293 cells was transfected with 50 ng of the tree different lengths of the 5´UTRs incorporated in pGL3-Basic, 50 ng pcDNA3-HA(empty vector) and10 ng of pcH110 β-gal using Metafecten®Pro. Luciferase and β-galactosidase values were measured using the Topix®Luciferase assay kit. Results show luciferase/β-galactosidase ratio. The results are relative to basal expression. The basal expression is pGL3-Basic without 5´UTR and not starved. The cells where starved for 3 or 6 hours by use of Hanks medium (Each group with 3 parallels, total n=12). **B)** Impact on luciferase expression using the pGL3-Control vector. Hek293 cells was transfected with 50 ng of the tree different lengts of the 5´UTRs incorporated in pGL3-Control, 50 ng pcDNA3-HA (empty vector) and10 ng of pcH110 β-gal using Metafecten®Pro. Luciferase and β-galactosidase values were measured using the Topix®Luciferase assay kit. The results are relative to basal expression. The basal expression is pGL3-Control without p6 5´UTR and not starved. The cells where starved in 3 or 6 h with use of Hanks medium (each group with 3 parallels, total n=12).

ATF4 seems to act on the p62 5´UTRs

Since the p62 5´UTRs repressed expression of Luciferase protein, we next wanted to see whether ATF4 could modulate this expression. Luciferase assay was used as method, and the different p62 5´UTRs cloned into pGL3-Basic vectors were co-transfected with pcH110 β-gal and 50 ng of an ATF4 expression plasmid in Hek293 cells.

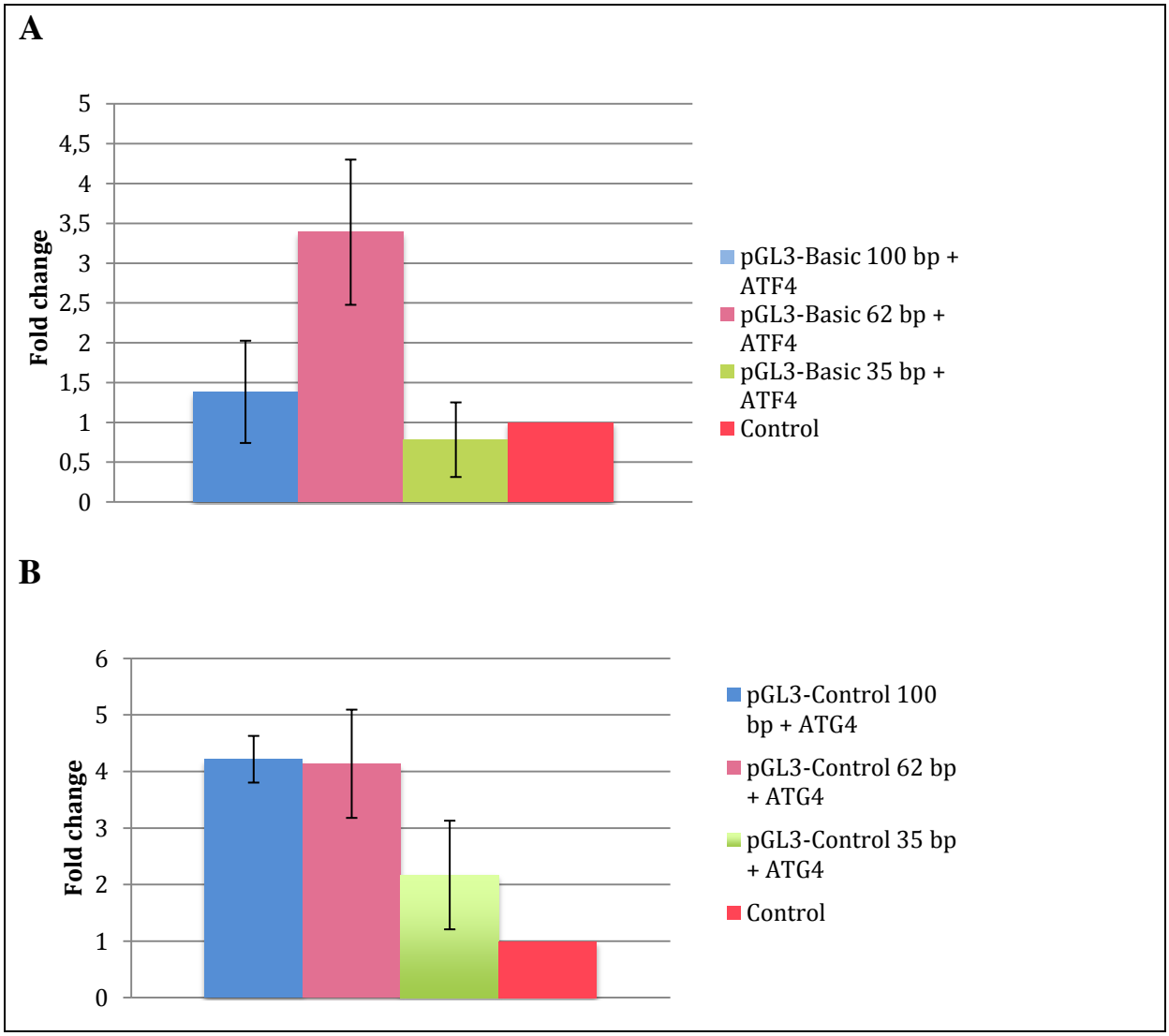

Figure 16. ATF4 enhance expression of luciferase proteins containing the p62 5´UTRs in their mRNA.

A) ATF4 enhances the expression of luciferase containing p62 5`UTRs. The results are relative to control without ATF4. Hek293 cells was transfected with 50 ng of the tree different lengths of the 5´UTRs incorporated in pGL3-Basic vector, then 50 ng ATF4 expression plasmid was co-transfected and further 50 ng pcDNA3-HA (empty vector) and 10 ng of pcH110 β-gal was adding using Metafecten®Pro. Luciferase and β-galactosidase values were measured using the Topix®Luciferase assay kit. The results are relative to basal expression; the basal expression is pGL3-Basic with the different length of p62 5`UTRs without ATF4 ( †ns, each group with 4 parallels, total n=16). **B)** ATF4 siginificantly enhances expression of luciferase containing p62 5`UTRs**.** The results are relative to control without ATF4. Hek293 cells was transfected with 50 ng of the tree different lengths of the 5´UTRs incorporated in pGL3-Control vector, then 50 ng ATF4 expression plasmid was co-

transfected and further 50 ng pcDNA3-HA (empty vector) and 10 ng of pcH110 β-gal was adding using Metafecten®Pro. Luciferase and β-galactosidase values were measured using the Topix®Luciferase assay kit. The results are relative to basal expression; the basal expression is pGL3-Basic with the different length of p62 5`UTRs without ATF4 (†ns, each group with 3 parallels, total n=12).

ATF4 seems to stimulate expression of luciferase protein containing the p62 5´UTRs, and especially the p62 bp version of the p62 5´UTRs (Figure 16A). Interestingly, for the 5´UTRs in the pGL3- Control vector which give high expression of the luciferase protein, the stimulating effect was even more pronounced (Figure 16B). This indicate that ATF4 may regulate p62 expression upon starvation via the p62 5`UTR, either directly or indirectly.

The p62 / SQSTM1 expression in Hek293 cells during starvation

Finally we examined that p62 / SQSTM1 in the Hek293 cells is regulated in the same way as in HepG2 and MEF cells during starvation conditions used in the study to Sahani et al, 2014. In the Sahani study p62 / SQSTM1 where up regulated again after a prolonged period of starvation (Sahani et al., 2014). For this purpose the Hek293 cells were starved in Hanks for different time periods, and the cell extracts harvested and analyzed by Western Blotting.

The results are shown in Figure 16, and demonstrate reduction in p62 levels upon starvation for 1-4 hours, then an increase after 6 hours starvation similarly as described in MEF and HepG2 cells (Sahani et al., 2014). However, inspection of the LC3B II level showed that the induction of autophagy was modest.

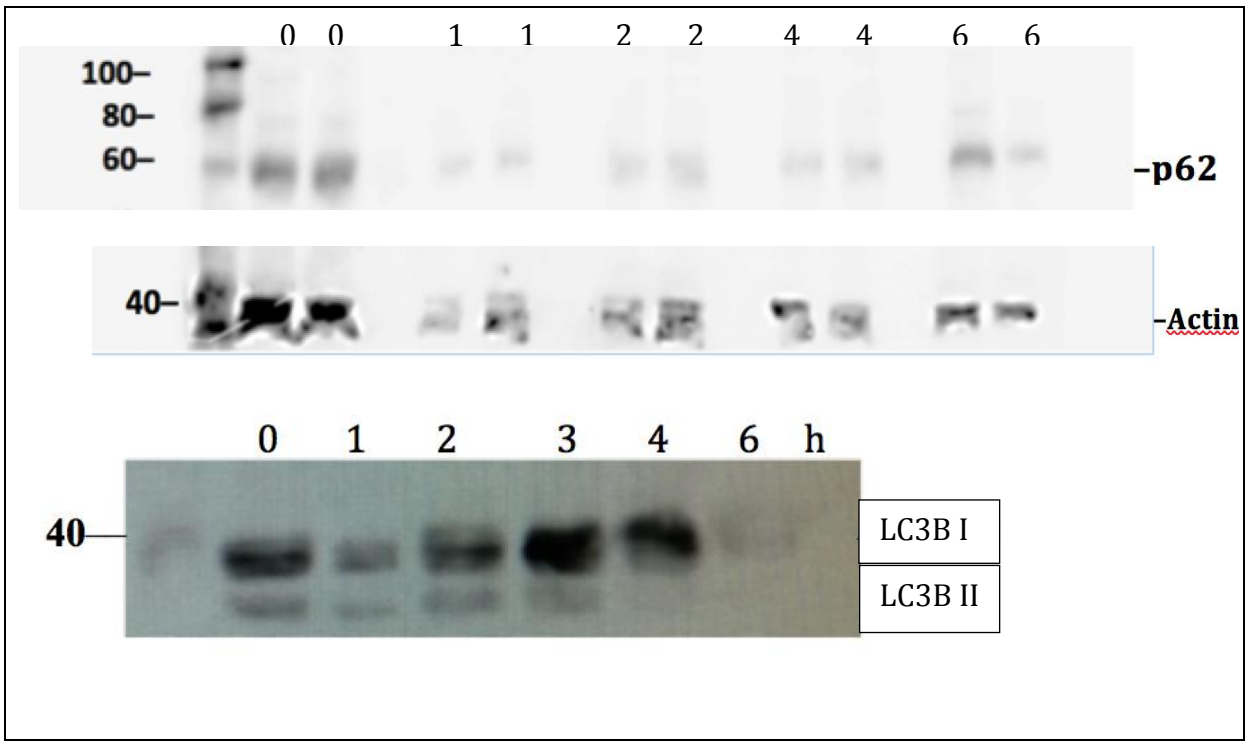

Figure 17. Expression of p62 seems to decreases upon long-term starvation
The cells where starved for respectively 1,2,4, or 6 hours with Hank's medium. The cell lysate was run on SDS-PAGE gel and proteins blotted on a membrane subjected to treatment with the antibodies mouse anti-p62 and rabbit anti-LC3B and rabbit anti-actin. The blot was developed using a Lumi-Imager. The LC3BII was used to examine whether autophagy was activated.

Discussion

Overexpression of ATF4 does not induce the p62 promoter

The transcription factor ATF4 has previously been shown to upregulate the expression of proteins involved in autophagy during amino acid starvation (B'Chir et al., 2013, Kilberg et al., 2009). ATF4 itself is upregulated during starvation and so is p62/SQSTM1 (Sahani et al., 2014, Kilberg et al., 2009). Here we have shown that overexpression of ATF4 in Hek293 cells does not induce expression from the p62 promoter.

Analysis of the p62 5`UTR revealed that it is unusually short, either 35, 62 and 100 nucleotides, and that it may form one or two hairpin structures. Luciferase assay showed that the p62 5`UTRs significantly repressed expression of the luciferase gene both during normal conditions and up on starvation. Interestingly this repression was reversed by ATF4 overexpression under normal conditions. A previous study in MEF cells have indicated that the p62/SQSTM1 promoter is induced by ATF4 upon Leucine deprivation, and that this induction is dependent on the transcription factor CHOP together with ATF4 (B'Chir et al., 2014). Our study in Hek293 cells showed no induction of the p62 promoter upon ATF4 over expression, even if there are several potential binding sites. However we did not test cotransfection of CHOP together with ATF4. Hence, the lack of induction in the Hek293 cells may be due to little or no CHOP present in the Hek293 cells. To test this, CHOP should be expressed together with ATF4 in further studies.

Finally, we showed that also in Hek293 cells the p62 expression seems to be upregulated during prolonged starvation.

Impact of the p62 5`UTRs and ATF4 on the luciferase expression

There are several regulatory elements in the 5'UTR sequence that can make the translation more efficient (Chatterjee and Pal, 2009). Because the 5`UTR section is important in the regulation of translation (Sonenberg and Hinnebusch, 2009), and that different genes have been reported with different structures of their 5`UTRs (Araujo et al., 2012) it was interesting in the present study to find that the p62 5'UTR is unusually short, 35, 62 and 100 bp respectively, compared with 5’UTRs of other mRNAs reported on average between 100 - 200

bp (Liu et al., 2012, Pesole et al., 2001). Current understanding of the action of a short 5`UTR is limited. However, in the sparse literature it is assumed a short 5'UTR part implicates less chance to attain an efficient translation. Despite this general assumption, short 5'UTRs sequences can also form secondary structures (Chatterjee and Pal, 2009). They may contain a specific element called TISU (Translation Initiator of short 5'UTR) which can control both transcription and translation (Elfakess and Dikstein, 2008).

To examine if the p62 5'UTRs used in present study could form secondary structures it was performed a structural analysis using *mfold program UNAfold web server*. The program predicted that the 5`UTR part of p62 actually may form Hairpin structures. The 100 bp 5`UTR may form two Hairpin, and the 62 bp one (Figure 12B). Hairpins are the most common secondary structures found in most RNA folding prediction studies (Svoboda and Di Cara, 2006). Hairpin structures are different, and they vary in length, magnitude of loop and nucleotide sequence. These various parameters permit specific interaction with different proteins. Functionally may RNA hairpins regulate gene expression (Svoboda and Di Cara, 2006). It is found a correlation between effectiveness of translation and mRNA secondary structure in initiation region. The hairpin structure commonly includes a ribosome binding site (de Smit and van Duin, 1990). Hairpin structures may allow the ribosome to bind better and fascilitate the translation, or they may inhibit binding of the ribosome and inhibit translation.

Two aspects considered of importance of the hairpin structure in translation is the stability as well as the placement in the 5'UTR. Location of hairpin structure close to the cap end commonly indicates a stable structure and this is found to inhibit translation. In contrast, structures located further away seems to be less stable, and unlike these may not slow down translation (Araujo et al., 2012). In the present study we found that p62 5'UTR significantly repress expression of the luciferase protein. However, the mechanism for this repression was not resolved, and is therefore an interesting topic for the future study. For example, it may be intriguing to mutate the hairpin structures in 5`UTRs to examine if structural changes at different location will increase the expression of p62 or vice versa. In this way one might be able to conclude whether hairpin or other structural elements in the p62 5'UTR regulate the translation of p62.

The p62 / SQSTM1 regulation in our Hek293 cells during starvation

The Western blots of p62 and LC3B was used to determine the effect of starvation on the autophagy process in the Hek293 cells. It seem like the induction of autophagy, here monitored by LC3B lipidation, is relatively modest. However p62 protein level is decreased after 1-4 hours of starvation. There after it seems to be restored, similarly as shown for MEF and HepG2 cells previously (Sahani et al., 2014). The results presented here could indicate that the increase in p62 expression during prolonged starvation, may at least in part be mediated by ATF4 acting in an unknown way on the p62 5`UTR. However, this has to be studied more closely in the future experiments. One way would be to knock out or down regulate ATF4, and then monitor autophagy and p62 protein levels during starvation.

Clinical or pharmacological significance of the findings

The mammalian autophagy receptor p62/SQSTM1 has several physiological functions as in tumorgenesis, bone metabolism, neurodegenerative diseases, and aging (Hernandez et al., 2004, Dikic et al., 2010). Since autophagy is an essential mechanism contributing to the cell maintains its homeostasis, it will be of great value to get more knowledge of how the p62 is involved. Expanded knowledge may improve the understanding of a variety of diseases and which further can pave the way for developing new drug targets and more effective drugs. It may also lead to development of new biomarkers and diagnostic tools.

Conclusions

In this study we have found that the p62 5`UTR seems to significantly repress protein expression. This may be due to Hairpin structures predicted to form in this region. Interestingly, ATF4 did not induce expression from the p62 promoter, but seemed to reverse the repression of the 5`UTR. Here, the restoration of the p62 expression upon prolonged starvation may be due to ATF4 acting on p62 5`UTR in a directly or indirectly way.

References

ARAUJO, P. R., YOON, K., KO, D., SMITH, A. D., QIAO, M., SURESH, U., BURNS, S. C. & PENALVA, L. O. 2012. Before It Gets Started: Regulating Translation at the 5' UTR. *Comp Funct Genomics,* 2012**,** 475731.

ARBER, W. & LINN, S. 1969. DNA modification and restriction. *Annu Rev Biochem,* 38**,** 467-500.

ASHFORD, T. P. & PORTER, K. R. 1962. Cytoplasmic components in hepatic cell lysosomes. *J Cell Biol,* 12**,** 198-202.

B'CHIR, W., CHAVEROUX, C., CARRARO, V., AVEROUS, J., MAURIN, A. C., JOUSSE, C., MURANISHI, Y., PARRY, L., FAFOURNOUX, P. & BRUHAT, A. 2014. Dual role for CHOP in the crosstalk between autophagy and apoptosis to determine cell fate in response to amino acid deprivation. *Cell Signal,* 26**,** 1385-91.

B'CHIR, W., MAURIN, A. C., CARRARO, V., AVEROUS, J., JOUSSE, C., MURANISHI, Y., PARRY, L., STEPIEN, G., FAFOURNOUX, P. & BRUHAT, A. 2013. The eIF2alpha/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res,* 41**,** 7683-99.

BARBERIS, A. & PETRASCHECK, M. 2003. Transcription activation in eukaryotic cells. *eLS*.

BERNARD WEISS, A. J.-S., THEODORE R. LIVE, GEORGE C. FAREED AND CHARLES C. RICHARDSON 1968. Enzymatic Breakage and Joining of Deoxyribonucleic Acid. VI. Further purification and properties of polynucleotide ligase from Escherichia coli infected with bacteriophage T4. *The journal of biological chemistry***,** 4543-4555.

BIRNBOIM, H. C. & DOLY, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res,* 7**,** 1513-23.

BJORKOY, G., LAMARK, T., BRECH, A., OUTZEN, H., PERANDER, M., OVERVATN, A., STENMARK, H. & JOHANSEN, T. 2005. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol,* 171**,** 603-14.

BROWN, E. J., ALBERS, M. W., SHIN, T. B., ICHIKAWA, K., KEITH, C. T., LANE, W. S. & SCHREIBER, S. L. 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature,* 369**,** 756-758.

CASAMASSIMI, A. & NAPOLI, C. 2007. Mediator complexes and eukaryotic transcription regulation: an overview. *Biochimie,* 89**,** 1439-46.

CHATTERJEE, S. & PAL, J. K. 2009. Role of 5'- and 3'-untranslated regions of mRNAs in human diseases. *Biol Cell,* 101**,** 251-62.

CHEN, R., ZOU, Y., MAO, D., SUN, D., GAO, G., SHI, J., LIU, X., ZHU, C., YANG, M., YE, W., HAO, Q., LI, R. & YU, L. 2014. The general amino acid control pathway regulates mTOR and autophagy during serum/glutamine starvation. *J Cell Biol,* 206**,** 173-82.

CIECHANOVER, A., ELIAS, S., HELLER, H., FERBER, S. & HERSHKO, A. 1980. Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. *J Biol Chem,* 255**,** 7525-8.

COOPER, G. M. & HAUSMAN, R. E. 2000. *The cell: A Molecular Approach*, Sinauer Associates Sunderland.

CUERVO, A. M. 2004. Autophagy: many paths to the same end. *Molecular and cellular biochemistry,* 263**,** 55-72.

DE SMIT, M. H. & VAN DUIN, J. 1990. Secondary structure of the ribosome binding site determines translational efficiency: a quantitative analysis. *Proc Natl Acad Sci U S A,* 87**,** 7668-72.

DIKIC, I., JOHANSEN, T. & KIRKIN, V. 2010. Selective autophagy in cancer development and therapy. *Cancer research,* 70**,** 3431-3434.

ELFAKESS, R. & DIKSTEIN, R. 2008. A translation initiation element specific to mRNAs with very short 5'UTR that also regulates transcription. *PLoS One,* 3**,** e3094.

ELLIS, R. J. 2006. Molecular chaperones: assisting assembly in addition to folding. *Trends in biochemical sciences,* 31**,** 395-401.

FUJITA, N., MORITA, E., ITOH, T., TANAKA, A., NAKAOKA, M., OSADA, Y., UMEMOTO, T., SAITOH, T., NAKATOGAWA, H., KOBAYASHI, S., HARAGUCHI, T., GUAN, J. L., IWAI, K., TOKUNAGA, F., SAITO, K., ISHIBASHI, K., AKIRA, S., FUKUDA, M., NODA, T. & YOSHIMORI, T. 2013. Recruitment of the autophagic machinery to endosomes during infection is mediated by ubiquitin. *J Cell Biol,* 203**,** 115-28.

GEETHA, T. & WOOTEN, M. W. 2002. Structure and functional properties of the ubiquitin binding protein p62. *FEBS Lett,* 512**,** 19-24.

GERTZ, E. M., YU, Y. K., AGARWALA, R., SCHAFFER, A. A. & ALTSCHUL, S. F. 2006. Composition-based statistics and translated nucleotide searches: improving the TBLASTN module of BLAST. *BMC Biol,* 4**,** 41.

HERNANDEZ, F., DıAZ-HERNANDEZ, M., AVILA, J. & LUCAS, J. J. 2004. Testing the ubiquitin–proteasome hypothesis of neurodegeneration in vivo. *Trends in neurosciences,* 27**,** 66-69.

HOCHSTRASSER, M. 1992. Ubiquitin and intracellular protein degradation. *Curr Opin Cell Biol,* 4**,** 1024-31.

ITAKURA, E. & MIZUSHIMA, N. 2011. p62 Targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding. *J Cell Biol,* 192**,** 17-27.

JAEGER, P. A. & WYSS-CORAY, T. 2009. All-you-can-eat: autophagy in neurodegeneration and neuroprotection. *Mol Neurodegener,* 4**,** 16.

JAIN, A., LAMARK, T., SJOTTEM, E., LARSEN, K. B., AWUH, J. A., OVERVATN, A., MCMAHON, M., HAYES, J. D. & JOHANSEN, T. 2010. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J Biol Chem,* 285**,** 22576-91.

JOHANSEN, T. & LAMARK, T. 2011. Selective autophagy mediated by autophagic adapter proteins. *Autophagy,* 7**,** 279-96.

JOUNG, I., STROMINGER, J. L. & SHIN, J. 1996. Molecular cloning of a phosphotyrosine-independent ligand of the p56lck SH2 domain. *Proc Natl Acad Sci U S A,* 93**,** 5991-5.

KADONAGA, J. T. 2004. Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. *Cell,* 116**,** 247-57.

KETTERN, N., DREISEIDLER, M., TAWO, R. & HOHFELD, J. 2010. Chaperone-assisted degradation: multiple paths to destruction. *Biol Chem,* 391**,** 481-9.

KILBERG, M. S., BALASUBRAMANIAN, M., FU, L. & SHAN, J. 2012. The transcription factor network associated with the amino acid response in mammalian cells. *Advances in Nutrition: An International Review Journal,* 3**,** 295-306.

KILBERG, M. S., PAN, Y. X., CHEN, H. & LEUNG-PINEDA, V. 2005. Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. *Annu Rev Nutr,* 25**,** 59-85.

KILBERG, M. S., SHAN, J. & SU, N. 2009. ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol Metab,* 20**,** 436-43.

KIRKIN, V., LAMARK, T., SOU, Y. S., BJORKOY, G., NUNN, J. L., BRUUN, J. A., SHVETS, E., MCEWAN, D. G., CLAUSEN, T. H., WILD, P., BILUSIC, I., THEURILLAT, J. P., OVERVATN, A., ISHII, T., ELAZAR, Z., KOMATSU, M., DIKIC, I. & JOHANSEN, T. 2009. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Mol Cell,* 33**,** 505-16.

KLIONSKY, D. J., ABDALLA, F. C., ABELIOVICH, H., ABRAHAM, R. T., ACEVEDO-AROZENA, A., ADELI, K., AGHOLME, L., AGNELLO, M., AGOSTINIS, P., AGUIRRE-GHISO, J. A., AHN, H. J., AIT-MOHAMED, O., AIT-SI-ALI, S., AKEMATSU, T., AKIRA, S., AL-YOUNES, H. M., AL-ZEER, M. A., ALBERT, M. L., ALBIN, R. L., ALEGRE-ABARRATEGUI, J., ALEO, M. F., ALIREZAEI, M., ALMASAN, A., ALMONTE-BECERRIL, M., AMANO, A., AMARAVADI, R., AMARNATH, S., AMER, A. O., ANDRIEU-ABADIE, N., ANANTHARAM, V., ANN, D. K., ANOOPKUMAR-DUKIE, S., AOKI, H., APOSTOLOVA, N., ARANCIA, G., ARIS, J. P., ASANUMA, K., ASARE, N. Y., ASHIDA, H., ASKANAS, V., ASKEW, D. S., AUBERGER, P., BABA, M., BACKUES, S. K., BAEHRECKE, E. H., BAHR, B. A., BAI, X. Y., BAILLY, Y., BAIOCCHI, R., BALDINI, G., BALDUINI, W., BALLABIO, A., BAMBER, B. A., BAMPTON, E. T., BANHEGYI, G., BARTHOLOMEW, C. R., BASSHAM, D. C., BAST, R. C., JR., BATOKO, H., BAY, B. H., BEAU, I., BECHET, D. M., BEGLEY, T. J., BEHL, C., BEHRENDS, C., BEKRI, S., BELLAIRE, B., BENDALL, L. J., BENETTI, L., BERLIOCCHI, L., BERNARDI, H., BERNASSOLA, F., BESTEIRO, S., BHATIA-KISSOVA, I., BI, X., BIARD-PIECHACZYK, M., BLUM, J. S., BOISE, L. H., BONALDO, P., BOONE, D. L., BORNHAUSER, B. C., BORTOLUCI, K. R., BOSSIS, I., BOST, F., BOURQUIN, J. P., BOYA, P., BOYER-GUITTAUT, M., BOZHKOV, P. V., BRADY, N. R., BRANCOLINI, C., BRECH, A., BRENMAN, J. E., BRENNAND, A., BRESNICK, E. H., BREST, P., BRIDGES, D., BRISTOL, M. L., BROOKES, P. S., BROWN, E. J., BRUMELL, J. H., et al. 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy,* 8**,** 445-544.

KLIONSKY, D. J., CUERVO, A. M., DUNN, W. A., JR., LEVINE, B., VAN DER KLEI, I. & SEGLEN, P. O. 2007. How shall I eat thee? *Autophagy,* 3**,** 413-6.

KOMATSU, M., KAGEYAMA, S. & ICHIMURA, Y. 2012. p62/SQSTM1/A170: physiology and pathology. *Pharmacol Res,* 66**,** 457-62.

KOMATSU, M., KUROKAWA, H., WAGURI, S., TAGUCHI, K., KOBAYASHI, A., ICHIMURA, Y., SOU, Y. S., UENO, I., SAKAMOTO, A., TONG, K. I., KIM, M., NISHITO, Y., IEMURA, S., NATSUME, T., UENO, T., KOMINAMI, E., MOTOHASHI, H., TANAKA, K. & YAMAMOTO, M. 2010. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol,* 12**,** 213-23.

LECKER, S. H., GOLDBERG, A. L. & MITCH, W. E. 2006. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *J Am Soc Nephrol,* 17**,** 1807-19.

LEVINE, B. & KROEMER, G. 2008. Autophagy in the pathogenesis of disease. *Cell,* 132**,** 27-42.

LIU, H., YIN, J., XIAO, M., GAO, C., MASON, A. S., ZHAO, Z., LIU, Y., LI, J. & FU, D. 2012. Characterization and evolution of 5' and 3' untranslated regions in eukaryotes. *Gene,* 507**,** 106-11.

MA, X. M. & BLENIS, J. 2009. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol,* 10**,** 307-18.

MIZUSHIMA, N., YOSHIMORI, T. & LEVINE, B. 2010. Methods in mammalian autophagy research. *Cell,* 140**,** 313-26.

MIZUSHIMA, N., YOSHIMORI, T. & OHSUMI, Y. 2011. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol,* 27**,** 107-32.

MOORE, P. A., ROSEN, C. A. & CARTER, K. C. 1996. Assignment of the Human FKBP12-Rapamycin-Associated Protein (FRAP) Gene to Chromosome 1p36 by Fluorescencein SituHybridization. *Genomics,* 33**,** 331-332.

MOSCAT, J. & DIAZ-MECO, M. T. 2009. p62 at the crossroads of autophagy, apoptosis, and cancer. *Cell,* 137**,** 1001-4.

NODA, N. N., OHSUMI, Y. & INAGAKI, F. 2010. Atg8-family interacting motif crucial for selective autophagy. *FEBS Lett,* 584**,** 1379-85.

PANKIV, S., CLAUSEN, T. H., LAMARK, T., BRECH, A., BRUUN, J. A., OUTZEN, H., OVERVATN, A., BJORKOY, G. & JOHANSEN, T. 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem,* 282**,** 24131-45.

PESOLE, G., MIGNONE, F., GISSI, C., GRILLO, G., LICCIULLI, F. & LIUNI, S. 2001. Structural and functional features of eukaryotic mRNA untranslated regions. *Gene,* 276**,** 73-81.

RENART, J., REISER, J. & STARK, G. R. 1979. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci U S A,* 76**,** 3116-20.

ROEDER, R. G. 2005. Transcriptional regulation and the role of diverse coactivators in animal cells. *FEBS Lett,* 579**,** 909-15.

ROGOV, V., DOTSCH, V., JOHANSEN, T. & KIRKIN, V. 2014. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Mol Cell,* 53**,** 167-78.

SAHANI, M. H., ITAKURA, E. & MIZUSHIMA, N. 2014. Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. *Autophagy,* 10**,** 431-41.

SANGER, F., NICKLEN, S. & COULSON, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A,* 74**,** 5463-7.

SEIBENHENER, M. L., GEETHA, T. & WOOTEN, M. W. 2007. Sequestosome 1/p62--more than just a scaffold. *FEBS Lett,* 581**,** 175-9.

SHAPIRO, A. L., VINUELA, E. & MAIZEL, J. V., JR. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem Biophys Res Commun,* 28**,** 815-20.

SONENBERG, N. & HINNEBUSCH, A. G. 2009. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell,* 136**,** 731-45.

SVOBODA, P. & DI CARA, A. 2006. Hairpin RNA: a secondary structure of primary importance. *Cell Mol Life Sci,* 63**,** 901-8.

TERMAN, A. & BRUNK, U. T. 2005. Autophagy in cardiac myocyte homeostasis, aging, and pathology. *Cardiovascular Research,* 68**,** 355-365.

VOGEL, C., ABREU RDE, S., KO, D., LE, S. Y., SHAPIRO, B. A., BURNS, S. C., SANDHU, D., BOUTZ, D. R., MARCOTTE, E. M. & PENALVA, L. O. 2010. Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. *Mol Syst Biol,* 6**,** 400.

WOOTEN, M. W., HU, X., BABU, J. R., SEIBENHENER, M. L., GEETHA, T., PAINE, M. G. & WOOTEN, M. C. 2006. Signaling, polyubiquitination, trafficking, and inclusions: sequestosome 1/p62's role in neurodegenerative disease. *J Biomed Biotechnol,* 2006**,** 62079.