Autophagy: Investigating the possible interaction between co-chaperone BAG3 and the Ubiquitin-binding protein p62

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1 ABSTRACT

Introduction: Autophagy is an evolutionary conserved intracellular catabolic process which involves the degradation of a cell's own components. The degradation happens in two (2) main pathways: the ubiquitin-proteasome system that degrades short-lived proteins, and the autophagy system that degrades long-lived proteins and damaged organelles. There are three known different pathways of autophagy being; (1) macroautophagy (2) microautophagy and (3) chaperone-mediated autophagy

The ubiquitin-binding protein p62 (SQSTM1) is the scaffold protein that binds to proteins that are to undergo macroautophagy, while BAG3 is known for its involvement in chaperone-mediated autophagy (CMA). Any possible interaction between Bag3 and p62 of which would indicate convergence of CMA and macroautophagy where p62 is a major factor

Aims: To investigate any interaction between BAG3 and p62 or their co-localization in HeLa cells.

Methods: Protein-protein interaction using GST pulldown and visualization of interaction with Coomassie blue staining and western blot. The investigation of co-localization was done through fluorescence confocal microscopy

Results: BAG3 does not co-localize with p62wt or p62 (R7A/D69A)/ p62 (R21A/D69A), but has some interaction and co-localization with p62 (d123-339) and p62 (124-440). This suggests that BAG3 interact with p62 in the region amino acids 339-440. This is the region containing LIR, KIR and UBA domains of p62. Surprisingly, we found interaction between the p62 deletions constructs p62 (d123-339) and p62 (124-440) but not with the wild type p62. This may lie in the fact that p62 forms polymers making the reaction sites unavailable, or that the small constructs of p62 do not fold properly and get bound to BAG3 for destination to CMA.
A further investigation is required using in vitro translation and radioactive analysis of BAG3 and p62 interaction, and also in vivo co-immunoprecipitation could be performed.

2 INTRODUCTION

Eukaryotic cells have developed efficient protein quality control systems and react to the presence of misfolded proteins. During protein synthesis, transcription of RNA from DNA to translation of proteins from RNA is well regulated to ensure accurate synthesis. As proteins are synthesized, they would undergo post-translational modification, in which amino acids are either acetylated, phosphorylated, methylated, carboxylated or hydroxylated and consequently would then fold either spontaneously or require assistance of certain molecules to fold into the correct functional conformation.

Cell viability is dependent on an efficient protein quality control system in which molecular chaperones, the ubiquitin-protease system (UPS) and the lysosomes play an essential role[(1)]. Protein quality control involving molecular chaperones recognize misfolded proteins thereby preventing their aggregation, and associated co-chaperones that modulate substrate sorting between renaturation and proteasomal degradation[(2)]. Those proteins that cannot be re-modified will then be degraded by the cell degradation systems. In eukaryotic cells, proteins are degraded via two (2) main pathways: the ubiquitin-proteasome system that degrades short-lived proteins, and the autophagy system that degrades long-lived proteins and damaged organelles [(3)].

2.1 Autophagy

Autophagy is an evolutionary conserved intracellular catabolic process involving the degradation of a cell’s own components to recycle nutrients, remodel and dispose of unwanted cytoplasmic constituents[(4)]. It is a tightly-regulated process that plays a normal part in cell growth, development, and homeostasis, helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products. It is a major mechanism by which a starving cell reallocates nutrients from unnecessary processes to more-essential processes. This ancient pathway, conserved from yeast to humans, is now emerging as a central player in the immunological control of bacterial, parasitic and viral infections. The process of autophagy may degrade intracellular pathogens, deliver endogenous antigens to MHC-class-II-loading compartments, direct viral nucleic acids to Toll-like receptors and

regulate T-cell homeostasis [5].There are three known different pathways of autophagy being; (1) macroautophagy (2) microautophagy and (3) chaperone-mediated autophagy[3, 6].

Macroautophagy is the sequestrating of organelles and long-lived proteins in a double-membrane vesicle, called an autophagosome or autophagic vacuole. Autophagosomes form from the elongation of small membrane structures while engulfing a portion of cytoplasm and then they fuse with lysosomes, thereby forming autolysosomes, where the cytosolic contents are degraded by lysosomal hydrolases (figure 1). In microautophagy, cytosolic components are transferred into the lysosome by direct invagination of the lysosomal membrane and subsequent budding of vesicles into the lysosomal lumen. Chaperone-mediated autophagy (CMA) is a proteolytic pathway that plays a role in protein quality control[7], and involves the chaperone molecules heat shock protein (Hsp) family groups Hsp70 and Hsp90 and co-chaperones. Co-chaperones modulate chaperone activity through conformational shifts[8]. The chaperone/co-chaperone complex binds to consensus peptide sequence then moves to the lysosomes, where the protein is degraded. In CMA the substrates are translocated across the lysosomal membrane on a one-by-one basis, whereas in the macroautophagy and microautophagy the substrates are engulfed or sequestered in-bulk. CMA is characterized by its selectivity regarding the specific degrading of only certain proteins and not organelles [9]. An efficient protein quality control involving the cooperative action of chaperones and co-chaperones is important in the presence of genetically mutated proteins that are prone to aggregation and that cannot be correctly folded.

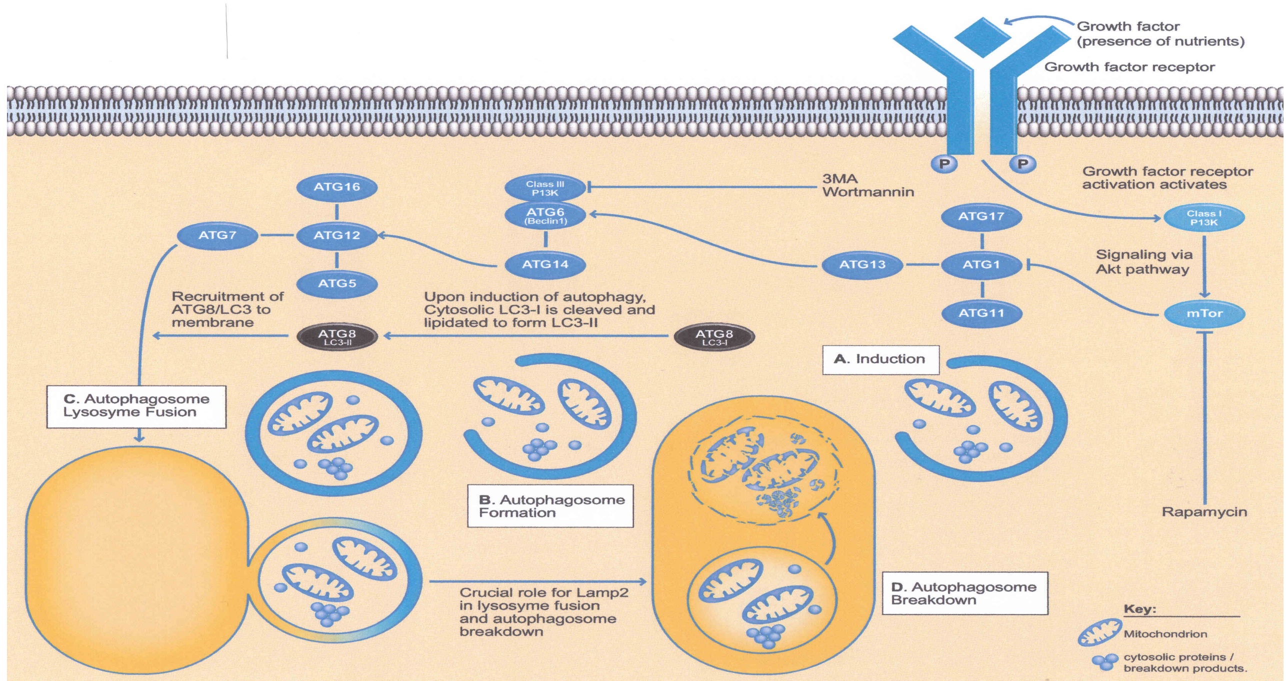

Figure 1: The Autophagic pathway: In the presence of adequate nutrients, growth factors are able to activate the Class I P13K proteins which in turn signal via the AKT pathway to activate mTOR. This leads to an inhibition of ATG1 - the key signal in autophagy induction. If there are inadequate nutrients or in the presence of mTOR inhibitors e.g. Rapamycin, then mTOR is not activated, and ATG1 is able to recruit ATG11, ATG13 and ATG 17 to form a complex which signals induction of autophagy. Formation of the autophagosome is dependent on the formation of 2 complexes - ATG6 (Beclin1) which interacts with the Class III P13K proteins complexes with ATG14, the second complex involves ATG12, ATG16, ATG5 and ATG7. This complex is critical for the recruitment of ATG8 (LC3). Upon induction of autophagy, cytosolic LC3-I (ATG8) is cleaved and lipidated to form LC3-II. LC3 is a marker for the autophagosome membrane. Fusion between the autophagosome and the lysosyme and subsequent breakdown of the autophagic vesicle is less well defined, though there is an essential role for the LAMP2 protein in this process (Adapted from www.abcam.com).

Autophagy is essential in helping to maintain the balance between the increase and decrease in the number of a cell population [3, 10]. It is active at a basal level in most cells and contributes to the routine turnover of cytoplasmic components. Three predominant cellular functions can be assigned to autophagy: (1) response to nutrient starvation (2) housekeeping process whereby long-lived proteins and organelles are recycled and (3) developmental processes like nucleus expulsion during erythrocyte development where autophagic process results in the functional biconcave shape.

Autophagy is generally activated by conditions of nutrient deprivation but has also been associated with physiological processes such as development, differentiation, neurodegenerative diseases, infection and cancer. During nutrient deficiency, autophagy

functions as a pro-survival mechanism; however, excessive autophagy leads to autophagic cell death, a process morphologically distinct from apoptosis.

Autophagy is important for the degradation of bulk cytoplasm, long-lived proteins, and entire organelles [(3)].

2.2 The ubiquitin-binding adaptor/scaffold protein/Sequestosome 1(p62)

The ubiquitin-binding protein p62 (SQSTM1) is evolutionary conserved in eukaryotic cells, and functions as a scaffold protein in a range of signaling pathways associated with cell stress[(11)], survival and inflammation and also controls transcriptional activation and protein recruitment to endosomes [(12)]. The p62 protein was initially identified as a protein that binds to SH2 domain of the tyrosine kinase Lck [(13)]. It functions as a receptor protein for aberrant proteins (ref). The protein contains a PB1 domain at its N-terminus followed by a ZZ-type zinc-finger motif, a LC3 interacting region, and a UBA domain at its C-terminus (figure 2) [(14)]. The p62 interacts with ubiquitin through the UBA domain, and self-assembles through PB1 domain to form large protein aggregates [(15)].

In the ubiquitin-proteasome system, p62 acts as a shuttling factor that transports ubiquitinated proteins to the proteasome, by interaction through the PB1 domain with the proteasome subunits [(13)]. The p62 binds to ubiquitin and ubiquitin-like protein LC3 linking substrate sequestration and the recruitment of proteins to autophagy [(11, 16, 17)]. The p62 also works as a key factor in cell signaling transduction (ref). In the NF-kB signaling pathway, p62 controls osteoclastogenesis, T-cell differentiation and tumor progression [(14)].

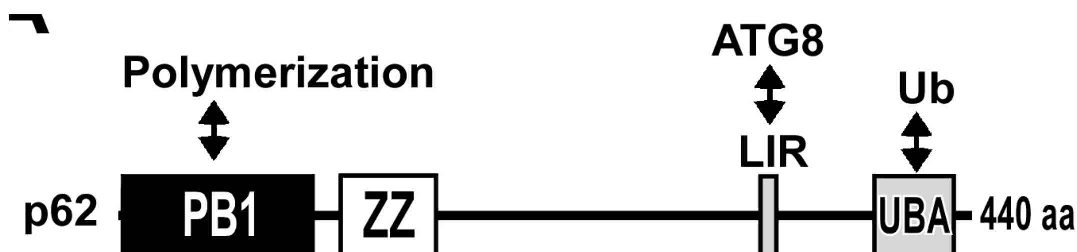

Figure 2: p62 protein with domains PB1, ZZ, LIR and UBA

2.3 Bcl-2-associated Athanogene (BAG) 3 protein

BAG3 is a 74 kDa protein with 575 amino acids belonging to the evolutionary conserved family of Bcl-2-associated athanogene (BAG) domain containing proteins [(18)]. It is constitutively expressed in muscle cells, a few other normal cell types and in some tumors. BAG3 is a cytoplasmic protein concentrated in rough endoplasmic reticulum, but a nuclear localization can be observed in some cells. A family of co-chaperone proteins that share the BAG domain are characterized by their interaction with a variety of partners (heat shock proteins, steroid hormone receptors, Raf-1 and others), involved in regulating a number of cellular processes[(18)], including proliferation and apoptosis [(19)].

BAG3 contains in addition to BAG domain a WW domain and a proline rich region with SH3-binding motifs, suggesting that it may interact with proteins relevant to signal transduction, recruiting Hsp70 to signaling complexes and altering cell responses [(20)]. Binding of the BAG domain to the ATPase domain of Hsp70 regulates the ATP-driven activity of the chaperone complex [(1)]. Through the proline rich repeat BAG3 appears to be involved in regulating cell adhesion and migration by forming an epidermal growth factor through binding to SH3 domain of PLC-gamma[(20)]. BAG3 in cooperation with sHsp regulates protein degradation that affects many cells. Protein degradation through both the proteasome and autophagy pathway is regulated by BAG3 and its interactions [(21)].

Bag3 as co-chaperone proteins act as a molecular switch mechanism to determine whether proteins are degraded in proteasomal or autophagic manner. BAG3, also known as CAIR-1 or Bis[(22)], forms a complex with the heat shock protein (Hsp) 70[(23)] assisting polypeptide folding and mediate protein delivery to proteasome and is able to modulate apoptosis by interfering with cytochrome c release (figure 3), apoptosome assembly and other events in the death process [(18)]. Over-expression of BAG3 interferes with the degradation of polyubuquitinated Hsp70 clients' proteins and participates, along with HspB8 in the degreadation of misfolded and aggregated proteins via macroautophagy [(1)].

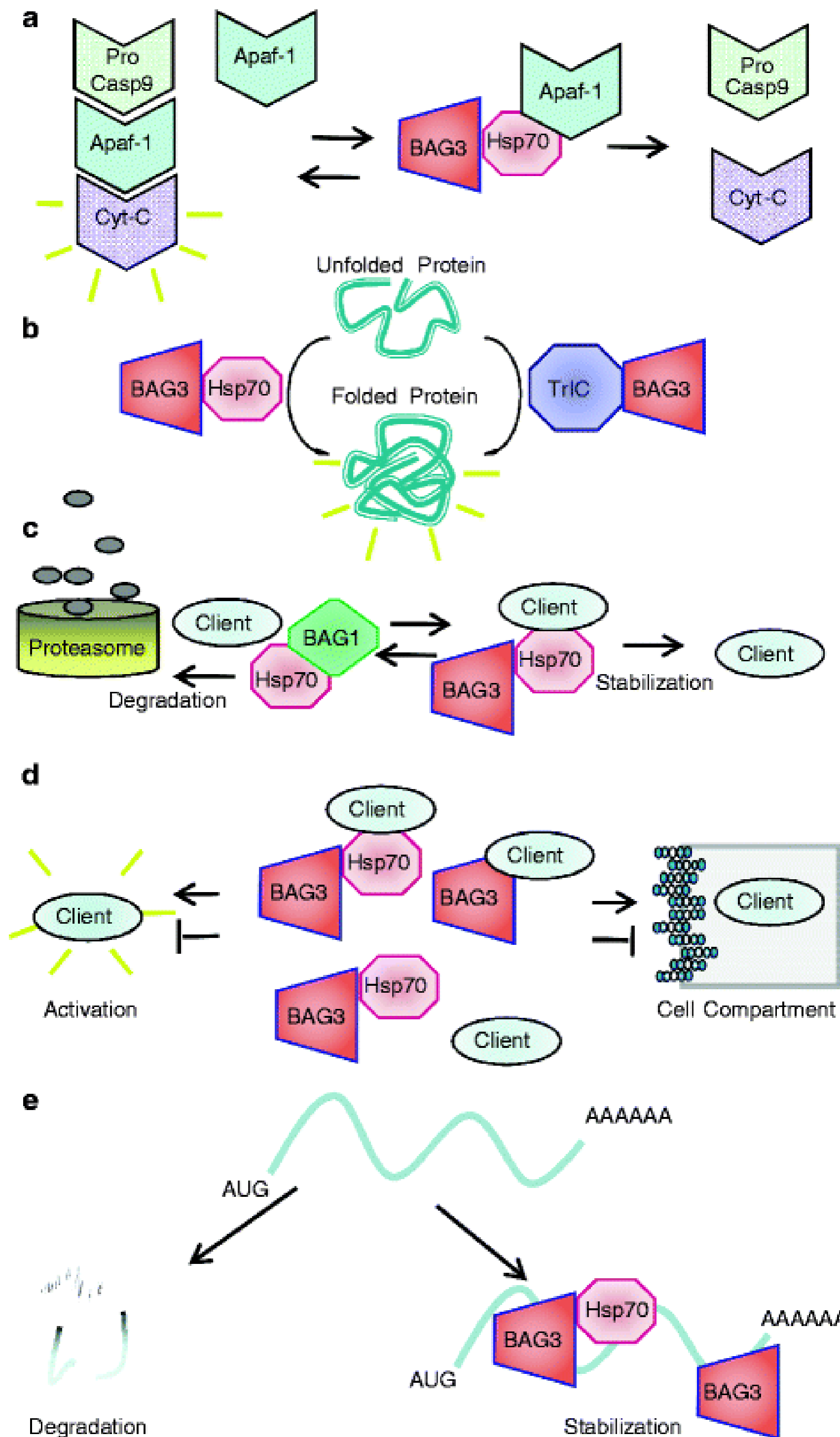

Figure 3: Functions of BAG3 co-chaperone in cooperation with Hsp70 chaperone protein[(24)], a) cell signaling through cytochrome c, b) protein control of misfolded proteins c) protein degradation through proteasome modulated by BAG3, while BAG1 modulate stabilization of client protein d) protein compartmentalization e) protein stabilization

The expression of BAG3 is stimulated during cell response to stressful conditions, such as exposure to high temperature, heavy metals, and certain drugs [(18)]. BAG3 protein has been reported to modulate apoptosis in human leukemia and is specifically expressed in thyroid

carcinomas and not in normal thyroid tissue or goiter [(25)]. Bag3 is also involved in protein quality control in aging. The specific role of BAG3 in protein degradation during the aging process is to regulate macroautophagic pathways, for the degradation of polyubiquitinated proteins [(26)]. A recent study has demonstrated that in aged cells a switch from BAG1 to BAG3 resulted in increased macroautophagy for turnover of polyubiquitinated proteins [(1)].

There are indications that BAG3 through the HspB8-Bag3 complex can initiate macroautophagy [(21)]. In the complex HspB8 is responsible for recognizing misfolded proteins, whereas Bag3 stimulate macroautophagy deciding the fate of chaperone-bound substrates. Protein degradation through both the proteasome and autophagy pathways is regulated by Bag3 and its interactions.

2.4 KEAP1 and LC3 relation to p62

Kelch-like ECH protein 1 (keap1) is primarily a cytoplasm protein that interact with p62 through the keap1 interacting region (KIR) motif on p62 [(17)]. There is also a specific interaction between p62 and Light Chain 3 (LC3) through the LC3 interacting region (LIR) motif found on p62. In this study keap1 and LC3 were used as positive controls [(17)].

2.5 Autophagy and Disease

The autophagic response has been described in various pathophysiological situations, including neurobiology, cancer and cardiovascular disease[(27)]. Studies have shown that autophagy plays a role in the removal of misfolded proteins which accumulate in the cell, such as the polyglutamine aggregates formed in Huntington's chorea[(3)].

While autophagy is required for homeostasis in all cell types, non-dividing cells, such as neurons, are particularly sensitive to changes in autophagic degradation. Most of neurons must survive for the lifetime of the organism, and the maintenance of organelle function and clearance of aberrant or damaged proteins are critical processes regulated by autophagy[(28)]. Cardiac myocytes are also terminally differentiated cells, so correct functioning of the autophagic pathway is essential for maintenance of myocyte homeostasis.

Extensive data suggest that such aggregate-prone proteins mediate toxicity primarily via gain-of-function mechanisms associated with their propensity to aggregate[(29)]. As autophagy is necessary for the clearance of aggregate-prone proteins that are toxic, the concept of autophagy failure as a mechanism predisposing to cell death is relevant to pathogenesis in a range of diseases. The dysfunction in autophagy has been implicated in the pathogenesis of various diseases, like cancer, infectious diseases and neurodegenerative disorders [(16, 30)].

Defects in autophagy would result in impaired recycling of damaged organelles and degradation of long-lived proteins and aggregates resulting in their accumulation in the cell hence interfering with the normal function of the cell processes [(31, 32)]. One of the common pathological features of most adult-onset human neurodegenerative diseases is the formation of intra-cytoplasmic aggregates within neurons and other cell types [(3)]. This is seen in Alzheimer disease, in tauopathies where tau accumulates in the cytoplasm, in Parkinson disease where synuclein is the major component of the aggregates [(33)], and in polyglutamine expansion diseases like Huntington disease, where the mutant protein is the primary constituent of the aggregates [(29, 34, 35)].

The recently discovered HspB8-Bag3 complex[(2)] participates in protein quality control through a mechanism that requires the activation of the eIF2alpha signaling pathway and that leads to protein synthesis inhibition and autophagy stimulation. Both processes help to protect the cells against the accumulation of aggregate-prone proteins, which may be relevant in many protein-conformational neurodegenerative disorders [(36)]. It has been reported that over-expression of the HspB8-Bag3 chaperone complex suppresses mutated huntingtin aggregation via autophagy. HspB8 and Bag3 induce the phosphorylation of the alpha-subunit of the translation initiator factor eIF2, which in turn causes a translational shut-down and stimulates autophagy [(37)]. HspB8 forms a stable complex with Bag3 in cells and that the formation of the complex is essential for the activity of HspB8. The HspB8 activity is intrinsically dependent on Bag3, a protein that may facilitate the disposal of doomed proteins by stimulating macroautophagy [(38)]. It has been shown that the proline-rich region of Bag3 is essential for the Bag3-mediated stimulation of mutated huntingtin clearance [(2)].

Besides the physiological function of autophagy in maintaining cellular homeostasis, autophagy is a newly recognized facet of innate and adaptive immunity[(4)], but certain viruses such as hepatitis C virus (HCV) and hepatitis B virus (HBV) have developed strategies to

subvert or use autophagy for their own benefit [39]. Several studies have assessed the autophagic pathway in hepatocytes infected with HCV both in vitro and in liver biopsies from chronic hepatitis C patients and these studies consistently demonstrated an accumulation of autophagic vacuoles in HCV-infected hepatocytes, but was not able to enhance autophagic protein degradation[40].

Chronic alcohol use may cause several types of liver injury: fatty liver (also called steatosis), hepatic fibrosis, cirrhosis and alcoholic hepatitis. There is suggestion that alcohol consumption suppresses liver cell autophagy [39]. It has been demonstrated that hepatocytes from patients with alcoholic steatohepatitis contain protein aggregates called Mallory-Denk bodies which are constituted by cytokeratins in association with other proteins including ubiquitin and p62 [13]. The decrease in protein catabolism likely contributes to the formation of Mallory-Denk bodies[39]. Recent studies have demonstrated that accumulation of p62 results in hyper-activation of the transcription factor (nuclear factor erythroid 2-related factor 2 (Nrf2) that causes liver changes such as hepatomegaly, liver cell swelling, and aminotransferase elevation [39].

In general, cancer has been genetically linked to autophagy malfunction. The regulation of autophagy overlaps closely with signaling pathways that regulate tumorigenesis, hence autophagy is a tumor suppressor mechanism [3, 13].

3 AIMS AND OBJECTIVES

Aim of study

Increased macroautophagic flux has been suggested to be regulated by Bag3 in concert with the ubiquitin-binding protein p62/SQSTM1. Gamerdinger et al [26] states that Bag3 mediated recruitment of the macroautophagy pathway is an important adaptation of the protein quality control system to maintain protein homeostasis in the presence of enhanced pro-oxidant and aggregation-prone milieu characteristic of aging. There is also a suggestion that HspB8/Bag3 complex stimulate macroautophagy[2].

The study aims to investigate any interaction between Bag3 and p62 of which would indicate convergence of CMA and macroautophagy where p62 is a major factor. This would suggest selective autophagy.

Objectives: To investigate the interaction of Bag3 protein a co-chaperone with p62 an autophagy-receptor protein by studying their protein-protein interaction and co-localization in mammalian cell.

4 MATERIALS AND METHODS

4.1 Materials

Table 1: Entry and Destination vectors used in cloning

Name	Expression in	Supplier	Antibody resistance
p-DEST 15	E.coli	Invitrogen	Ampicillin
p-DEST-EGFP	mammalian		
p-DONR 221	E.coli	Invitrogen	Ampicillin

Table 2: Primary antibodies for Western Blotting

Name	Dilution	Supplier
Mouse-anti-MBP	1:3000	Santa Cruz Biotechnology
Rabbit-anti-GST	1:3000	Santa Cruz Biotechnology
Mouse-anti-Biotin	1:2000	Santa Cruz Biotechnology

Table 3: Secondary antibodies for Western Blotting

Name	Dilution	Supplier
Anti-rabbit IgG-HRP	1:2000	BD Pharmingen™
Anti-mouse IgG-HRP	1:2000	BD Pharmingen™

Table 4: Restriction enzymes

Name	Supplier
BsrGI	New England BioLabs
Hind III	New England BioLabs

Table 5: Clones used in this study

Clone Name	Resistance Marker	Constructed by/Supplier
pTH1	Amp	Invitrogen
pDestTH1-p62(wt)	Amp	A.Jain
pTH1-p62 (R21A/D69A)	Amp	T.Lamark
pTH1-p62 (124-440)	Amp	T.Lamark
pTH1-p62(D123-319)	Amp	T.Lamark
pDEST15-keap1	Amp	A.Jain
pDEST15-LC3	Amp	

Table 6: Theoretical size of the translated proteins

Protein	Theoretical MW(kDa)
GST	~26
GST-BAG3	~100
GST-Keap1	~95
GST-LC3	~44
MBP	~42
MBP-p62wt	~104
MBP-p62 (R21A/D69A)	~104
MBP-p62 (d123-319)	~84
MBP-p62 (124-440)	~77

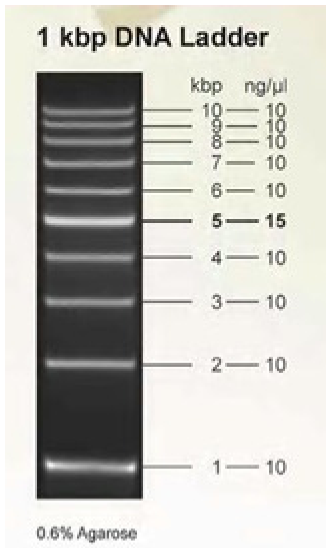

Figure 4: 1 kbp DNA ladder

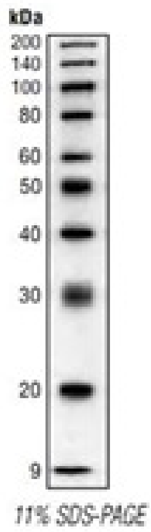

Figure 5: Biotinylated Protein Ladder

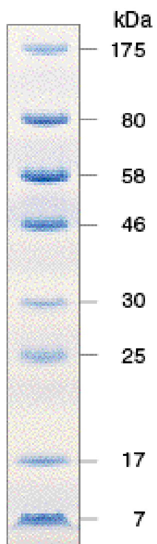

Figure 6: Pre-stained protein marker (New England Biolabs)

4.2 General methods

4.2.1 Transformation of competent Escherichia Coli

Transformation is the genetic alteration of bacteria by introducing foreign or recombinant DNA.

Competent cells were thawed on ice. A proper amount of plasmid was added to 120 µl competent cells, and the solution was kept on ice for 20 min followed by 50 seconds heat-shock at 42 degrees in a water bath. The transformation mixture was then kept on ice for 2 min. 780 µl SOC medium was added and the mixture was incubated at 37 ºC with shaking for 60-90 min. Mixture was then spun down in a microcentrifuge at 5000 rpm for 10 min. The pellets were resuspended in 100 µl SOC medium and spread on an agar plate (LA plate) with ampicillin (100µg/ml).The plate was incubated at 37 ºC overnight. Colonies on the plate the following day, indicate a successful transformation. Some colonies (one colony per tube) were transferred to tubes containing 5 ml of LB+ amp media, and were incubated with shaking at 37 degrees overnight.

Purification of the plasmids was done after each transformation by following the Plasmid mini Kit protocol. To verify if transformation was successful the purified plasmids were test cut with restriction enzymes and then run on an agarose gel.

SOC medium

20 g Bacto Trypton

5 g Bacto yeast extract

0.5 g NaCl

10 ml 250 mM KCl

Add dH_2O to 1 liter

Adjust pH to 7.0 with NaOH

Supplemented prior to use with 5 g $MgCl_2$

20 mM glucose

LA plate

10 g Bacto trypton

5 g Bacto yeast extract

10 g NaCl

Add dH_2O to 1 liter

Adjust pH to 7.5 with NaOH

LB (Luria-Bertani) medium

10 g Bacto trypton

5 g Bacto yeast extract

10 g NaCl

Add dH_2O to liter

pH adjusted to 7.5 with NaOH

4.2.2 Plasmid Purification

Plasmid purification is a method of isolating plasmids from a bacterial culture.

Plasmid miniprep

This method can be used to quickly check whether the plasmid is correct in any of several bacterial clones. The yield is a small amount of impure plasmid DNA, which is sufficient for analysis by restriction digest and for some cloning techniques. The plasmid mini kit 1 (Omega bio-tek) was used. The kit uses the alkaline-SDS lysis method of bacteria and columns containing a Hibind matrix that specifically binds DNA under the conditions used.

From the overnight cultures – 1.5 ml was transferred to eppendorf tubes. The tubes were centrifuged at 13000 rpm for 1 min, the supernatant was discarded, 250 µl of solution I added then tubes were vortexed. 250 µl solution II was added and tubes flipped upside down 4-6 times. The tubes were incubated at room temperature for 2 min to ensure complete lysis. 350 µl of solution III was added, the tubes flipped upside down a few times before centrifugation at 13000 rpm for 10 min. The supernatant was transferred to the HiBind DNA micro columns following the kit, placed in collection tubes. The columns were centrifuged at 13000 rpm for 1 min. The flowthrough was discarded and the columns washed with 500 µl Buffer HB. The

columns were centrifuged at 13000 rpm for 1 min. The flowthrough was discarded and the columns were centrifuged at 13 000 rpm for 1 min. The flowthrough was discarded and ethanol added. The columns were centrifuged at 13000 rpm for 1 min. The flowthrough was discarded and the columns were centrifuged at 13000 rpm for 1 min to dry the columns. The columns were placed in new eppendorf tubes, 50 µl of Elution Buffer was added and the columns centrifuged at 13000 rpm for 1 min to release the DNA from the columns.

TE buffer

10mM TrisHCl-pH 8.0

1mM EDTA

4.2.3 Determination of DNA concentration

The concentration of DNA extracted usingthe Plasmid Miniprep procedure was measured by using a NanoDrop ND-100 Spectrophotometer.

4.2.4 Agarose gel Electrophoresis

Agarose gel electrophoresis separates DNA fragments based on size and enables determination of the sizes of DNA fragments. Agarose is a linear polysaccharide that polymerizes into rigid gel with pores. The concentration of agarose in the gel determines the size of these pores. A current will be applied to the gel and the negatively charged DNA fragments will moved towards the positive electrode. The smallest DNA fragments will be closer to the positive electrode since they move more easily through the agarose pores than larger DNA fragments.

A 1 % agarose solution was made by adding 0.5 g agarose to 50 ml 0.5 x TBE buffer. This was cooked in a microwave oven to boiling point. When the agarose was dissolved the solution was cooled to ~ 60 ºC and 2.5 µl EtBr (ethidium bromide) was added. The solution was poured into a casting frame with a comb to make wells. The gel was left to polymerize for ~ 40 min. The gel was then placed in a tray and 0.5 x TBE was added until the gel was covered completely. The samples to be run were mixed with 1 µl 6 x T buffer (gel loading

buffer) for each 5 µl DNA solution and loaded into the wells. 6 µl 1 kb PLUS ladder was placed in the first well as standard to be able to determine the sizes of the DNA fragments in the other lanes. The gel was run at 90 V for ~ 45 min.
The bands in the gel were visualized by exposing the gel to UV light 302 nm using Gel Logic Imaging System with Kodak GL 200 Camera (Kodak)

0.5 x TBE-buffer
5.4 g Tris-HCl
2.75 g Bromic acid
2 ml 0.5 EDTA, pH 8.0
Add dH_2O to 1 liter

6 x T gel loading buffer
0.25 % Bromophenol blue (Baker)
60 mM EDTA pH 8.0
0.6 % SDS
40 % (w/v) sucrose
Sterile filtered

1kb PLUS ladder
1 µl 1 kb PLUS stock (1.0 µg/µl) (Invitrogen)
24 µl TE buffer, pH 8.0
5 µl 6 x T gel loading buffer

4.2.5 Preparation of freeze stocks

Freeze stocks are created to store bacterial cultures after being transformed with a specific plasmid. This enables later enrichment of larger volumes of bacterial cultures followed by isolation of their plasmids.

The following components were mixed into a sterile 2 ml tube
1200 µl overnight culture
300 µl 50 % glycerol
The freeze stocks were kept at -70 ºC.

4.3 Recombinant DNA technology

4.3.1 Digestion of DNA with restriction enzymes

Restriction enzymes recognize specific DNA sequences and cut double stranded DNA at or close to their recognition sequence. The enzyme makes incisions, one through each of the phosphate backbones of the double helix without damaging the bases. Restriction cutting of plasmids with site specific restriction enzymes enables investigation of whether the correct plasmid has been purified or not.

Following components were mixed in a 1.5 ml eppendorf tube

200-300ng plasmid

1 µl restriction enzyme

2 µl 10 x enzyme specific buffer

Add dH_2O to a total volume of 20 µl

The digestion mix was incubated in a 37 ºC water bath for 60-90 min. 10 µl of the digestion mix was mixed with 2 µl 6 x T gel loading buffer and run on an agarose gel.

4.3.2 Polymerase chain reaction

PCR is method used to amplify a specific DNA sequence. Two primers complementary to the 3' end of the desired sequence, polymerase, free nucleotides, polymerase buffer and water are mixed. The mixture is heated to around 95 C, which will denature the double stranded DNA. The temperature is reduced to 55-60 C for the primers to anneal to the DNA strands. The temperature is then raised to 72 C for the polymerase to add nucleotides to the 3' end of the primers. The cycle is repeated as many times as necessary to obtain the desired amount of PCR product.

PCR program

1. 94 °C 2 min
2. 94 °C 30 sec
3. 55 °C 30 sec
4. 72 °C 1 min
5. Go to step 2 39 times

6. 72 °C 5 min
7. 4 °C forever

4.3.3 GATEWAY cloning technology

Gateway cloning technology is a powerful methodology that greatly facilitates protein expression, cloning of PCR products, and analysis of gene function by replacing the restriction endonucleases and ligase with site recombination. The technology provides a versatile system for transferring DNA segments between vectors. DNA can be transferred from an Entry Clone into numerous vectors or from the Expression vector back into Entry Clones. There are two (2) reactions that constitute GATEWAY cloning system, being LR reaction and BP reaction. The LR reaction is a recombination reaction between an Entry Clone and a Destination vector (pDest), mediated by LR clonase mix of recombination proteins. This reaction transfers DNA segments in the Entry Clone to the Destination vector, to create an Expression Clone. The BP reaction is essentially the reverse of the LR reaction were the gene in the Expression Clone is transferred into a Donor vector, to produce an Entry Clone. The reaction is catalyzed by BP Clonase mix of recombination proteins.

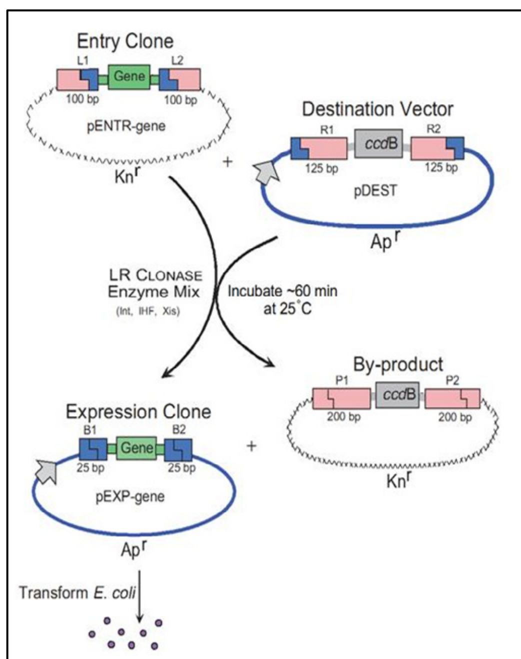

Figure 7: **GATEWAY Cloning Reactions: The LR Reaction.** An Entry Clone, containing a gene flanked by recombination sites, recombines with a Destination Vector to yield an Expression Clone and a by-product plasmid. The result is that a gene sequence in the Entry Clone is transferred into an Expression Vector, donated by the Destination Vector. The by-product plasmid contains the *ccd*B gene, and hence gives rise to no colonies when using standard strains of *E. coli* (Ap^r- ampicillin resistance, Kn^r- kenamycin resistance) (Adapted from Life Technologies, www.lifetech.com/gateway)

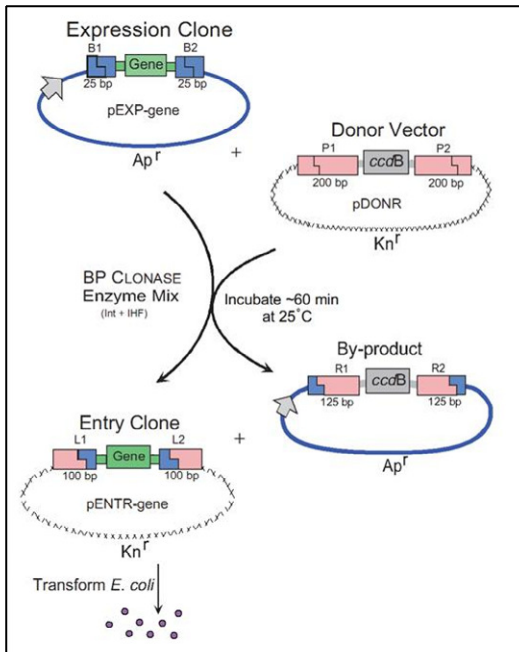

Figure 8: **GATEWAY Cloning Reactions: The BP Reaction.** A gene in an Expression Clone can be transferred into an Entry Vector by the BP Reaction. Only plasmids without the *ccd*B gene that are also kanamycin resistant (Knr) will yield colonies.

(Adapted from Life Technologies, www.lifetech.com/gateway)

In this study an LR reaction was performed with an Entry clone pDONOR 221-BAG3 and a Destination vector pDEST15 to produce an Expression vector. For the mammalian expression route the pDest EGFP destination vector was used together with the Entry Clone pDONOR 221-BAG3.

Creating Expression Clones (Transferring Genes from Entry Clones to Destination Vectors) via LR Reaction

The reaction of an Entry Clone with a Destination vector creates a new Expression Clone

Materials needed

- LR reaction buffer
- Destination vector ~ 150 ng/µl
- Entry Clone
- LR Clonase Enzyme mix
- Proteinase K solution
- Competent cells (DH5α)
- S.O.C medium
- LB plates containing 100µg/ml ampicillin

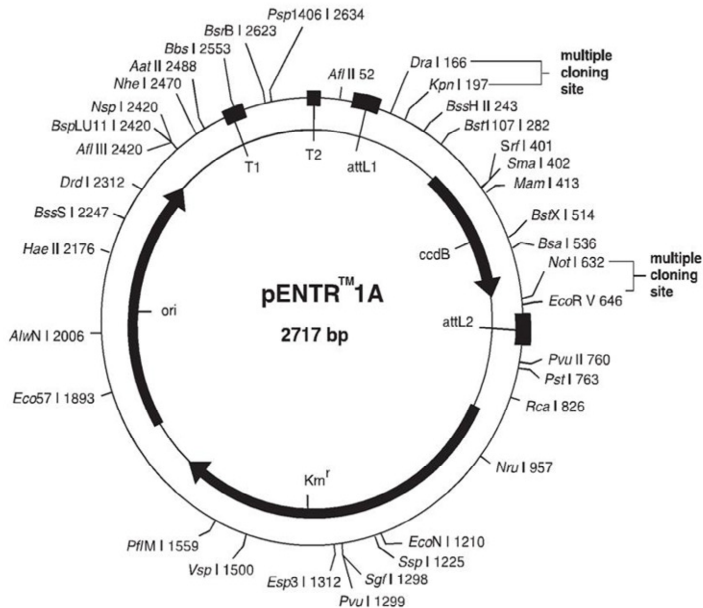

Figure 9: Map of a typical Entry Vector: all entry vectors consist of the same vector backbone but differ in the sequences and cloning sites

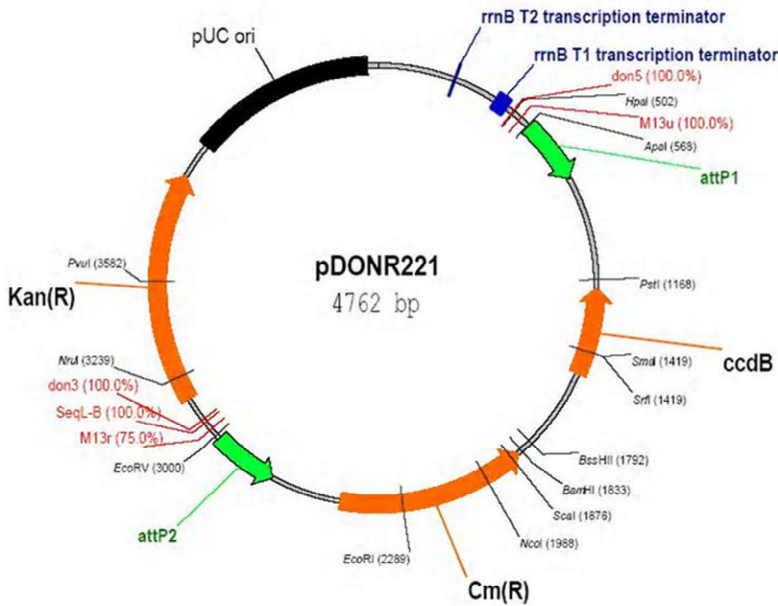

Figure 10: Map of pDONOR221

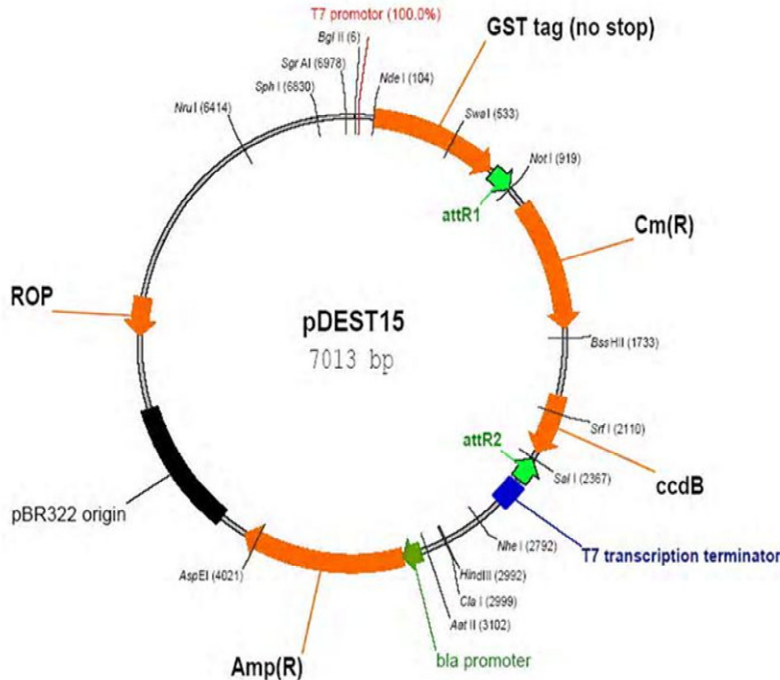

Figure 11: Map of pDEST15

4.4 Protein Methods

4.4.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE Gels are gel matrices which are used to separate proteins by size in the presence of electric current. Low percentage gels separate larger proteins whereas higher percentage gels separate smaller proteins better. To neutralize the charges on the proteins, SDS-loading buffer is added to the protein samples. SDS binds to proteins every few amino acids and neutralizes the charge differences that proteins have. This allows proteins to be separated by size and not by charge

The amount of sample proteins to be separated determines the size of combs to be used on the gel to create wells where the samples would be added. A separating gel (10 % acrylamide) is used at the bottom, and a concentrating gel (4% acrylamide) is used to pack proteins in together after loading. Polymerization of the gels is increased by the catalysts APS (Ammonium persulfate) and TEMED which speed up the polymerization reaction (formation and solidification) of the polyacrylamide gel. Every sample added to SDS-loading buffer is carefully and slowly added to prevent leaking out of the lane. A pre-stained protein marker with SDS-loading buffer is also loaded to the first lane well to provide protein sizemeasure.

2x SDS-PAGE loading buffer:

4% SDS

100mM Tris, pH 7.6

0.2% Bromophenol Blue

20% Glycerol

200mM DTT

Electrophoresis buffer:

3.03 g Tris base

14.4 g Glycin

1.0 g SDS

Dissolve in deionized water and fill to 1000 ml

SDS-PAGE separating Gel (10%):

4.9 ml water

2.5 ml acryl amide (40 %)

2.5 ml separating gel buffer (1.5 M Tris, pH 8.8 plus 10% SDS)

10 µl TEMED (TetraMethylEthyleneDiamine)

100 µl 10% APS (Ammonium persulfate)

Total volume: 10 ml

SDS-PAGE concentrating Gel (4 %):

6.4 ml water

1.0 ml acryl amide (40 %)

2.5 ml concentrating gel buffer (0.5 Tris pH 6.8 plus 10% SDS)

10 µl TEMED

100 µl 10% APS

4x separating gel buffer

181.65g Tris-base

4g SDS

Add dionized water to 1000ml

Adjust pH to 8.8 with HCL

4x concentrating gel buffer

60.55g Tris-base

4g SDS

Add deionized water to 1000ml

Adjust pH to 6.8 with HCL

4.4.2 Coomassie blue Staining of proteins in SDS-PAGE gel

Coomassie blue staining of proteins in polyacrylamid gels allows for visualization of the protein bands. The coomassie dye binds non-specifically to virtually all proteins.

After electrophoresis, the SDS-PAGE gel is removed from the electrophoresis apparatus and place in a petri-dish. The gel is fixed with gel-fixing solution on a table shaker for 30 min. The gel-fixing solution is removed, and then coomassie blue stain is added, the petri-dish is placed back on the table shaker for 1 hour. The staining solution is removed, and Destain I is added and the petri-dish is place again on the table shaker for 30 min. Thereafter Destain I was removed and Destain II was added and the gel incubated not less than 45 min. The gel can stay on Destain II for a longer period. The gel was photographed using a Gel Logic 200 Imaging System with Kodak GL 200 Camera.

Gel-fixing solution:

40% methanol

10% acetic acid

50% water

Coomassie Blue Stain:

0.125% coomassie blue R-250 dye

50% methanol

10% acetic acid

39.875% deionized water (dH2O)

Destain Solution I:

50% methanol

10% acetic acid

40% dH2O

Destain solution II:

5% methanol

7% acetic acid

88% dH2O

4.4.3 Western Blot

Western blotting is a technique used to identify and locate proteins based on their ability to bind to specific antibodies. The technique can detect a protein of interest from a mixture of a great number of proteins. This would give information about the size of the protein with comparison to a size marker or ladder in kDa, and also give information on protein expression

Western blot analysis can analyze any protein sample whether from cells or tissues, but also can analyze recombinant proteins synthesized in vitro. Western blot is dependent on the quality of antibody that is being used to probe for protein of interest, and how specific it is for the protein.

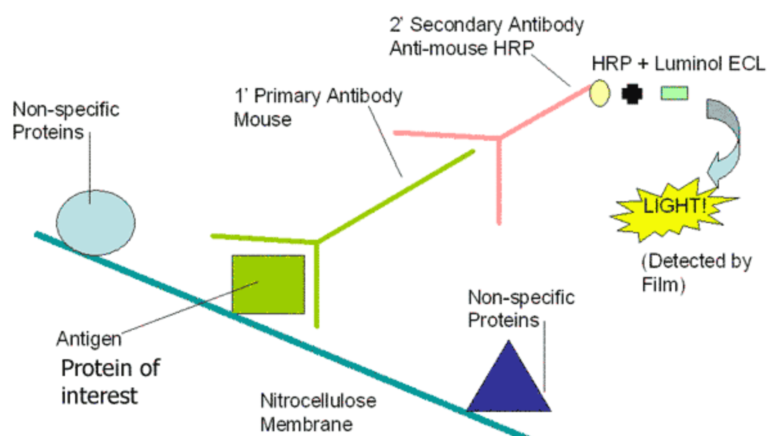

Figure 12: A primary antibody binding to a protein of interest on the membrane, and a secondary antibody binding to the primary antibody. The secondary antibody is then attached to a luminating chemical that when exposed to light can be detected on a film. (Adapted from http://www.molecularstation.com/protein/western-blot/#1)

Samples are prepared from tissues or cells that are homogenized in a buffer that protects the protein of interest from degradation. The sample is separated using SDS-PAGE and then

transferred to a membrane for detection. The membrane is incubated with a generic protein (such as milk proteins) to bind to any remaining sticky places on the membrane. A primary antibody is then added to the solution which is able to bind to its specific protein. A secondary antibody-enzyme conjugate, which recognizes the primary antibody, is added to find locations where the primary antibody bound.

Towbin buffer

6.06 g Tris-base

28.8 g glycine

400 ml Methanol

Add 1.6 L water

5% dried milk solution

2.5g dried milk (Mager Pulver by Saliter)

50 ml TBS-T

TBS-T

75 ml 2M NaCl

10 ml 1M TrisHCl pH 8.0

1 ml Tween20

914 ml Deionized water

Western Blot Procedure

After proteins to be analyzed have been run on SDS-PAGE, along a biotinylated protein ladder (Cell Signaling), the proteins are transferred to a membrane of the size 6.5 cm x 8.5 cm. The membrane is pre-soaked together with 2 filter papers and 2 sponges in Towbin buffer. The membrane, gel filter papers and sponges were placed as shown below:

Western Blot transfer order:

(-) *electrode side*

sponge on black

filter paper

Gel

Nitrocellulose (membrane)

filter paper

sponge

(+) *electrode side*

The sandwich was then locked inside a holder and placed in a blotting chamber with the membrane towards the positive electrode. Blotting was done at 100V for 2 hours in Towbin buffer

Western Blot antibody detection after protein transfer

When transfer is completed the membrane is then blocked in a 5% dried nonfat milk solution for 1 hour. The blocked membrane is then transferred to 50 ml tube, and incubated with a 2 ml TBS-T with diluted primary antibody on a rotating wheel at 4°C overnight. The following day, the membrane is transferred to a tray and washed 4 times with 1xTBS-T for 5 min each. The membrane is then transferred again into a 50 ml tube and incubated in 2 ml TBS-T diluted with secondary antibody place on a rotating wheel for 1 hour. The membrane is thereafter washed 4 times 5 min each with 1xTBS-T.

750µl of solution A and 750µl solution B (Western Blot Luminol Reagent, Santa Cruz Biotechnology) are mixed on a tray and the membrane is soaked in for 1 min. The membrane is then wraped with plastic foil and the signals detected by the Lumi-Imager(TM) machine (Mannheim Boheringer).

4.4.4 Production of GST-fusion proteins and MBP-fusion proteins

The T7 late promoter system in E.coli was used to produce the GST-fusion proteins and the MBP-fusion proteins for the study. The T7 gene in the E.coli strain in under control of lac promoter. When adding Isopropyl-beta-D-thiogalactopyranoside (IPTG) to bacterial cultures, the synthesis of T7 RNA polymerase will be promoted. T7 RNA polymerase will bind the T7 promoter in the pDEST expression vector and drive the expression of the target cDNA.

Procedure

Freeze stocks of E.coli with plasmids that code for GST-fusion and MBP-fusion proteins were spread on LB agar plate with ampicillin and incubated overnight at 37 ºC. A colony was

picked and added to 5 ml LB+amp and incubated overnight at 37 ºC. 5ml overnight culture was then added to pre-warmed 100ml 2xTY and incubated at 37°C in a shaker until OD_{600} is between 0.5-0.9. When the required range of OD600 is reached, 1M IPTG (50µl to 100ml culture) is added and then incubated for protein expression for 3-4 hours at room temperature in a shaker. After incubation the culture were placed on ice for 5 min then centrifuged at 4°C at 5000 rpm for 10 min (Sorvall GST Rotator). The cells are re-suspended in 4ml ice-cold lysis buffer added lysozyme and DTT, and then kept on ice for 20 min. Thereafter the re-suspended solution is transferred into a 15 ml tube and 10% TritonX100 (150µl to 1.5 ml lysate) was added before the solution is frozen at 70°C.

Lysis buffer

50mM TrisHCl pH 8.0
250mM NaCl
1mM EDTA
10µl/ml 1M DTT
35µg/ml lysozyme
dH_2O

10% Triton-X100

10% triton X-100 (BDH chemicals Ltd) in 1xPBS

1x PBS^-

0.1mM Na-phosphate buffer, pH 7.2
0.7 % NaCl

4.4.5 Purification of GST-fusion and MBP-fusion Proteins

The frozen lysate is thawed then sonicated 8 times for 30 seconds each. The sonicated lysate is centrifuged at 10000 rpm at 4°C.

GST beads are prepared by washing with 1xPBS and the MBP beads are washed with MBP buffer. Thereafter the supernatant are added to the beads, with GST-tagged protein lysate to GST beads and the MBP-tagged protein lysate to MBP beads and then incubate at 4°C for 1

hour. The beads where then washed, GST-tagged washed with 1xPBS and MBP-tagged washed with MBP buffer.

2xSDS buffer and DTT were added to 10µl of beads (GST or MBP) then heated in boiling water for 5 minutes, then centrifuged and the proteins were run on SDS-PAGE and coomassie stained. The GST-fusion protein were stored on the beads at 4ºC while the MBP proteins were purified and stored at -20ºC

4.4.6 Elution of MBP-tagged fusion proteins

MBP buffer

20mM TrisHCl pH 7.4
200mM NaCl
1mM EDTA
dH2O
1mM DTT (Add fresh)
1:10 protease inhibitor cocktail (Add fresh)

MBP Elution buffer

20mM TrisHCl pH 7.4
200mM NaCl
1mM EDTA
10mM Maltose
1mM DTT (Add fresh)
1:10 protease inhibitor cocktail (Add fresh)

Procedure

1. Remove supernatant from the MBP beads with MBP-fusion proteins attached
2. Add MBP elution buffer, volume=volume of beads
3. Incubate at room temperature for 10 minutes, while shaking
4. Centrifuge at 13000 rpm for 10 seconds, and then transfer the supernatant into a new eppendorf tube for storage.

5. Repeat step 2-4

4.4.7 GST pulldown

GST pulldown is a method to study protein-protein interactions. Using recombinant DNA techniques GST fusion proteins can be constructed and expressed in E.coli, and recovered on the gluthathione-conjugated beads. The interaction between a GST fusion protein and another protein (in this study MBP fusion protein) can be studied by incubating the bead-bound GST fusion protein with a putative binding partner, followed by recovery of the complex on the beads by centrifugation and washing. Coomassie blue staining and western blot were used for detecting the proteins.

An appropriate amount of bead-bound GST-fusion proteins (4-10µl) were transferred to eppendorf tubes. The beads were washed 2 times with 500µl NET-N buffer and centrifuged after each wash at 13 000 rpm for 10 sec. After the fourth wash 100µl NET-N buffer was added to the beads. Eluate of MBP fusion proteins that were purified following the MBP purification method were added to the bead-bound GST fusion proteins and the beads were kept on a rotating wheel for 1 hour at 4ºC. The tubes were then centrifuged at 13000 rpm for 20 sec, and the supernatant were removed. The beads were washed five times with ice cold NET-N buffer. 12µl of 2xSDS loading buffer with 200mM DTT was added to the beads, the samples were place in boiling water for 5 min, then centrifuged for 10 sec at 13000 rpm and run on 10 % SDS-PAGE gel along with inputs of the MBP fusion proteins and a protein marker.

NET-N buffer (50 mM NaCl)
42.8 ml dH_2O
1ml 1 M TrisHCl-pH8.0
2.5 ml 2 M NaCl
2.5 ml 10 % NP-40
600 µl EDTA
600 µl EGTA

4.5 Mammalian Cell Culture Procedures

4.5.1 Cell Culture

HeLa cells were grown in a 5 % CO_2 humidified incubator at 37 degrees in Eagle's minimum essential medium (MEM) supplemented with 10 % Fetal Bovine Serum (FBS), penicillin and streptomycin

4.5.2 Splitting and determining of HeLa cells

HeLa cells need to be split to prevent the cells from detaching and dying. A proliferation HeLa cell in culture will divide every 21-22 hours. The cells stop growing when they are confluent due to contact inhibition. During the splitting the number of HeLa cells can be determined.

The MEM with 10 % FBS was removed by suction and the cells were washed twice with 10 ml preheated (37 ºC) 1 x PBS^-. 1 ml trypsin was added and distributed evenly over the attached cells. The culture flask was placed at 37 degrees for a couple of minutes to allow the cells to detach. The reaction was stopped by adding 9 ml preheated MEM with 10 % FBS.
Some of the cell solution was transferred to a Bucker's counting chamber. The amount of cells in one square was counted and the number of cells per ml was calculated by multiplying with 10^4. Depending on when the cells would be needed or when the next splitting would take place, a fraction (1/2, ¼, 1/8 or 1/16) of the cell solution would be transferred to a new culture and MEM with 10 % FBS added to 10 ml. The flask would be incubated at 37 ºC at 5 % CO_2 until next splitting.

4.5.3 Lipofectamine transfection

This is a method of introducing DNA into eukaryotic cells. It involves liposomes that are spheres of cationic lipids that will surround the DNA molecules to be introduced to the cells. The lipids will then fuse with the cell membrane releasing the DNA into the cell. The lipofectamine ™ Reagent and PLUS™ Reagent from Invitrogen were used.

A day before transfection, the HeLa cells were washed twice with 1x PBS^-, trypsinated with 1 ml trypsin and re-suspended in 9 ml MEM. The cells were counted using microscope. 10 000 cells were seeded in each well in 8-well slide plates in a total volume of 200 µl.

Miniprep of overnight cultures of E.coli with plasmids coding for mcherry-BAG3, EGFP-p62wt, EGFP-p62 (d123-339), EGFP-p62 (124-440) and EGFP-p62 (R7A/D69A) was done and the concentration measured for each purified plasmid DNA. 100ng plasmid was used for each plasmid for transfection. 8 eppendorf tubes were used, with 4 containing single plasmid, and the other for containing mcherry-BAG3 plus each of the EGFP-p62 variants. DME (Dulbecco's Modified Eagle Medium) without antibiotics which kept at 37ºC was then added to each tube resulting in a total volume of 25 µl and incubated for 10 minutes at room temperature. Another tube with lipofectamine and DME without antibiotic was also prepared, then added to the plasmid-DME mixture and incubated at room temperature for 15 minutes. The MEM from the cells on the 8-well-plate was removed and plasmid/lipofectamine mixture of 200µl was added to each well and then incubated at 37ºC for 2½ hours. The DME solution is then removed and 200 µl MEM + 10 % FCS was added and the plates were incubated at 37ºC overnight.

Trypsin in 1 % PBS^- (Phosphate Buffer Saline)
2,5 g/l trypsin
0,5 g/l Na_2EDTA
pH 7.5

4.6 Confocal Microscopy

Confocal microscopy was pioneered by Marvin Minsky in 1955 of which one would perform point-by-point image construction by focusing a point of light sequentially across a specimen and then collecting some of the returning rays [(41)]. This would enable one to obtain high resolution images of real-life objects. A confocal microscope creates sharp images of a specimen that would otherwise appear blurred when viewed with a conventional microscope allowing better observation of fine details and the possibility to build three-dimensional (3D) reconstructions of a volume of specimen [(41)]. With a confocal microscopy the images are

achieved by excluding most of the light from the specimen that is not from the microscope's focal plane. Only the in-focus light is detected, while the out-of-focus light is blocked out using a pinhole. A pinhole is placed at the image plane and an electronic detector is placed behind the pinhole allowing one point in the specimen to be focused upon time.

The majority of confocal microscopes image either by reflecting light off the specimen or by stimulating fluorescence from dyes applied to the specimen. In this study a multicolor fluorescence is used to distinguish between proteins within a cell which were imaged as separate colors.

The Zeiss LSM Image Browser can be used for

- Viewing, comparing, sorting and printing LSM 5 images
- Presenting LSM 5 images in the gallery, form or table mode in the LSM 5 database
- Changing image contrast and brightness
- Displaying LSM 5 images in superimposed and split image mode
- Displaying up to 8 channels simultaneously
- Assigning each channel a predefined color
- Set zoom (automatic fit, resize and zoom factors in power-of-two steps)

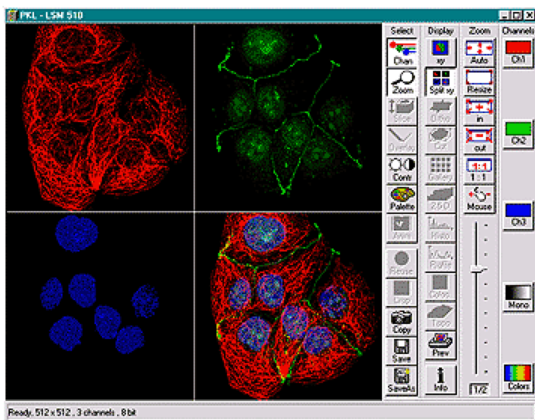

Figure 13: LSM 510 software program for viewing images from confocal microscope with efflorescence. Adapted from Carl Zeiss (www.zeiss.com)

4.5.4 Fluorescence tags – GFP and Cherry

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength [(41)]. In this study a green fluorescent protein (GFP) and a red fluorescent protein (mcherry) were used.

5. RESULTS

This study involves the analysis of the interaction between proteins p62 and BAG3 involving protein-protein interaction in GST pulldown and co-localization in HeLa cells using confocal microscope visualization.

5.1 Construction of BAG3 insert into plasmids for *E.coli* expression and Mammalian expression

LR reaction was performed with an entry clone pDONR 221-BAG3 and destination vector p-DEST 15 enabling bacterial expression of BAG3 fused to GST, and pDEST-EGFP for expression of BAG3 fused to GFP in mammalian cells. The generated vectors were verified with restriction enzyme digestion. pDEST-EGFP-BAG3 was digested with BsrGI, which should release the insert BAG3 of ~1700 base pair. pDEST15 was digested with Hind III, which should lineate the plasmid of ~5000 base pair. Figure 14 shows that both vectors were generated successfully.

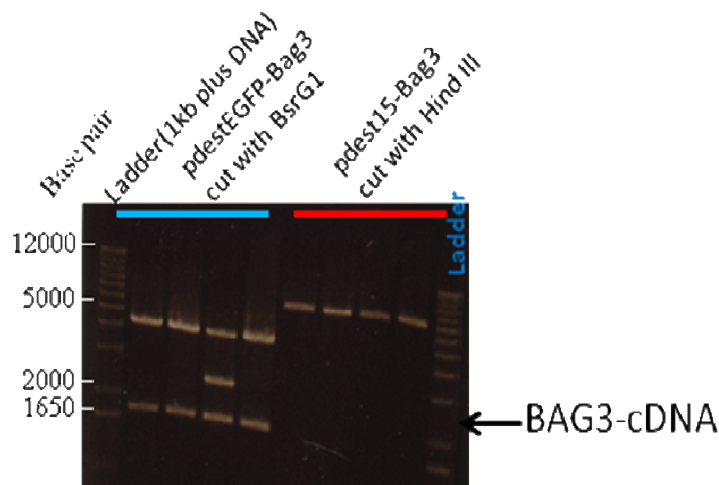

Figure 14: Restriction digestion of the isolated plasmids confirms that pDEST-EGFP-BAG3 and pDEST-BAG3 were successfully generated

5.2. Expression and Purification of GST and MBP tagged proteins from *E.coli*

Overnight cultures were prepared from already designed freeze stocks of p62 with its variants (p62 wild type, p62 (R21A/D69A), p62 (d123-339) and p62 (124-440)) together with the GST

as a negative control and GST-Keap1 as a positive control. GST-BAG3 was also cultured. The overnight cultures were induced by IPTG to produce proteins. GST tagged proteins were purified from the extract using glutathione sepharose beads, while p62 variants which were MBP tagged were extracted using amylose beads. After purification the MBP tagged proteins were eluted from the beads, while the GST tagged proteins were stored on the beads to be used in GST pulldown. The purified proteins were run on an SDS-PAGE gel to verify expression and purification. Figure 15 shows the successful expression of the proteins, except for Keap1 which has a lower MW than calculated.

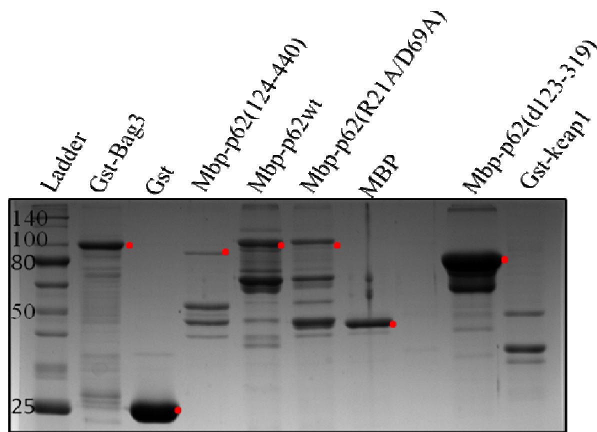

Figure 15: GST and MBP fusion proteins expressed and purified from *E.coli*. The red dots mark the extracted proteins. Almost all the proteins are at the expected theoretical molecular weight (MW), except for GST-keap1 which is at a much lower band than the theoretical MW. SDS-PAGE was done on the beads bound proteins after extraction.

The study of protein-protein interaction between proteins BAG3 and p62 involves the use of different p62 variants being: p62 wild type (p62wt), p62 (R21A/D69A) a mutant that is unable to dimerize, p62 (d123-339) which has deleted base pair from 123-339, and a small construct p62 (124-440). The smaller constructs are used to map the putative BAG3 interaction and to obtain better expression in *E.coli*. The full length p62 with MBP tag is large and is not well expressed in *E.coli*. Keap1 is shown to interact with p62 (Asish et al, 2010) and is here used as a positive control

5.3 No detectable interaction between BAG3 and p62 in a GST-MBP pulldown interaction

In order to test for any interaction between p62 and BAG3, GST pulldown assays were performed using GST and MBP tagged recombinant proteins. The MBP-fusion proteins were

added to GST, GST-BAG3 and GST-Keap1 immobilized on beads. After incubation, the beads were washed and then bound proteins resolved by SDS-PAGE. The page gels were stained by Coomassie to visualize the bound proteins. Figure 16 shows that there is no interaction between GST and the p62 variants (figure 16, lane 2-4). The positive control Keap1 shows interaction with p62 (d123-339) (figure 16, lane 9). There is no indication of interaction between BAG3 and the two p62 variant proteins (p62 (d123-339) and p62 (124-440)) as only the band of BAG3 at 100 base pair is prominent (figure 16, lane 5 and 6). These results indicate no interaction between BAG3 and p62.

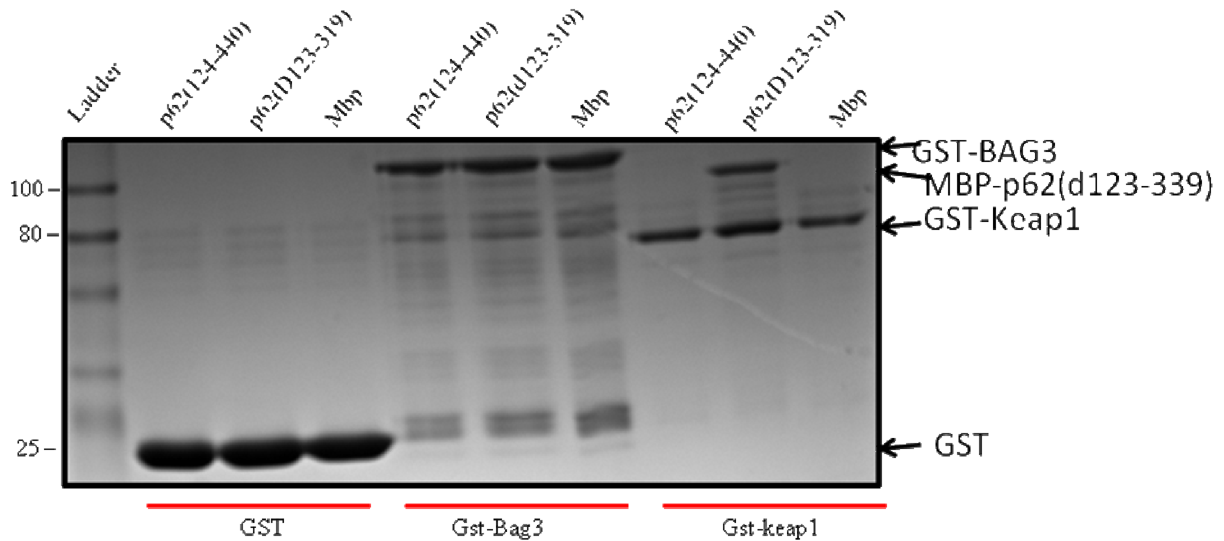

Figure 16: No detectable interaction between BAG3 and p62. GST is a negative control and Gst-keap1 a positive control. MBP is also used as a control as p62 proteins are tagged with MBP.

To further investigate the interaction between p62 and BAG3, a GST pulldown experiment was repeated with LC3 as a positive control for p62 interactions and here the full length of p62 were also used (p62wt and p62 (R21A/D69A)). Figure 17 shows that there is no interaction between p62 and BAG3 could be detected.

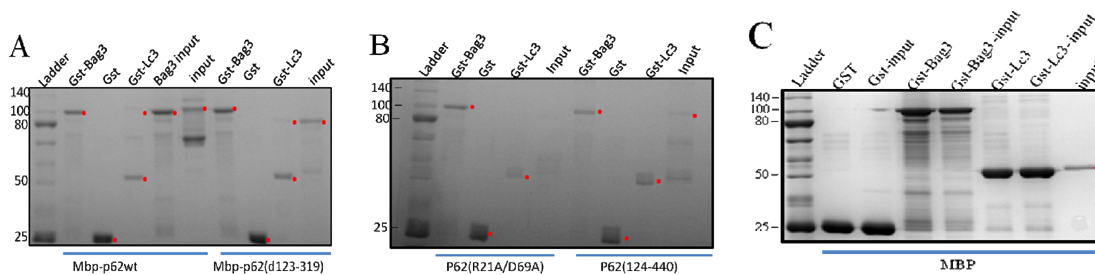

Figure 17: MBP-tagged proteins were incubated with GST fusion proteins as indicated above. Bound proteins were separated by SDS-PAGE and visualized by Coomassie staining. A) No detectable interaction between p62wt/p62 (D123.319) and BAG3. B) No detectable interaction between p62 (R21A/D69A) or p62 (124-440)

and BAG3. Red dots show the protein bands after protein-protein interaction. C) BAG3, GST and LC3 do not bind to MBP.

5.4 Weak interaction mapped between BAG3 and p62 (124-440)

Detection by Coomassie staining is not a very sensitive method. Hence, to investigate whether there is a weak interaction between p62 and BAG3, a new GST pulldown were set up, but now the bound proteins separated by SDS-PAGE were visualized by western blot.

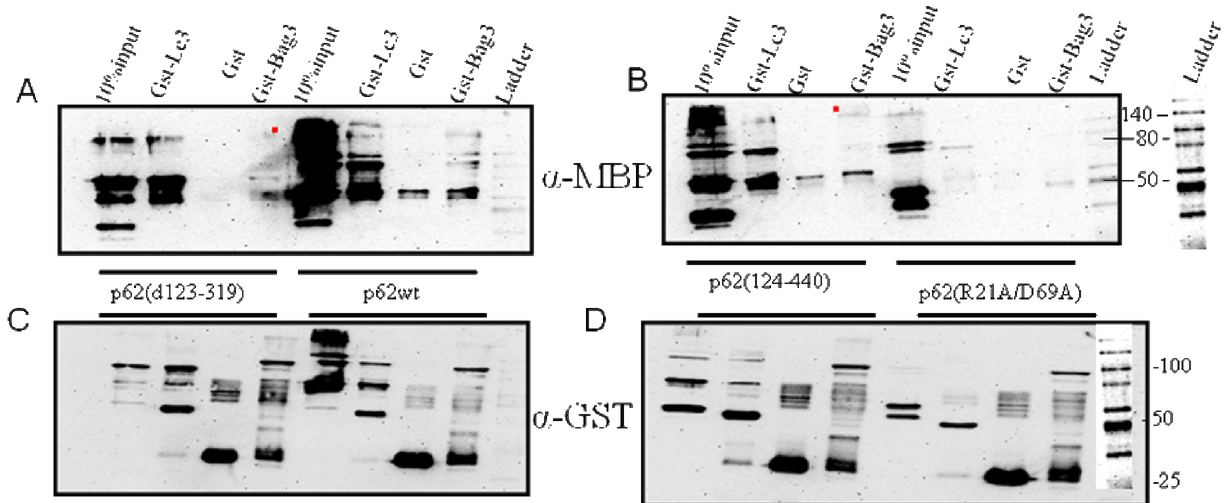

Figure 18: Analysis of Bag3 protein interaction with p62 on western blot with anti-MBP (A and B) and anti-GST (C and D), and anti-biotin against the ladder. The red dots show the faint bands, in A band for p62 (d123-339), and in B band for p62 (124-440).

Western Blot was also performed as it was not easy to differentiate the bands for Mbp-p62wt and Mbp-p62 (R21A/D69A) and GST-BAG3 from each other on the Coomassie stained gels. A western blot was performed first with antibody against MBP (figure 18, A and B) to reveal those proteins that interacted with GST-BAG3 and the negative and positive controls. Then antibody against GST was performed to visualize the GST tagged proteins (Figure 18, C and D). In this analysis LC3 was used as the positive control. There is a weak band identifying MBP-p62 (d123-339) co-precipitated with GST-BAG3 (red dot) (figure 18A), and co-precipitated p62 (124-440) with GST-BAG3 (figure 18B). This suggests that p62 interacts weakly with a region between amino acids 340-440 of p62. This region contains the domains PB1, ZZ, LIR, KIR and UBA (figure 2) which are involved in p62 interactions with other proteins.

5.5 Both Bag3 and p62 are mainly localized in the cytoplasm of HeLa cells.

Plasmids expressing Cherry tagged Bag3 and EGFP tagged p62 variants were transfected into HeLa cells to allow for protein production with florescence. A laser scanning confocal microscope was used to detect the proteins. Figure19A shows Bag3 protein localization which is strongly expressed within the cytoplasm (red color) but sparsely in the nucleus. In figure 19B we see p62 wild type (p62wt) both in the cytoplasm and nucleus forming dot like formations.

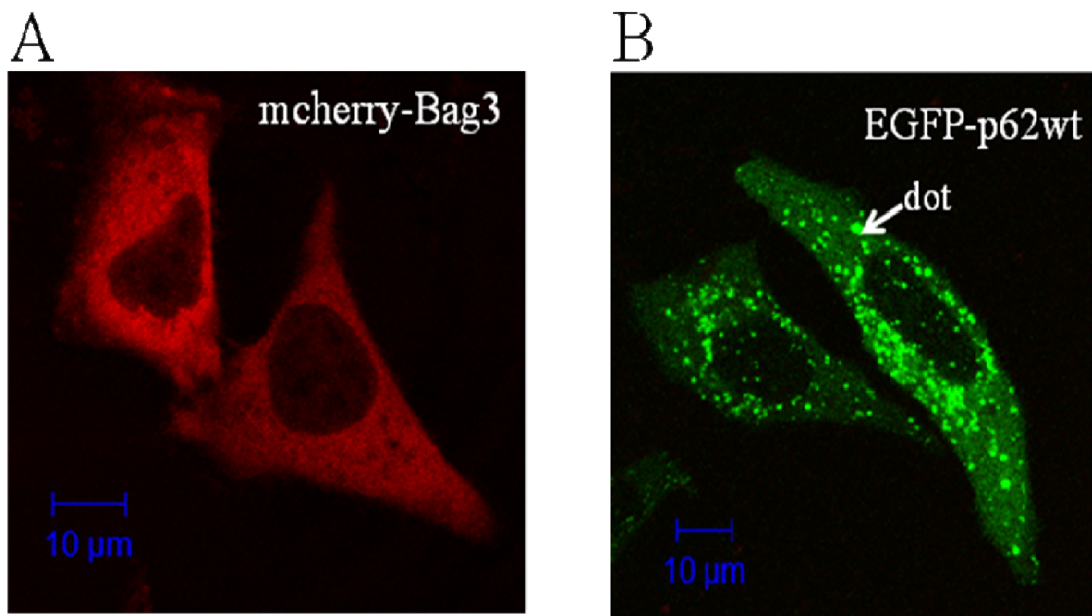

Figure 19: **A)** pDEST-mcherry-BAG3 transfected into HeLa cells. The red fluorescence in HeLa cells is the expression of BAG3 protein and its localization is mostly concentrated in the cytoplasm with small traces in the nucleus. There are signs of aggregation, but with no clear boundaries around the aggregates as seen with p62wt. **B)** pDEST-EGFP-p62wt transfected into HeLa cells. The green fluorescence in the HeLa cells is the expression of p62wt protein which is well distributed in the cytoplasm, and forms aggregates which are in the form of dots that are visible both in the cytoplasm and nucleus. The dots are brightly shining as compared to the other green from the cell.

5.6 Bag3 is not recruited to p62 dots when co-expressed with p62wt

To test whether p62 and BAG3 co-localize in HeLa cells, plasmids expressing EGFP-p62wt and mcherry-BAG3 were co-transfected into HeLa cells. As shown in figure 20, BAG3 is not co-localized with p62 in the p62 dots.

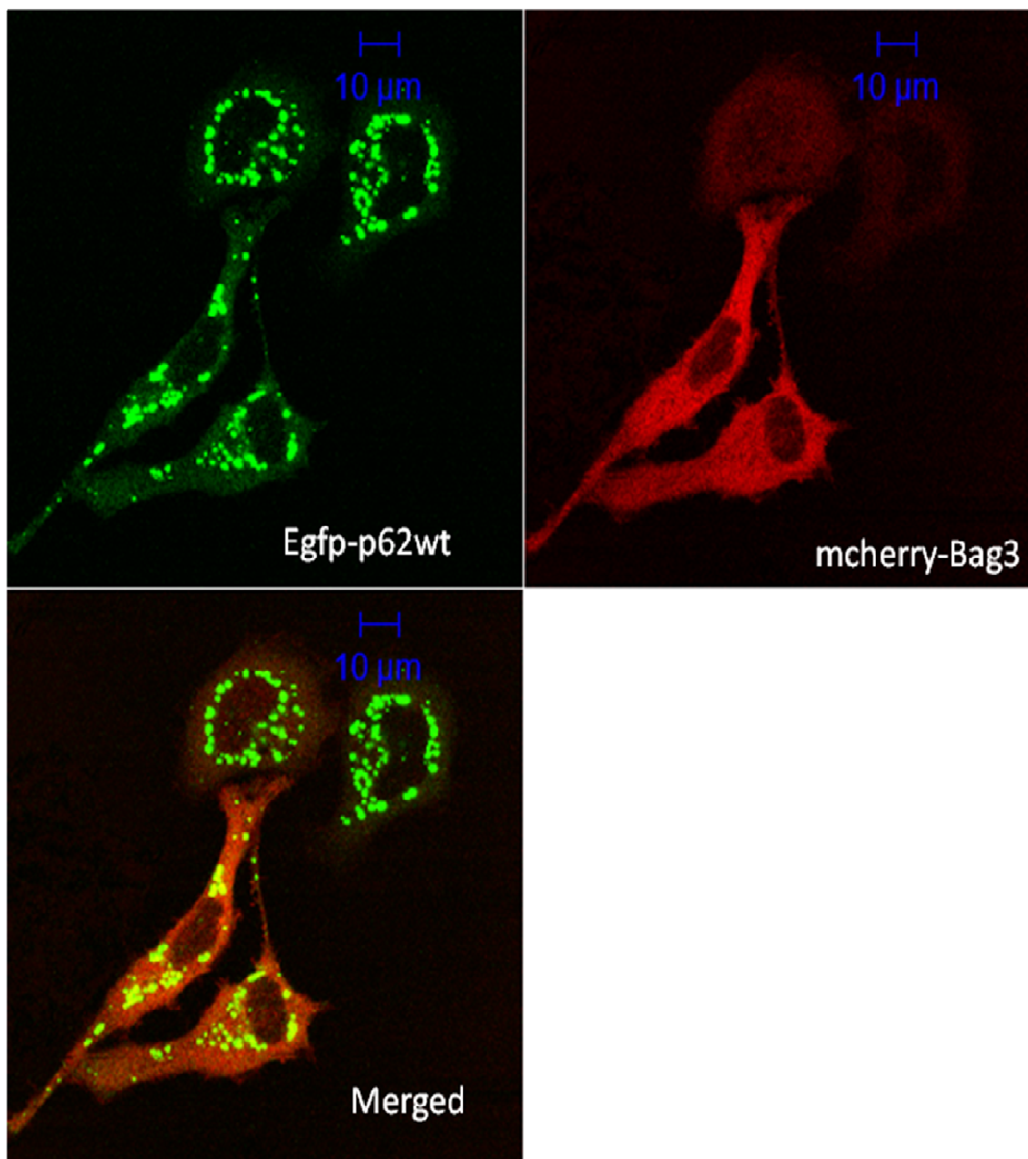

Figure 20: pDEST-mcherry-BAG3 and pDEST-EGFP-p62wt co-transfected into HeLa cells. Using the LSM image browser, the red and green efflorescence we split and merged to determine the location of each protein separately or together. BAG3 seems not to be recruited to the p62 bodies (dots).

5.7 BAG3 is partly co-localized with p62 (124-440)

The GST-pulldown suggested that BAG3 bind weakly to p62 (124-440), to test whether BAG3 co-localized with p62 (124-440) in cells, HeLa cells were co-transfected with plasmids expressing EGFP-p62 (124-440) and mcherry-BAG3. Dots formation is a treat of p62, but we see here that both BAG3 and p62 (124-440) show dot formation (fig 23), the merged image show that there is co-localization, the arrow shows the same dot in both the merged and split forms.

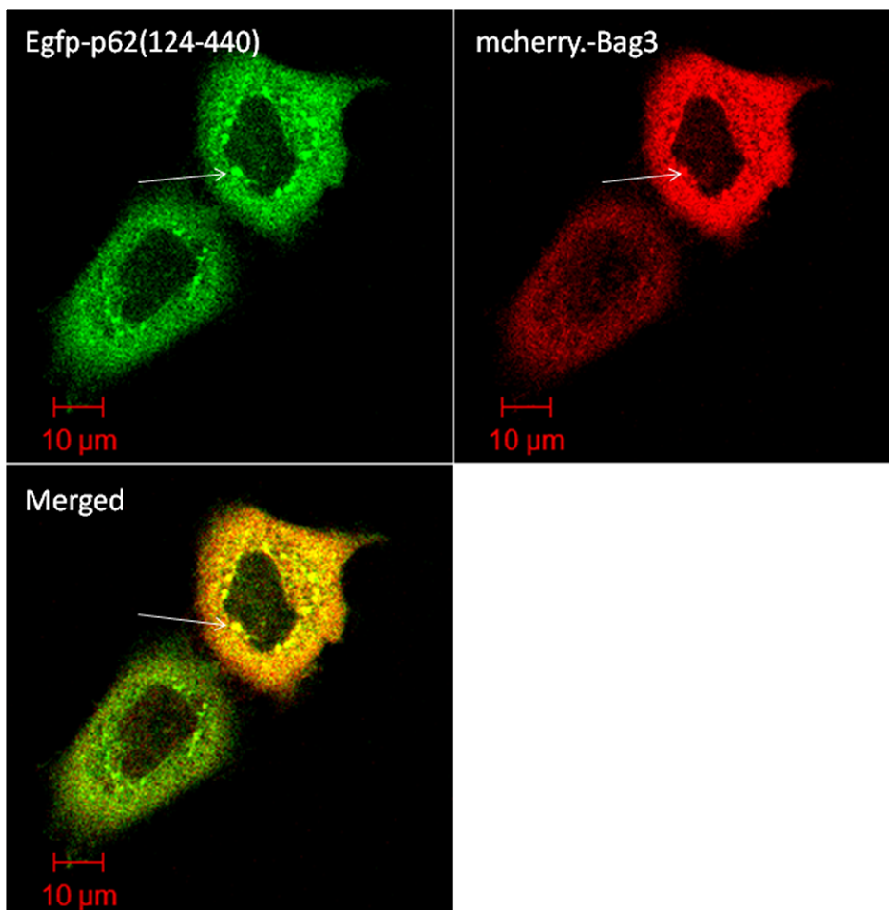

Figure 21: pDEST-mcherry-BAG3 and pDEST-EGFP-p62 (124-440) transfected into HeLa cells. The arrows in the figure show the same dot both in the merged and split images.

5.8 BAG3 do not co-localize with p62 (R7A/D69A)

To test whether BAG3 co-localized with the monomer form of p62, HeLa cells were co-transfected with EGFP-p62 (R7A/D69A) and mcherry-BAG3. As shown in figure 22, Bag3 was not recruited to the p62 dots, but both proteins are distributed throughout the cytoplasm.

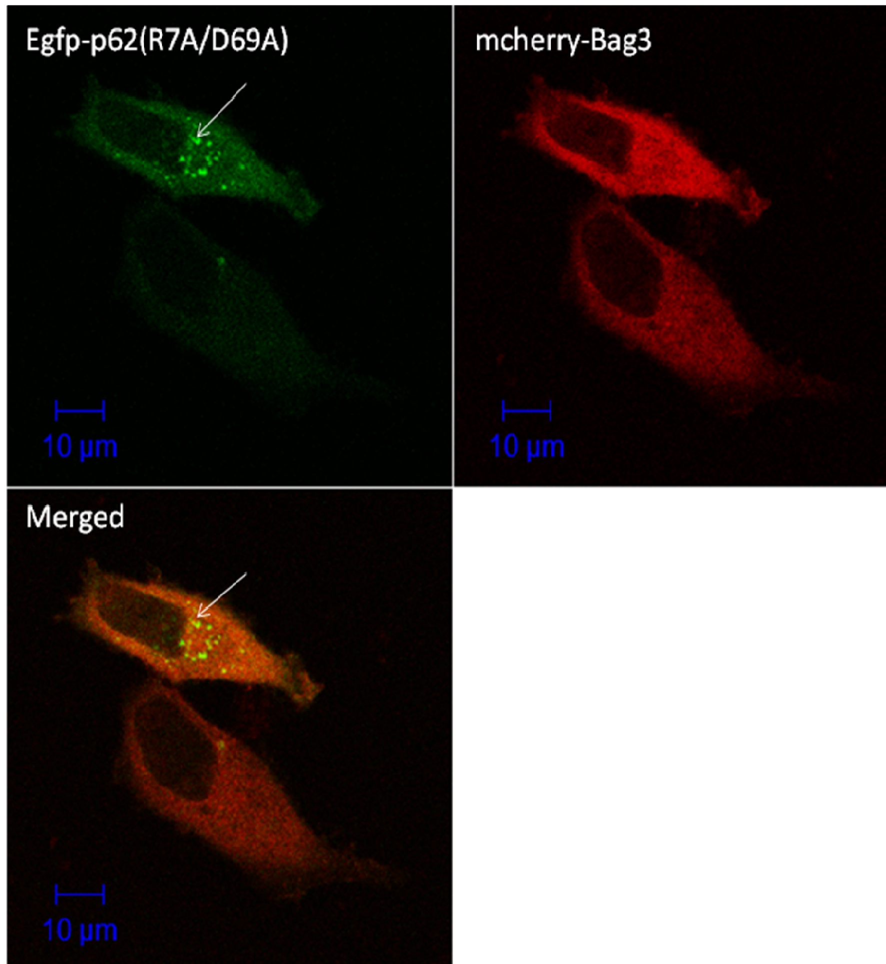

Figure 22: pDEST-mcherry-BAG3 and pDEST-EGFP-p62 (R7A/D69A) monomer were co-transfected into HeLa cells. The arrows show dot formations which can only be seen in the egfp-p62 (R7A/d69A) and merged image.

5.9 BAG3 is recruited to p62 (d123-339) dots

Finally, we investigated the co-localization of p62 (d123-339) and BAG3. BAG3 and p62 (d123-339) are highly co-localized in several p62 dots throughout the cytoplasm of the HeLa cells co-transfected with mcherry-BAG3 and EGFP-p62 (d123-339) (figure 23).

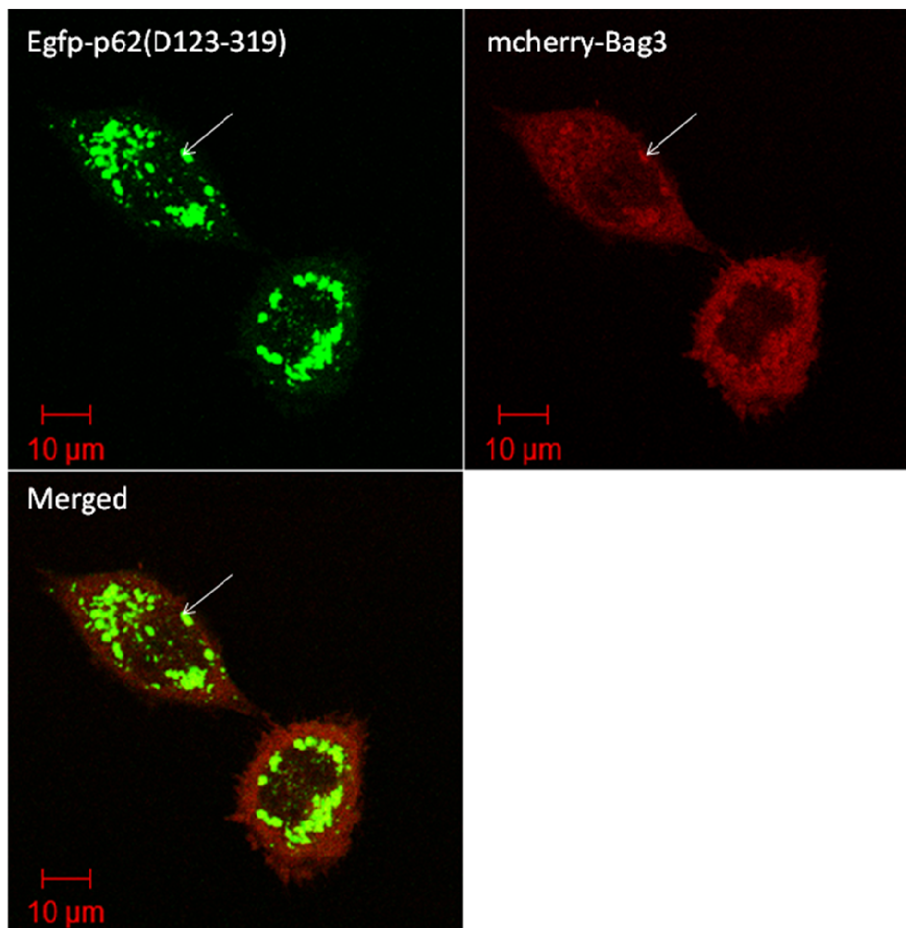

Figure 23: pDEST-mcherry-BAG3 and pDEST-EGFP-p62 (d123-319) transfected into HeLa cells. There is sign of dot formation for BAG. The arrows in the figure show the same dot in split images and merged. There is a strong concentration of red color around the dots, which are aggregated of BAG3 an indication that here BAG3 is recruited into the dot.

6. DISCUSSION

There is evidence that shows that both BAG3 and p62 are involved in autophagy. It has been reported that BAG3 in concert with ubiquitin-binding protein p62 regulate the increase of the macroautophagic flux [(1)]. To investigate any interaction between BAG3 and p62, a study of protein-protein interaction between BAG3 and p62 was initiated, and also a study that looks at co-localization in mammalian cells.

A weak interactions between BAG3 and p62 (d123-339) and p62 (124-440) were detected in the GST-pulldown assay. Both p62 (d123-339) and p62 (124-440) contains the LIR motif of p62wt that is involved in interaction with other proteins. Normally p62wt forms polymers but still with exposed reaction motif for binding with other proteins. The p62 (R21A/D69A) is a monomer, but does not also interact with BAG3 suggesting that the interaction, if any between p62 and BAG3 is weak as it only involves the smaller constructs of p62 that still contains the reaction motifs. Maybe the full length p62 does have an open/close structure that could explain the non interaction with the BAG3.

The weak interaction is strengthened by the co-localization analysis. A partly co-localization is observed between p62 (d123-339) with BAG3 (figure 23). Interestingly, p62 (124-440) seems to recruit BAG3 into autophagy dots (figure 21). The same results have been observed in the protein-protein interaction as resolved by western blot. This may indicate that p62 (124-440) is misfolded and is recruited by BAG3 which directs it to autophagy. BAG3 is not recruited either by p62wt or p62 (R7A/D69A).

7. COUNCLUSION

There is no interaction between full length p62 and BAG but there is some interaction between BAG3 and the small constructs of p62. Any further investigation of BAG3 and p62 interaction should involve the use of in-vitro translation with radioactive proteins that would allow a sensitive analysis of this putative weak interaction in vitro. In vivo co-immunoprecipitation would give a sensitive analysis of proteins in their biological environment.

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