NF-κB regulates protein quality control after heat stress through modulation of the BAG3–HspB8 complex

Mathieu Nivon1,2,3, Michel Abou-Samra1,2,3, Emma Richet4, Boris Guyot1,2,3, André-Patrick Arrigo1,2,3 and Carole Kretz-Remy1,2,3,*

1Université de Lyon, Lyon, F-69361, France
2Université Lyon1, Villeurbanne, F-69622, France
3CNRS, UMR5534, Centre de Génétique et de Physiologie Moléculaire et Cellulaire, Villeurbanne, F-69622, France
4Division of Ocular Biology and Therapeutics (ORBIT), Institute of Ophthalmology, 11-43 Bath Street, London, EC1V 9EL, UK

*Author for correspondence (Carole.Kretz@univ-lyon1.fr)

Summary

We previously found that the NF-κB transcription factor is activated during the recovery period after heat shock; moreover, we demonstrated that NF-κB is essential for cell survival after heat shock by activating autophagy, a mechanism that probably helps the cell to cope with hyperthermic stress through clearance of damaged proteins. In this study, we analyze the involvement of NF-κB in basal and heat-stress-induced protein quality control, by comparing the level of multiubiquitylated and/or aggregated proteins, and proteasome and autophagic activity in NF-κB-competent and NF-κB-incompetent cells. We show that NF-κB has only a minor role in basal protein quality control, where it modulates autophagosome maturation. By contrast, NF-κB is shown to be a key player in protein quality control after hyperthermia. Indeed, NF-κB-incompetent cells show highly increased levels of multiubiquitylated and/or aggregated proteins and aggresome clearance defects; a phenotype that disappears when NF-κB activity is restored to normal. We demonstrate that during heat shock recovery NF-κB activates selective removal of misfolded or aggregated proteins – a process also called ‘aggrephagy’ – by controlling the expression of BAG3 and HSPB8 and by modulating the level of the BAG3–HspB8 complex. Thus NF-κB-mediated increase in the level of the BAG3–HspB8 complex leads to upregulation of aggrephagy and clearance of irreversibly damaged proteins and might increase cell survival in conditions of hyperthermia.

Key words: NF-κB, Heat shock, Autophagy, Aggrephagy, Protein quality control, Protein aggregation, BAG3, HspB8

Introduction

To be functional, most proteins must adopt a defined three-dimensional structure called the native fold. Protein folding begins as soon as proteins are synthesized at ribosomes; newly made and partially folded polypeptides often expose hydrophobic domains that can lead to inappropriate protein association and/or aggregation (Cabrita et al., 2010; Harms et al., 2001; Saibil, 2008). Protein aggregation is dependent upon particular mutations (Wetzl, 1994) or errors during translation (Cabrita et al., 2010), but can also be promoted by environmental changes such as pH, ionic strength (Chi et al., 2003), UV irradiation (Borkman et al., 1996), oxidative (Norris and Giasson, 2005) or heat stresses (Pinto et al., 1991). Because aggregates can trap transcription factors and/or cytosolic signaling components, they are often toxic for the cell. Therefore, cells are equipped with various protein quality control (PQC) mechanisms to minimize misfolding, mainly through chaperone machinery (Saibil, 2008), and eliminate misfolded and/or aggregated proteins, principally by the ubiquitin proteasome system (UPS) (Goldberg, 2003) and lysosomal compartments (Clague and Urbe, 2010). When refolding and proteolytic processes are overwhelmed, misfolded proteins can form toxic aggregates (Garcia-Mata et al., 1999). Cells respond to the presence of aggregates by addressing them to the microtubule-organizing center (MTOC) where they form a unique large ‘storage-bin’ structure called aggresome (Garcia-Mata et al., 1999). Thanks to a vimentin coat surrounding the aggresome, non-native proteins are then unable to interact with well-functioning cell components (Johnston et al., 1998). The only known process that can lead to aggresome degradation is the autophagy–lysosomal pathway (Filimonenko et al., 2010).

Autophagy is a cellular process of self-eating (Mehrpour et al., 2010). Double-membrane vesicles called autophagosomes are formed initially, which engulf parts of the cytoplasm and then fuse with lysosomes, leading to the formation of autophagolysosomes where enclosed material will be degraded by lysosomal proteases (Mehrpour et al., 2010). This phenomenon is regulated by ATG gene products and requires the interaction of class III phosphatidylinositol 3-kinase (PI3K) with the tumor suppressor beclin1 and two ubiquitin-like conjugation systems, Atg12–Atg5 and LC3–phosphatidylethanolamine (LC3-II) (Xie and Klionsky, 2007). Autophagy is under the control of various signal transduction pathways (Kamada et al., 2000; Pattingre et al., 2008). It is active at basal levels in most cell types and regulates long-lived proteins and organelle turnover (Mehrpour et al., 2010). Autophagy is also induced to maintain cellular ATP energy during starvation (Kuma et al., 2004) or to degrade protein aggregates during proteinopathic diseases such as muscular disorders or neurodegeneration (Martinez-Vicente and Cuervo, 2007).
Recently, autophagy was reported to be activated by heat stress (Komat et al., 2004) and we demonstrated that this activation requires the activation of NF-kB transcription factor (Nivon et al., 2009).

NF-kB belongs to the Rel/NF-kB transcription factor family. The most frequent heterodimer association, consisting of RelA (also known as p65) and p50 is called NF-kB (Ghosh et al., 1998). It is an inducible transcription factor that is inactivated by binding to an inhibitory subunit of the IkB family. Most members of the IkB family inhibit NF-kB by blocking both its DNA binding and transactivation abilities (Hayden and Ghosh, 2004). Hence, activation of NF-kB requires the dissociation of the NF-kB–IkB complex. Most inducers (Pahl, 1999) activate the NF-kB–IkB dissociation through the ‘canonical’ pathway resulting in IKK kinase phosphorylation, which in turn leads to phosphorylation and ubiquitylation of IkB before it is degraded by the 26S proteasome (Hayden and Ghosh, 2004). However, some inducers activate NF-kB through non-canonical pathways. For instance, treatment with UV or amino acid analogs induces IkB degradation without any prior phosphorylation (Kretz-Remy et al., 1998; Li and Karin, 1998). We also showed that NF-kB activation by heat stress does not require activation of IKK or phosphorylation and degradation of IkB, but is concomitant with a dissociation of the NF-kB–IkB complex (Kretz-Remy et al., 2001).

Heat shock induces many cellular alterations such as protein misfolding and aggregation (depending on heat stress severity) (Pinto et al., 1991), cell cycle arrest (Kuhl and Rensing, 2000), modulation of membrane fluidity (Balogh et al., 2005), redox state (Davidson and Schiestl, 2001) and transcription modulation (Park et al., 2005). Indeed, during heat stress, heat shock genes are preferentially transcribed (Morimoto and Santoro, 1998). The resulting heat shock proteins (Hsps) are molecular chaperones that help the cell to cope with misfolded proteins by either refolding them or addressing them to the degradative systems (Young et al., 2004). Recently, we have demonstrated that hyperthermia induces another survival pathway: autophagy activation by NF-kB (Nivon et al., 2009). Moreover, we observed that aberrantly folded and/or aggregated proteins play an essential role in triggering activation of NF-kB and autophagy during heat shock recovery. This suggests that NF-kB might be involved in the cellular response to intracellular protein aggregation.

Here, we thus examined the involvement of NF-kB in basal and heat-stress-induced PQC by comparing protein aggregation, and proteasome and autophagic activity in NF-kB-competent and p65/RelA-depleted cells. NF-kB involvement in basal PQC was quite modest, because NF-kB was observed to only partially modulate autophagosome fusion to lysosomes. By contrast, after heat stress, NF-kB-depleted cells accumulated much more protein aggregates than NF-kB-competent cells, yet the kinetics of aggresome formation was similar in both cell types. However, we observed that aggresome clearance was greatly delayed or altered in NF-kB-depleted cells. This impairment was not dependent on modification of the chaperone machinery or proteasome activity, but rather the consequence of a lack of autophagy stimulation. Indeed, we demonstrated that in NF-kB-depleted cells, the expression of BAG3 and HSPB8 genes and the formation of the BAG3–HspB8 complex, which has been described to selectively activate autophagic removal of misfolded proteins, are downregulated.

**Results**

**NF-kB is involved in basal protein quality control**

We previously reported that autophagy activation by NF-kB is essential for cell survival after heat shock and that aberrantly folded or aggregated proteins trigger heat-shock-induced NF-kB and autophagy (Nivon et al., 2009). We thus hypothesized that autophagy modulation by NF-kB might help the cell to clear misfolded or aggregated proteins induced by heat shock and thus involving NF-kB transcript factor in PQC. We first assessed whether NF-kB could be involved in basal PQC. To this end, we measured the level of protein aggregates conjugated to mult ubiquitin in control (HeLa and HeLa-cont#1) and p65-depleted HeLa cells (p65-KD#1 and p65-KD#2). As shown in Fig. 1A, under normal growth conditions the level of mult ubiquitylated proteins was higher in p65-depleted cells than in control cells (see total cell extracts). Moreover, restoring standard p65 levels by transfecting p65-KD#2 cells with the p65 expression vector (p65-KD#2 + p65FL) reversed this increased mult ubiquitylation of proteins (Fig. 1B). The distribution of mult ubiquitylated proteins into Triton-soluble and -insoluble fractions was next analyzed. Whereas in control cells most of the mult ubiquitylated proteins were soluble into Triton, in p65-depleted cells, mult ubiquitylated proteins were predominantly Triton-insoluble (Fig. 1A). The same characteristics of accumulation and solubility were observed with the ubiquitin-binding protein p62/SQSTM1, a protein that is overexpressed in aggregate-prone conditions and whose accumulation is described to be a good marker of protein aggregation (Tanida and Waguri, 2010). To confirm those results, filter trap analysis of protein aggregation in control and p65-depleted cells was performed (Fig. 1C,D). Four different dilutions of total protein extracts from each cell line were slot-blotted onto a nitrocellulose membrane. Soluble proteins passed through the membrane whereas protein aggregates were trapped on the membrane and were immunodetected with antibodies against mult ubiquitin or p62. In p65-depleted cells, we observed a 1.5- to 3-fold higher detection of protein aggregates conjugated to mult ubiquitin or associated to p62 in control cells (Fig. 1C). Moreover, this increase in SDS-insoluble aggregates was reversed when the p65 level was restored to standard levels (Fig. 1D, see p65-KD#2 + p65FL). Hence, our results indicate that p65-depleted cells possess a higher content of protein aggregates under basal conditions when compared with control cells.

Such a phenotype can be the consequence of any modification of the cellular refolding machinery or of protein degradation pathways. We previously reported that absence of p65 does not alter Hsp6 expression and global folding activity in HeLa cells (Nivon et al., 2009). Moreover, in this study, we observed that p65 depletion did not modulate distribution of the main Hsps (Hsp27, Hsp70 and Hsp90) into Triton-soluble and -insoluble fractions (Supplementary material Fig. S1), suggesting that accumulation of mult ubiquitylated and aggregated proteins is not a consequence of alteration of Hsps levels and/or chaperone activity. We thus checked whether mult ubiquitylated protein accumulation was a consequence of an impairment of protein degradation. To this end, we analyzed proteasome and autophagy activities in control and p65-depleted cell lines under basal growth conditions. As shown in Fig. 2A, p65 depletion only slightly increased chymotrypsins- (p65-KD#2) and caspase-like (p65-KD#1 and p65-KD#2) activities of the 26S proteasome, but did not modify its trypsin-like activity. The distinct modulation
observed between p65-KD#1 and p65-KD#2 cell lines might be related to the differences in p65 levels, because KD#1 cells show less depletion of p65 (50% depletion) than KD#2 ones (90% depletion) (Nivon et al., 2009). Next, we focused on autophagy and analyzed LC3 protein levels and more precisely formation of LC3-II, which is formed upon activation of autophagy (Tanida et al., 2004). We observed that the level of LC3-II protein was 1.5- to 2.2-fold higher in p65-KD cell lines, with the highest increase observed in cells with the most complete p65 depletion (p65-KD#2 cells) (Fig. 2B). Moreover, the increase in LC3-II was augmented (3.9-fold) when a more complete knockdown of p65 was achieved, by expression of shRNAs directed against P65 mRNA in the p65-KD#2 cell line (Fig. 2C, see p65-KD#2 + shp65). By contrast, restoring the p65 level in p65-KD#2 cells reversed the increase in LC3-II (Fig. 2D, see p65-KD#2 + p65FL), confirming that the effect on LC3-II is specific to NF-κB. An increase in LC3-II can be explained by: (1) a higher induction of autophagic process or (2) an inhibition of the maturation and degradation of autophagic vesicles. To distinguish between those two mechanisms, control and p65-depleted cells were treated with rapamycin, an autophagy inducer, or bafilomycin A1, an inhibitor of autophagosome maturation (Fig. 2E). Rapamycin treatment increased LC3-II to the same extent (threefold) in the four cell lines studied, indicating that autophagy is inducible to the same extent in p65-depleted and control cells. Upon bafilomycin treatment, the increase of LC3-II observed after blockade of autophagosome maturation in comparison with non-treated cells was slightly less intense in p65-KD cell lines than in control cell lines. This suggests that a partial blockade of autophagosome maturation could be responsible for the slight increase in the level of LC3-II observed in p65-deficient cells. Taken together, our results suggest that under basal growth conditions NF-κB is involved in a modest regulation of PQC, facilitating autophagosome fusion to lysosomes and thus clearance of misfolded or aggregated proteins.

NF-κB is involved in protein quality control during heat shock recovery

We then assessed whether and how NF-κB was involved in PQC after a protein aggregation stress. Indeed, in our previous paper we showed that p65-depleted cells are more sensitive to hyperthermia treatment, a well-known inducer of protein aggregation; this sensitivity is linked to an impairment of autophagy induction after heat shock. In addition, we demonstrated that protein aggregation is necessary for induction of NF-κB-dependent autophagy suggesting that NF-κB might have a role in PQC after heat shock (Nivon et al., 2009). We thus analyzed protein aggregate formation after hyperthermia treatment in control and p65-depleted HeLa cells. We observed that heat shock induced a transient accumulation of multiubiquitylated proteins in control cells (Fig. 3A). This accumulation started immediately after heat shock (R0), increased until 6 hours of recovery (R6) and decreased to reach basal levels 24 hours after heat shock (R24); thus multiubiquitylated protein accumulation was cleared 24 hours after the onset of the heat stress. By contrast, in p65-depleted cells, accumulation of multiubiquitylated proteins was still detected 24 hours after heat shock. The same observations were made with the ubiquitin-binding protein p62 (Fig. 3A). However, when the p65 level was restored, accumulation of multiubiquitylated proteins was then nearly identical to that observed in control HeLa cells (supplementary material Fig. S2). Hence, our results suggest that p65-deficient cells have a
decreased ability to cope with multiubiquitylated protein accumulation induced by heat shock treatment.

To confirm these results, we performed filter-trap analysis with multiubiquitin labeling (Fig. 3B). In control cell lines (HeLa and HeLa-Cont#1), the level of multiubiquitylated aggregates was increased eightfold during the first 6 hours of recovery after hyperthermia and then returned to basal levels after 24 hours of recovery. In p65-depleted cells, the global kinetics of accumulation of protein aggregates was similar. However, the increase was much more important and was only partially absorbed after 24 hours of recovery (fourfold more protein aggregates in p65-KD#2 cells than in HeLa cells).

To describe more accurately this phenomenon, we analyzed the distribution of multiubiquitylated proteins and p62 protein in Triton-soluble and -insoluble fractions (Fig. 3C). Most multiubiquitylated proteins in untreated control HeLa cells were soluble, and p62 was also mainly found in the soluble fraction. Immediately after heat shock, p62 and almost all multiubiquitylated proteins shifted to the insoluble fraction. After 6 hours of recovery, p62 and multiubiquitylated proteins were found equally in soluble and insoluble fractions, and 24 hours after the heat treatment, most p62 and multiubiquitylated proteins were back to soluble fraction. By contrast, in p65-depleted cells, the insolubilization induced by heat shock was much more intense; furthermore, after 24 hours of recovery after heat shock, the vast majority of p62 and multiubiquitylated proteins was still found in the insoluble fraction. Consequently, these results confirm that p65-depleted cells deal less efficiently with aggregated proteins induced by heat shock. Moreover, analysis of the distribution of main heat shock proteins in soluble and insoluble fractions, led to the observation of a small enrichment of Hsp90 and Hsp27 in the insoluble fractions of p65-depleted cells submitted to heat shock in comparison with control cells (supplementary material Fig. S3). Therefore, these results support a delayed and/or altered clearance of protein aggregates in the absence of NF-κB.

As mentioned above, we demonstrated that p65 depletion inhibits heat-induced autophagy (Nivon et al., 2009). Interestingly, autophagy and UPS are interconnected; for instance, autophagy blockade has been shown to inhibit proteasome activities due to proteasome saturation (Korolchuk et al., 2009). Subsequently, we quantified proteasome activities during heat shock recovery in control and p65-depleted cell lines. We observed an increase of proteasome activities in both cell lines immediately after heat shock (R0) and thereafter an inhibition of its three activities until 24 hours of recovery post heat shock (supplementary material Fig. S4), indicating that proteasome modulation after heat shock is not dependent on NF-κB. In conclusion, our results demonstrate that NF-κB is
involved in protein quality control and clearance of protein aggregates during heat shock recovery. However, this control does not seem to involve modulation of chaperone and proteasome activities, but only impairment of autophagy induction by heat shock.

Because heat shock is known to induce misfolded or aggregated proteins and thus aggresome formation (French et al., 2001; Pinto et al., 1991), which is reported to activate autophagy (Gamerdinger et al., 2011; Garcia-Mata et al., 2002; Yamamoto and Simonsen, 2010), we analyzed the kinetics of aggresome formation during heat shock recovery. p62 is described to be associated with the aggresome in a structure surrounded and isolated from the cytosol by a vimentin cage. Aggresome formation after hyperthermia was thus monitored by immunofluorescence analysis of p62 (Fig. 4) and vimentin (supplementary material Fig. S5) in control and p65-depleted HeLa cells. As seen in Fig. 4A,B, in untreated (NT) control cells, p62 distribution was diffused into the cytoplasm. Immediately after heat shock (R0), cells became rounded and p62 relocalized around the nucleus. At 6 hours of recovery, antibodies against p62 labeled a unique structure near the nucleus and colocalized with pericentrin (a component of the MTOC), indicating the formation of an aggresome. At 24 hours after the heat stress (R24), the aggresome was cleared and cells exhibited the same phenotype than untreated cells. In the first 6 hours of recovery in p65-depleted cells, observations were very similar to those performed in control cells. However, at the R24 point, aggresome structure was still present in a large number of cells (10- to 20-fold more p65-depleted cells containing aggresome structure than control cells) (Fig. 4B) and was still detectable 72 hours after heat shock (data not shown). Immunofluorescence experiments performed with antibody against vimentin showed similar results, validating the aggresome-like structure of p62-containing aggregates (supplementary material Fig. S5). By contrast, when the p65 level was restored in p65-KD#2 cells (p65-KD#2 + p65FL), clearance of the aggresome became almost identical to that observed in control cells (Fig. 4A,B). Hence p65 depletion does not modify aggresome formation, but rather modifies its clearance. Consequently, the inhibition of autophagy induction by heat shock, in the absence of NF-κB, does not seem to be caused by any disruption of aggresome formation. It rather seems that autophagy might not be activated by aggregates and/or aggresome formation, or that targeting of aggregates or the aggresome to autophagosomes is strongly delayed or deficient.
NF-κB modulates BAG3 and HSPB8 expression levels and BAG3–HspB8 complex formation during heat shock recovery

The BAG3–HspB8 complex is known to selectively activate autophagic removal of misfolded proteins. Indeed, HspB8 was shown to recognize and link to misfolded proteins and to interact with BAG3, which in turn induces engulfment of aggregates and aggresome by autophagosomes (Carra, 2009). Therefore, we wondered whether NF-κB was involved in the modulation of HSPB8 and BAG3 expression and/or levels of the BAG3–HspB8 complex.

We first analyzed HSPB8 and BAG3 mRNA levels in control and p65-deficient cells submitted or not to a heat shock treatment followed by a 3 hour recovery period at 37˚C by quantitative RT-PCR (Fig. 5A). We observed that the level of BAG3 mRNA was increased more than 18-fold after heat shock treatment in control HeLa cells, whereas this increase was reduced by 37% in p65-depleted cells. A more complete knockdown of p65 in p65-KD#2 cell line (p65-KD#2 + shp65), reduced the heat shock stimulation by 68%, whereas the rescue of p65 level (p65-KD#2 + p65FL) completely restored the induction of BAG3 expression by heat shock. Heat shock stimulation causes an 8.5-fold increase in HSPB8 mRNA in control cells, with a 40% decrease in p65-deficient cells. This reduction reached 72% in p65-KD#2 cells with a more efficient p65 knockdown and was completely reversed when the p65 level was returned to normal. This modulation of HSPB8 and BAG3 mRNA levels by p65 was confirmed by microarray analysis (data not shown). Hence, these results suggest an involvement of NF-κB in the modulation of expression of HSPB8 and BAG3 genes after heat shock. Consequently, we checked by ChIP analysis whether NF-κB could activate transcription of HSPB8 and/or BAG3 genes by binding to putative NF-κB binding sites in their promoters (supplementary material Fig. S6). We could not detect any specific binding of p65 to putative NF-κB binding sites of HSPB8 and BAG3 promoters after heat shock (Fig. S6B). By contrast, an increased p65 binding to the IL8 promoter after treatment with tumor necrosis factor alpha (TNFα; as a positive control) was detected (supplementary material Fig. S6A). However, after heat shock we observed an increased recruitment of RNApolIII to HSPB8 and BAG3 promoters, which contain putative NF-κB binding sites. Furthermore, this increase was not observed in p65-KD#2 cell line (supplementary material Fig. S6C). These results suggest that NF-κB might regulate...
HSPB8 and BAG3 at the transcriptional level. The location of NF-κB binding on the HSPB8 and BAG3 promoters will need to be elucidated.

Next, we checked whether the modulation of expression of BAG3 and HSPB8 was still detectable at the protein level (Fig. 5B,C). When analyzing the kinetics of HspB8 and BAG3 expression during heat shock recovery, we observed a gradual increase of these protein levels in control cells until 6 hours of recovery after heat shock and then a decrease at the R24 recovery time point. By contrast, in p65-deficient cells, no increase could be detected for HspB8 and only a faint increase was detected for BAG3 (Fig. 5B). Moreover, 6 hours after heat treatment in control, p65-KD#2 and p65-KD#2 + p65FL cells, we observed that reversing the p65 level to normal levels in the p65-KD#2 cell line restored induction of HspB8 and BAG3 protein levels by heat shock (Fig. 5C). Thus, our results indicate that the activation of HSPB8 and BAG3 expression levels in response to heat shock is dependent on NF-κB activity.

We then investigated whether the NF-κB-dependent-modulation of BAG3 and HspB8 protein levels after heat shock (Fig. 6A1) could interfere with the quantity of BAG3–HspB8 complexes formed during the recovery period after heat stress (Fig. 6A,B). This was assessed by co-immunoprecipitation analysis. Proteins from control and p65-depleted cells submitted to heat shock treatment followed by 6 hours of recovery were extracted and immunoprecipitated with antibody against BAG3, HspB8 and actin (as a loading control). (C) Total protein extracts of HeLa, p65-KD#2 and p65-KD#2 + p65FL cells, submitted or not (NT) to a 90 minute heat shock treatment at 43°C followed by 6 hours of recovery at 37°C (R6); immunoblot analysis of p65 is also shown as a control for restoration of p65 expression. *P<0.05, **P<0.01, ***P<0.001.

Fig. 5. NF-κB modulates BAG3 and HSPB8 expression. (A) HeLa, p65-KD#2, p65-KD#2 + shp65 and p65-KD#2 + p65FL cells were submitted or not (NT) to a 90 minute heat shock treatment at 43°C followed by a 3 hour recovery period at 37°C (R3). Then, quantitative RT-PCR analysis of BAG3 and HSPB8 mRNA levels was performed. Fold stimulation is the ratio between transcript levels in the various cell lines submitted or not to heat shock treatment and transcript levels in the non-treated HeLa cells. (B) Total protein extracts of HeLa and p65-KD#2 cells submitted or not (NT) to a 90 minute heat shock treatment at 43°C followed by 0 (R0), 6 (R6) or 24 hours (R24) of recovery at 37°C analyzed by immunoblots probed with antibodies against BAG3, HspB8 and actin (as a loading control). (C) Total protein extracts of HeLa, p65-KD#2 and p65-KD#2 + p65FL cells, submitted or not (NT) to a 90 minute heat shock treatment at 43°C followed by 6 hours of recovery at 37°C (R6); immunoblot analysis of p65 is also shown as a control for restoration of p65 expression. *P<0.05, **P<0.01, ***P<0.001.
because the increase in complex formation after heat shock is abrogated in p65-deficient cells. This result suggests that induction of autophagy is inhibited in p65-depleted cells submitted to heat shock because of downregulation of formation of the BAG3–HspB8 complex, which leads to an inhibition of the degradation of protein aggregates and aggresome-like structures.

**Discussion**

We have previously shown that NF-κB mediates cell survival after heat shock by activating the autophagic process. Moreover, we demonstrated that heat-shock-denatured proteins are key factors for NF-κB and autophagy activation during heat shock recovery (Nivon et al., 2009). This led us to hypothesize that NF-κB-dependent autophagy might help the cell to clear protein aggregates generated by heat shock conditions. Here, our interest was therefore to determine whether NF-κB is involved in PQC after heat stress, but also in basal growth conditions.

We first analyzed the involvement of NF-κB in basal PQC by quantifying the level of misfolded or aggregated proteins in p65-depleted cells under basal growth conditions. Indeed, any alteration of PQC processes usually results in accumulation of misfolded and aggregated proteins (Hamano et al., 2008; Korolchuk et al., 2009; Latonen et al., 2011; Welch, 2004; Wilde et al., 2011). We observed that NF-κB-deficient cells possess a higher content of multiubiquitylated and aggregated proteins than control cells. Moreover, restoring an identical p65 level to that measured in control cells, by overexpressing the p65 protein in p65-depleted cells, completely reversed the accumulation of aggregated proteins, demonstrating the specificity of the shRNA and of the phenotype observed. Our observations thus suggest that basal PQC is less efficient in NF-κB-depleted cells. Major players involved in PQC are: molecular chaperones, UPS and autophagy (Arrigo et al., 2007; Marques et al., 2006). We have shown here and in our previous paper that the expression and solubility of main Hsps and the overall folding capacity of the chaperone machinery are not modified by depletion of p65 (Nivon et al., 2009). We thus addressed whether UPS is altered, and observed a slight increase of chymotrypsin-like and caspase-like activities in p65-depleted cells. However, we suggest that this increase is more likely to be related to the small augmentation of the level of multiubiquitylated proteins observed in p65-depleted cells. We then measured autophagic activity. Indeed, an implication for autophagy in clearance of multiubiquitylated and aggregated proteins came from the observation that the conditional knockout of genes essential for autophagy (ATG5 or ATG7), in mouse liver and brain, led to hepatic dysfunction and neurodegeneration with accumulation of ubiquitin-positive-aggregates (Komatsu et al., 2006; Kuma et al., 2004). We observed a small but systematic and repeatable increase of the level of LC3-II in every NF-κB-deficient cell line analyzed. Fluctuations of the increased LC3-II levels strictly reflected the intensity of the p65 depletion obtained in the various cell lines, with the highest LC3-II increases observed in the cells showing the most complete p65 depletion. Thus NF-κB seems to specifically modulate basal autophagy and more precisely to alter autophagosome maturation rather than induction of autophagy as revealed upon treatment with bafilomycin A1. However, the modest effects observed highlight the only partial involvement of NF-κB in the modulation of basal autophagy. But because this process is described to achieve cellular homeostasis by regulating long-lived protein turnover and eliminating protein aggregates (Mehpour et al., 2010), a partial blockade of autophagosome maturation could nevertheless be responsible for the modest accumulation of aggregated proteins, as observed in p65-depleted cells. p65 has been described to modulate basal autophagy by regulating BECLIN1 transcription in HEK293 and Jurkat cells (Copetti et al., 2009). However, by performing microarrays and qPCR experiments, we did not observe any differential expression of BECLIN1 in control and p65-depleted HeLa cells, suggesting that BECLIN1 is not, in our conditions, a target for basal autophagy modulation by NF-κB. Our observation that NF-κB might partially modulate autophagosome maturation could imply a role for NF-κB in the modulation of lysosomal acidification or fusion to lysosomes. Indeed, bafilomycin A1, a specific inhibitor of the V0 subunit of vacuolar proton ATPase is described to prevent maturation of autophagosomes (Klionsky et al., 2008); it was also recently demonstrated that a deficiency of the vacuolar proton ATPase homolog VMA21, which is responsible for X-linked myopathy, induces an accumulation of autophagic vacuoles and interrupts autophagic flux (Ramachandran et al., 2009). Of interest, some preliminary results (microarrays and qPCR) suggested that ATP6V0D2, a subunit of the vacuolar proton ATPase, is differentially expressed between control and p65-depleted cells after heat shock. Whether a partial modulation of ATP6V0D2 by NF-κB could be responsible for NF-κB modulation of basal autophagy will be investigated.

Because heat shock induces protein misfolding or aggregation, which is toxic for the cell and because NF-κB-depleted cells are far more sensitive to hyperthermia treatment, we also assessed whether NF-κB was involved in PQC after heat shock. We observed a higher increase of multiubiquitylated or aggregated proteins in NF-κB-depleted cells and a delay in the clearance of those aggregates. Hence, NF-κB modulates clearance of misfolded or aggregated proteins. By measuring proteasome activity during heat shock recovery, we observed a similar proteasome blockade in control and NF-κB-deficient cells. This blockade upon heat treatment is a commonly described process that is probably due to sequestering of proteasome by aggregated proteins that it cannot degrade (Pajonk et al., 2005) and thus is not part of NF-κB modulation of PQC. In our previous paper, we demonstrated that autophagy induction by heat shock is drastically reduced in NF-κB-depleted cells (Nivon et al., 2009), which might explain the altered elimination of protein aggregates in NF-κB-depleted cells. Indeed, over the past few years it has become evident that autophagy is not only unspecific, but is also a selective degradative pathway involved in the removing of misfolded or aggregated proteins in the cytosol, which is then called aggrephagy (Yamamoto and Simonsen, 2010). However, it is also known that cells respond to protein aggregation by gathering aggregates into a unique structure termed the aggresome that can activate autophagy (Garcia-Mata et al., 1999). Therefore, we analyzed the kinetics of aggresome formation during heat shock recovery and observed that it is not altered in p65-depleted cells; by contrast, aggresome clearance is very clearly delayed or altered in NF-κB-deficient cells; indeed, 40% of those cells still contain an aggresome 24 hours after heat shock, compared with 3% of the control cells. Thus it seems that NF-κB regulates PQC by either modulating autophagy induction by aggregates or aggresomes, or by modulating aggresome targeting to autophagosomes. We consequently focused on BAG3, an Hsp70 co-chaperone, whose gene transcription is activated by heat shock (through HSF1 transcription factor).
BAG3 was recently described to be a molecular switch that determines whether proteins are degraded in a proteasomal or autophagic manner (Gamerding et al., 2009) and was found to increase autophagic activity (Carra, 2009). When interacting with HspB8, BAG3 was described to selectively activate autophagic removal of misfolded proteins and the aggresome (Carra et al., 2008b). We observed that heat shock increased BAG3 and HSPB8 mRNA levels in an NF-kB-dependent manner. Moreover, the highest p65 depletion obtained led to a reduction of heat shock stimulation of BAG3 and HSPB8 of 70%, whereas the rescue of NF-kB activity completely reversed the heat shock stimulation to normal levels; thus, we conclude that NF-kB is a major regulator of BAG3 and HSPB8 under heat shock recovery, but also that other minor players might be involved in regulation of these genes after heat shock. ChIP analysis on putative NF-kB binding sites of BAG3 and HSPB8 promoters did not allow us to detect any binding of p65. However, we could detect an NF-kB-dependent recruitment of RNApolII to the promoters during heat shock recovery. Our results are therefore in favor of a transcriptional regulation of BAG3 and HSPB8 by NF-kB. Furthermore, we observed that the heat shock induction of BAG3 and HSPB8 mRNA levels was not suppressed in the presence of the translation inhibitor cycloheximide (data not shown), suggesting a direct transcriptional regulation of BAG3 and HSPB8 by NF-kB during heat shock recovery. Localization of NF-kB binding sites in BAG3 and HSPB8 will be investigated further. Finally, we demonstrated that the modulation of BAG3 and HSPB8 expression by NF-kB was still detectable at the protein level and interfered with the formation of the BAG3–HspB8 complex. We suggest that this NF-kB-dependent upregulation of the formation of the BAG3–HspB8 complex probably leads to an increased clearance of protein aggregates and/or aggresome structures by autophagosomes. Indeed, BAG3–HspB8 was described to prevent aggregation of Htt43Q, a pathogenic form of Huntingtonin polypeptide (Carra et al., 2008a). Furthermore, knockdown of the endogenous BAG3–HspB8 complex resulted in decreased levels of LC3-II and increased Htt43Q aggregation, which strictly parallels our observations. Indeed, we showed that NF-kB deficiency induces a downregulation of formation of the BAG3–HspB8 complex after heat shock, associated with a decreased autophagic process and an increase in protein aggregation. The involvement of BAG3–HspB8 in aggrephagy regulation is thought to take place at two levels: selectivity and induction. HspB8, as a molecular chaperone, is able to recognize aggregating proteins and to bind to BAG3 (Fuchs et al., 2009), thus presenting substrate protein to the autophagic machinery for degradation (Carra et al., 2008b). The BAG3–HspB8 complex can therefore mediate autophagy activation in close proximity to protein aggregates and the aggresome, leading to aggrephagy (Gamerding et al., 2011). Of interest, HspB8 mutants are responsible for neuromuscular disorders (Fontaine et al., 2006) and BAG3 mutants induce severe myopathies (Selcen et al., 2009). Here we demonstrate that this complex is regulated by NF-kB after hyperthermia and thus regulates aggrephagy induction by heat shock. Induction of aggrephagy has also been described to be regulated by p62/SQSTM1, NBR1 (Neighbor of BRCAl gene) and Alfy (PIP3-binding autophagy-like FYVE domain protein) (Filimonenko et al., 2010; Kirkin et al., 2009; Pankiv et al., 2007). However, preliminary analysis of microarrays data did not reveal any differential expression of those genes in NF-kB–competent and -incompetent cells submitted to heat treatment.

To conclude, this study demonstrates that NF-kB is a key player in PQC by modulating the autophagic process. This modulation is modest and partial for basal autophagy and might take place at the autophagosome maturation step. By contrast, NF-kB seems to be a major regulator of aggrephagy after heat stress by upregulating expression of BAG3 and HSPB8 and the quantity of the BAG3–HspB8 complex formed; this complex is described to be involved in selective removal of misfolded or aggregated proteins by autophagy and thus might be involved in the clearance of toxic protein aggregates induced by heat shock. Whether these new NF-kB targets can be used for therapeutic modulation of autophagy by hyperthermia to decrease cell survival to heat shock during cancer treatment merits further investigation.

Materials and Methods

Cell culture, cell lines and heat shock treatment

HeLa cells were grown at 37°C and 5% CO2 in DMEM supplemented with 10% decomplemented FCS, 1 μg/ml fungizone and 50 U/ml penicillin-streptomycin. p65-depleted HeLa cells expressing shRNA directed against the p65 subunit of NF-kB (p65-KD#1, p65-KD#2 and p65-KD#3) and control HeLa cells expressing scrambled shRNA (HeLa-cont#1) have been already described. p65-KD cell lines differ by their content of p65: a 50% depletion of p65 was found in the p65-KD#1 cell line, with a 60% depletion of p65 in the p65-KD#3 cell line and a 90% depletion in the p65-KD#2 cell line (Nivon et al., 2009). Heat shock treatments were performed at 43°C for 90 minutes.

Reagents and plasmids

Bafilomycin A1, cycloheximide, IGEPAL, Hoechst 33258 and Triton X-100 were from Sigma. Rapamycin was from WVR International. MG132 was from Merck and lactacystin was from Calbiochem. The protease inhibitor Complete Mini, EDTA-free was from Roche. Fast flow Protein-G-Sepharose was from Amersham. Mouse monoclonal antibodies against HspB8 (Abnova), Hsp90, Hsp70, Hsp27 (Stressgen), actin (Chemicon), p62/SQSTM1 (Becton Dickinson Biosciences), multi-ubiquitin, kinase I (MBl), vimentin (Sigma) and BAG3 were used. Rabbit polyclonal antibodies used were against: pericentrin (Abcam), p65 (Upstate for western blots and Abcam p65-ChIP grade for ChIP analysis) and LC3 (Chamorel et al., 2008). Rabbit immunoglobulins were from Santa Cruz. p65FL expression vector was a gift from R. Dikstein (The Weizmann Institute of Science, Rehovot, Israel) and is described elsewhere (Yamit-Hezi and Dikstein, 1998). pSUPER RNAI vector generated with the ‘25’ sequence has been already described (Nivon et al., 2009) and allows expression of p65-shRNA directed against mRNAs encoding the p65 subunit of NF-kB.

Gel electrophoresis and immunoblotting

Briefly, 10 μg of total protein extracts were submitted to SDS-PAGE. After electrophoresis, proteins were transferred to Protran BA85 nitrocellulose membrane (Schleicher & Schuell). Blots were then incubated with primary antibody and horseradish-peroxidase-conjugated secondary antibodies and revealed with the ECL detection kit (Amersham Biosciences). For HspB8 and BAG3 western blotting, 20 μg (HspB8) or 3 μg (BAG3) of total protein extracts were used.

Triton-soluble and -insoluble protein fractionation

2 × 106 cells were plated in 100 mm dishes. On the next day, cells were lysed on ice for 30 minutes in 0.1% Triton X-100 in TEM Buffer (20 mM NaCl, 20 mM Tris·HCl, pH 7.4, 5 mM MgCl2, 0.1 mM EDTA), using a Dounce homogenizer. Then, total cell extracts were submitted to a 15 minute centrifugation at 16,000 g to separate soluble (supernatant) and insoluble (pellet) fractions. These fractions were submitted to SDS-PAGE and immunoblotting with antibody against either multiubiquitin or p62, as described above.

Filter-trap assay

SDS-insoluble protein aggregates conjugated to mult ubiquitin or containing p62 protein were analyzed by filter-trap assay as previously described (Carra et al., 2005; Nivon et al., 2009). Briefly, cells were scraped in FTA buffer (150 mM NaCl, 50 mM DTT, 10 mM Tris·HCl, pH 8) supplemented with 2% SDS. These fractions were then homogenized by three passages through 25-gauge needle. 2.5 g of total protein extracts were then diluted (1, 2, 1/4 and 1/8) and applied with mild suction into a slot blot apparatus onto a Protran BA83 nitrocellulose membrane.
(Schleicher & Schuell) pre-washed with 0.1% SDS-FTA buffer. The membrane was then washed with 0.1% SDS-FTA buffer and 0.1% Tween Tw20 in TBS (TBS: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl) and processed for immunoblotting.

**Fluorescence microscopy analysis**

Cells were grown on glass coverslips and submitted to hyperthermia treatment followed by various recovery periods at 37°C, rinsed in PBS, fixed for 10 minutes with 2% paraformaldehyde, pH 7.4, and permeabilized for 10 minutes with ice-cold methanol. Cells were then stained with primary antibodies (pericentrin, p62 or vimentin) and fluorescent secondary antibodies (goat anti-mouse Alexa Fluor 568 nm and goat anti-rabbit Alexa Fluor 488 nm). Hoechst 33258 reagent was used to stain nuclei (5 minutes, 1 ng/ml). The stained cells were observed with 365/12 excitation, 397 emission (Hoechst), 475/40 excitation, 530/50 emission (Alexa Fluor 488) and 545/25 excitation, 605/70 emission (Alexa Fluor 568) band filters and 63 × objectives. They were photographed with a Zeiss axiovert 200M microscope (Carl Zeiss). Images were digitized with a camera (Coolscan HQ2; Roper Scientific) and acquired with Metavue Imaging system; brightness and contrast of images were adjusted using Photoshop CS2 (Adobe).

**Proteasome assay**

Cells were grown in Phenol-Red-free medium, were plated in 96-well plates with 10,000 cells/well. On the next day, they were submitted to various treatments. Proteasome inhibitors MG132 and lactacystin (2 hours, 10 μM) were used as negative controls. Caspase-like, trypsin-like and chymotrypsin-like activities of the proteasome were quantified with fluorescent probes (Z-NAcLeu-L-Pro-AMC, Z-LLF-dimethylcarbamoyl-L-lysyl-lysine (LLF-DML) and Suc-LLVY-aminoluciferin, respectively). The probes were used according to the manufacturer’s instructions (Promega, Proteasome-Glo). Luminescence intensity was measured with a Victor 1420 Multilabel Counter (Perkin Elmer). The relative light units produced were reported to 50 μg of total cellular proteins.

**Real-time PCR**

For expression analysis of BAG3 and HSPB8, total RNA was isolated from 5 × 10⁸ HeLa cells using a Nucleospin RNAII extraction kit, according to the manufacturer’s instructions (Macherey-Nagel). For each condition, 1 μg of total RNA was reverse-transcribed using RevertAid H Minus M-MuLV reverse transcriptase and random hexamers as per the manufacturer’s recommendations (Fermentas). Real-time PCR was performed with the Mx3000P system (Stratagene) using Quantitect SYBR Green PCR kit and Quantitect primer assay (Qiagen) for BAG3, HSPB8 and GAPDH transcripts. Data were normalized to the GAPDH internal standard. Fold differences were calculated with the mathematical model described by Pfaffl (Pfaffl, 2010).

**ChiP**

4 × 10⁶ cells were used and fixed for ChiP analysis as previously described (Jibun et al., 2009) and according to the manufacturer’s instructions (Millipore). Immunoprecipitated and input DNA (prepared from aliquots taken before the immunoprecipitation step) were quantified by real-time PCR using a SYBR green PCR kit and Quantitect primer assay (Qiagen) for BAG3, HSPB8 and GAPDH transcripts. Filters and 63 filters (Alexa Fluor 488) and 545/25 excitation, 605/70 emission (Alexa Fluor 568) band filters and 63 × objectives. They were photographed with a Zeiss axiovert 200M photomicroscope (Carl Zeiss). Images were digitized with a camera (Coolscan HQ2; Roper Scientific) and acquired with Metavue Imaging system; brightness and contrast of images were adjusted using Photoshop CS2 (Adobe).

**Acknowledgements**

We wish to thank Dominique Guillet for excellent technical assistance. We thank Patrice Cordon for helpful discussion and critical analysis of our first experiments.

**Funding**

This work was supported by the Ligue contre le Cancer, comité du Rhône, the Région Rhône-Alpes, contrat Cible 2010 and BQR from Université Claude Bernard Lyon I to C.K. M.N. was supported by a doctoral fellowship from the French Department of Research and the French Medical Research Foundation (FRM).

**Supplementary material available online at**

http://jcs.biologists.orglookup/suppl doi:10.1242/jcs.091041/DC1

**References**

**Arrigo, A. P., Simon, S., Gilbert, B., Kretz-Remy, C., Nixon, M., Czekała, A., Guillet, D., Moulin, M., Diaz-Latoud, C. and Vicart, P. (2007).** Hsp27 (HspB1) and alpha-lactalbumin (HspB5) as therapeutic targets. FERS Lett. 581, 3665-3674.

**Balogh, G., Horvath, I., Nagy, E., Hoyz, Z., Benko, S., Bensaude, O. and Vigh, L. (2005).** The hyperfluidization of mammalian cell membranes acts as a signal to initiate the heat shock protein response. FEBS J. 272, 6077-6086.

**Borkman, R. F., Knight, G. and Obi, B. (1996).** The molecular chaperone alpha-crystallin inhibits UV-induced protein aggregation. Exp. Eye Res. 62, 141-148.

**Cabrera, L. D., Dubson, C. M. and Christodoulou, J. (2010).** Protein folding on the ribosome. Curr. Opin. Struct. Biol. 20, 33-45.

**Cara, S. (2009).** The stress-inducible HspB8-Bag3 complex induces the eIF2alpha kinase pathway: implications for protein quality control and viral factory degradation. Autophagy 5, 428-429.

**Cara, S., Sivillotti, M., Chavez Zobel, A. T., Lambert, H. and Landry, J. (2005).** HspB8, a small heat shock protein mutated in human neuromuscular disorders, has in vivo chaperone activity in cultured cells. Hum. Mol. Genet. 14, 1659-1669.

**Cara, S., Seguin, S. J., Lambert, H. and Landry, J. (2008a).** HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. J. Biol. Chem. 283, 1437-1444.

**Cara, S., Seguin, S. J. and Landry, J. (2008b).** HspB8 and Bag3: a new chaperone complex targeting misfolded proteins to macroautophagy. Autophagy 4, 237-239.

**Chaumorcel, M., Souquere, S., Pierron, G., Codogno, P. and Esclatine, A. (2008).** Human cytoskeletal virus controls a new autophagy-dependent cellular antiviral defense mechanism. Autophagy 4, 46-53.

**Chi, E. Y., Krishnan, S., Randolph, T. W. and Carpenter, J. F. (2003).** Physiological stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregates. Pharm. Res. 20, 1325-1336.

**Clague, M. J. and Urbe, S. (2010).** Ubiquitin: same molecule, different degradation pathways. Cell 143, 682-685.

**Copetti, T., Demarchi, F. and Schneider, C. (2009).** p65/RelA binds and activates the lsm1-1 promoter. Autophagy 5, 639-649.

**Davidson, J. F. and Schiestl, R. H. (2001).** Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in Saccharomyces cerevisae. Mol. Cell. Biol. 21, 8483-8489.

**Filimonenko, M., Isakson, P., Finley, K. D., Anderson, M., Jeong, H., Melia, T. J., Bortle, B. J., Myers, K. M., Birkeland, H. C., Lamark, T. et al. (2010).** The selective macroautophagic degradation of aggregated proteins requires the PIP3-binding protein Ali7. Mol. Cell 38, 265-279.

**Fontaine, J. M., Sun, X., Hoppe, A. D., Simon, S., Vicart, P., Welsh, M. J. and Berndorf, S. (2006).** Abnormal small heat shock protein interactions involving neuroprotective-associated HSPB2 (HSPB8) mutants. FASEB J 20, 2168-2170.

**French, B. A., van Leeuwen, F., Riley, N. E., Yuan, Q. X., Bardag-Gorce, G., Gaal, K., Lau, Y. H., Marezaun, N. and French, S. W. (2001).** Aggresome formation in liver cells in response to different toxic mechanisms: role of the ubiquitin-proteasome pathway and the frameshift mutant of ubiquitin. J Biol. Chem. 276, 1437-1444.

**Gamerdinger, M., Hajeja, P., Kaye, A. M., Wolfrum, U., Hartl, F. U. and Behl, C. (2009).** Protein quality control during aging involves recruitment of the macro-autophagy pathway by BAG3. EMBO J 28, 889-901.

**Gamerdinger, M., Kaye, A. M., Wolfrum, U., Clement, A. M. and Behl, C. (2011).** BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins. EMBO Rep. 12, 149-156.

**Garcia-Mata, R., Bubek, Z., Sorochen, E. J. and Sztul, E. S. (1999).** Characterization and dynamics of aggresome formation by a cytosolic GFP-chimeras. J. Cell Biol. 146, 1239-1254.

**Garcia-Mata, R., Gao, Y. S. and Sztul, E. (2002).** Hasilaces with taking out the garbage: aggregating aggresomes. Traffic 3, 388-390.

**Ghosh, S., May, M. J. and Kopp, E. B. (1998).** NF-κB and Rel proteins: evolutionarily conserved mediators of innate immune responses. Annu. Rev. Immunol. 16, 225-260.

**Goldberg, A. L. (2003).** Protein degradation and protection against misfolded or damaged proteins. Nature 426, 895-899.
NF-κB regulates protein quality control

Nixon, M., Richet, E., Codognon, P., Arrigo, A. P. and Kretz-Remy, C. (2009). Autophagy activation by NF-κB is essential for cell survival after heat shock. Autophagy 5, 766-783.

Norris, E. H. and Giasson, B. I. (2005). Role of oxidative damage in protein aggregation associated with Parkinson’s disease and related disorders. Antioxid. Redox Signal. 7, 672-684.

Pagliai, M. G., Lerose, R., Cigliano, S. and Leone, A. (2003). Regulation by heavy metals and temperature of the human BAG-3 gene, a modulator of Hsp70 activity. FERS Lett. 541, 11-15.

Pahl, H. H. (1999). Activators and target genes of Rel/NF-κB transcription factors. Oncogene 18, 6853-6866.

Pajonk, F., van Ophoven, A. and McBride, W. H. (2005). Hypothesis-induced proteasome inhibition and loss of androgen receptor expression in human prostate cancer cells. Cancer Res. 65, 4836-4843.

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., Overvatn, A., Bjorkoy, G. and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. 282, 24131-24145.

Park, H. G., Han, S. I., Oh, S. Y. and Kang, H. S. (2005). Cellular responses to mild heat stress. Cell. Mol. Life Sci. 62, 10-23.

Pattingre, S., Esteve, L., Biard-Piechaczyk, M. and Codogno, P. (2008). Regulation of macroautophagy by mTOR and Beclin 1 complexes. Biochemie 90, 313-323.

Pattf, M. W. (2010). The ongoing evolution of qPCR. Methods 50, 215-216.

Pinto, M., Morange, M. and Bensaude, O. (1991). Denaturation of proteins during heat shock. In vivo recovery of solubility and activity of reporter enzymes. J. Biol. Chem. 266, 13941-13946.

Ramachandran, N., Munteanu, L., Wang, P., Anbourg, P., Ribbione, J. J., Israelian, N., Narasain, T., Paroutis, P., Guo, R., Ren, Z. P. et al. (2009). VMA21 deficiency causes an autophagic myopathy by compromising V-ATPase activity and lysosomal acidification. Cell 137, 235-246.

Sahib, H. R. (2008). Chaperone machines in action. Curr. Opin. Struct. Biol. 18, 35-42.

Selcen, D., Muntoni, F., Burton, B. K., Pegoraro, E., Sewry, C., Bitt, A. V. and Engel, A. G. (2009). Mutation in BAG3 causes severe dominant childhood muscular dystrophy. Ann. Neurol. 65, 83-89.

Song, S., Kole, S., Precht, P., Pazin, M. J. and Bernier, M. (2010). Activation of heat shock factor 1 plays a role in pyrrolidine dithiocarbamate-mediated expression of the co-chaperone BAG3. Int. J. Biochem. Cell Biol. 42, 1856-1863.

Tanida, I. and Waguiri, S. (2010). Measurement of autophagy in cells and tissues. Methods Mol. Biol. 648, 193-214.

Tanida, I., Ueno, T. and Komamin, K. (2004). LC3 conjugation system in mammalian autophagy. Int. J. Biochem Cell Biol. 36, 2503-2518.

Welch, W. J. (2004). Role of quality control pathways in human diseases involving protein misfolding. Semin. Cell Dev. Biol. 15, 31-38.

Wetzel, R. (1994). Mutations and off-pathway aggregation of proteins. Trends Biotechnol. 12, 193-198.

Wilde, I. B., Brack, M., Winget, J. M. and Mayor, T. (2010). Proteomic characterization of aggregating proteins after the inhibition of ubiquitin proteasome system. J. Proteome Res. 10, 1062-1072.

Xie, Z. and Klionsky, D. J. (2007). Autophagosome formation: core machinery and adaptations. Nat. Cell Biol. 9, 1102-1109.

Yamamoto, A. and Simonsen, A. (2010). The elimination of accumulated and aggregated proteins: A role for aggrephagy in neurodegeneration. Neurobiol. Dis. 43, 17-28.

Yamamoto, A. and Kirkwood, T. L. (2005). A new role for p53 in aging. Science 307, 61-62.

Yamamoto, A. and Simonsen, A. (2005). Autophagy and neurodegeneration: an emerging view. Curr. Opin. Neurobiol. 15, 441-446.

Yamamoto, A. and Simonsen, A. (2005). Does neurodegeneration involve autophagy? Trends Biotechnol. 23, 82-86.

Yamamoto, A. and Simonsen, A. (2005). Autophagy and neurodegeneration: an emerging view. Curr. Opin. Neurobiol. 15, 441-446.

Yamamoto, A. and Simonsen, A. (2005). Autophagy and neurodegeneration: an emerging view. Curr. Opin. Neurobiol. 15, 441-446.