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Nucleocytoplasmic Dispersal of Autophagosome Marker LC3

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Abstract: **Autophagy is a cellular self-degradation system that transfers cytoplasmic components to the lysosome by means of autophagosome. A wide array of molecules has been linked to the formation of this structure. LC3, a structural protein of autophagosome membrane, is widely studied protein among others, due, mainly, to its participation at different stages of autophagy. For the same reason, it has been successfully used as a biomarker of autophagy. Recent progress has demonstrated LC3 cycling between nucleus and cytoplasm in an autophagic-dependent manner. In this regard, some intracellular mechanisms have also been proposed that underline nucleocytoplasmic shuttling of LC3, such as protein-protein interaction and deacetylation/acetylation reaction. The aim of this review is to discuss the distribution of LC3 in the nucleus and cytoplasm in the light of recent findings, and highlighting pathways regulating LC3 shuttling.**

Keywords: **Autophagy, LC3, Nucleocytoplasm, Nucleus, Cytoplasm, Dispersal.**

I. INTRODUCTION

Autophagy is a highly conserved, lysosomal-dependent destructive process, targeting unwanted materials, damaged organelles and recycles essential components for cellular survival [1], [2]. There are three types of autophagy, Chaperone-mediated autophagy, Microautophagy and Macroautophagy. The latter is found to be selective and widely studied; hereafter Macroautophagy is referred to as autophagy. Generally, the process begins by formation of phagophore membrane around targeted cellular components, then the phgophore becomes elongated and completed to form a double-membrane structure called autophagosome, which then fuses with lysosome, forming autolysosomes [3], [4].

Autophagy essentially functions during multiple physiological and pathological conditions. Starvation condition is among those cases, in which essential amino acids, aiding in the survival of the cells, are recycled into the cytoplasm of the cells [5]. Furthermore, autophagy has also a dual role in cancer, it could either avoid its occurrence through confining damaged cellular compartments, hence provides protection [6], [7], or promote survival of the tumor cells, thus aiding in the occurrence of cancer [8]. Additionally, it has neuroprotective roles in some neurodegenerative diseases, such as Parkinson's, Alzheimer's, Amyotrophic Lateral Sclerosis (ALS) and Huntington's disease [8], [9]. Moreover, autophagy also contributes in the degradation and presentation of intracellular microbes as a part of innate and adaptive immunity against infections [10], [11]. That is why broadening our understanding at cellular and molecular level is the key to gaining new insights on autophagy, that could implicate among different field of researches.

Until now, more 30 autophagy-related genes have been determined both in yeast and mammals [2], [12]. Microtubule Associated Protein 1 Light Chain-3 (MAP1-LC3), an Atg8 homolog of yeast, hereafter refers to as LC3, is the widely studied autophagic protein [13]. Originally, LC3 is believed to be implicated in assembly and disassembly of microtubules [14]. The protein can be found in two forms, LC3-I and LC3-II [15]. Although, the synthetic mechanism of this protein is not clear, its processing occurs in the cytoplasm through cleaving of the C-terminal region, forming LC3-I (18-16 kDa) which resides in the inner part of autophagosome. Later, the cleaved LC3 becomes activated to conjugate with Phosphotidyl Ethanolamine (PE), forming LC3-II (16-14 kDa) that locates in the outer side of autophagosomes. It is worth mentioning that at the end of autophagic process, LC-I is degraded within autophagosome contents and LC3-II is

recycled back to the cytoplasm. Thus, LC3 contributes in the entire process of autophagy. That is why it has been used successfully as a marker to monitor autophagy [16], [17]. At subcellular level, LC3 has been demonstrated at different locations, mainly in the Nucleus and Cytoplasm [26, 27, 31]. The last decade showed an extensive research in this field. Accordingly, our knowledge has also been broadening. Therefore, it is vital to review parts of this area in separate, as an attempt to gain better understanding as well as making connections among different aspects. In this regard, this review will shed a focused light on LC3 distribution in nucleus and cytoplasm with exploring current knowledges of pathways underlining LC3 distribution.

II. NUCLEOCYTOPLASMIC DISPERSAL OF LC3

Cytoplasm, a common reported place for LC3 localization, is the site where autophagosome growth and completion occurs. In the literature, the presence of LC3 in the cytoplasm is identified as a prerequisite for establishing autophagy [17], [18]. Despite that LC3 mainly involves in the autophagic process which occurs in the cytoplasm, its participation in other cellular activities has been identified as well. Among them; it is original identification in assembly and disassembly of Microtubule [14]. Beside this, it also regulates the level of fibrinonectin mRNA [19], [20]. Moreover, it has been defined as an interacting protein of the dendritic-specific Ca+2 sensing protein [21]. What is more, the LC3 protein also contributes in activation of membrane ruffling through interaction with a guanine nucleotide exchange factor, in an SOS1-dependent reaction [22]. All of these events have emphasized the importance of cytoplasm as a place for LC3 residence.

In spite of all the above mentioned functions of LC3, chief of which is autophagy, LC3 is demonstrated abundantly in the nucleus. Application of Florescence Microscopy for monitoring LC3, particularly, Green Florescent Protein (GFP) or Enhanced GFP (EGFP), a technique used to find localization site of interest proteins, became a widely used method. It has a specificity of preserving the biological function and properties of a labelled protein [23]. In that sense, GFP-LC3 was noted to be resided in the nucleus [24]. In Hela and Cos-7 cell lines, Drake et al. found nucleus enriched with EGFP-LC3 following induction of autophagy [26]. In human, there are three LC3 gene family members, LC3A, LC3B and LC3C. Additionally, two other variants of LC3A (Variant-1 and Variant-2) have also been reported in some published papers [28], [29], [30]. Among these family, LC3A (Variant-I), LC3B and LC3C are able to become conjugated with PE and create LC3-II [29]. Confocal Microscopical examination of LC3, under normal nutrient condition, can reveal nuclear localization of LC3A and LC3C. However, the LC3B can be noticed within both nucleolar region and cytoplasm [31]. Koukourakis et al. have demonstrated LC3 at the mentioned places using pre-validated specific anti-LC3 antibodies over a wide range of cell lines, including normal and cancerous cells such as Lung cancer A549 and H1299, Glioblastoma U87MG and T98G, Breast cancer, Embryonic MRC5 fibroblast, HeLa and COS-7 cell lines [31].

With an enormous increase in researches on autophagy, experts in the field began to evaluate the feasibility of using LC3 as a maker of autophagy, they outlined some alerting notes on common techniques used for interpreting LC3 localization. One of which is about the feasibility of using GFP-LC3. Despite the efficiency and broad use of this technique, it is reported that GFP-LC3 could interfere with other protein aggregates such as ubiquitinated proteins in the cystol, therefore, it makes LC3 interpretation much harder [18], [32], [33]. Similarly, it is also noted that EGFP-LC3 suppresses polyubiquitination, a post-translational modification process that involves adding a single or numerous ubiquitin molecules to a particular protein targeted for degradation. EGFP-LC3 blocks lys48, that targets proteins for degradation and lys63, which modulated polyubiquitination [25]. Therefore, experts recommend using more than one method in studying LC3 as an effort to make researches more reliable and valid. Alternatively, a transient expression of GFP or EGFP-LC3 is also recommended [18], [33]. In this regard, Drake et al. performed transient approach for EGFP-LC3 expression [26], making their used methodologies in line with current guidelines for monitoring autophagy [18].

Raising antibody against LC3 (Tagged Anti-LC3 Antibody) has been widely applied a long time ago for monitoring autophagy, most commonly, via using Immunotechniques (Immunohistochemistry, Immunocytochemistry and Immunoblotting) [33], [34] [35]. However, very few studies have validated the specificity of used antibodies prior to conducting LC3 investigations. In that respect, in the study by Koukourakis et al. antibody validation was conducted, making their findings more effective and reliable. Although, the study conducted without using any common methods for upregulating autophagy, such as starvation or targeting mTOR through Rapamycin, it provides strong evidence of LC3 localization among cells grown under basal condition [31]. Apparently, the induction of autophagy contributes in the translocating LC3 protein from nucleus to cytoplasm. Under certain conditions, such as starvation, the nuclear LC3 becomes activated and shifts into cytoplasm, where it interacts with Atg7, an autophagic gene encoding E1-like enzyme,

that is essential for biogenesis of autophagosome. In HEK293 cell line, using both endogenous (Antibody-based) and exogenous (EGFP-LC3), Huang et al. claimed that under normal nutrient condition, LC3 presents both in nucleus and cytoplasm. But under nutrient poor condition, a larger LC3 ratio was seen in the cytoplasm [27]. Whether all three family members of LC3 (A, B and C) translocate into cytoplasm or not after establishing autophagy remain unclear.

III. PATHWAYS REGULATING NUCLEOCYTOPLASMIC SHUTTLING OF LC3

A. Protein-protein interaction:

One possible method in controlling LC3 movement from nucleus to cytoplasm is proposed to be modulated through protein-protein interaction. Early evidence suggests that Atg4B, a protease that processes newly synthesized LC3, contributes in nucleus to cytoplasm translocation of LC3. Mutant Atg4B cells is found to be inhibiting autophagosome formation in the cytoplasm, possibly through accumulating free LC3 and restraining lipidation of LC3, hence avoiding autophagosome biogenesis [36]. Drake et al. have further supported this observation through demonstrating coexpression of EGFP-LC3 with a mutant form of Atg4B in cytoplasm. Beside this, EGFP-LC3 was shown to be relatively slow in movement and unequally distributed between nucleus and cytoplasm under basal nutrient condition [26]. Suggesting that a particular molecule may control LC3 movement, possibly Atg4B. These evidence exclude the probability of active and passive diffusion involvement in translocating LC3 from nucleus to cytoplasm.

B. Deacetylation and Acetylation reactions:

A highly possible mechanism behind regulating nucleocytoplasmic shuttling of LC3 is proposed to be Deacetylation and Acetylation reactions. Earlier researches highlighted the significant role of these two reverse reactions in autophagy [37], [38]. Sirtuins, specifically Sirt1, a family of NAD-dependent deacetylase, is the major component modulating nuclear LC3 deacetylation at K49 and K51 sites up on induction of autophagy. This consequently results in moving of LC3 to the cytoplasm. There, the deacetylated LC3 is enhanced to interact with Atg7, leading to establishing of autophagy under poor nutrient condition [27]. Initially, Sirt1 is thought to participate in controlling the interaction of Atg3-Atg8 induced by autophagy [39]. in a wide range of cell lines, nuclear LC3 deacetylation is demonstrated to be a prerequisite for initiation of autophagy, induced by limited nutrient condition. The Diabetes and Obesity Regulated nuclear factor (DOR) is responsible for transferring deacetylated LC3 from nucleus to cytoplasm [27]. DOR is identified as a nuclear protein that shuttles between nucleus and cytoplasm. Up on induction of autophagy, DOR is recruited to the cystol through interacting with LC3 protein. Hence, promoting autophagosome formation [40]. However, what exactly activates DOR remains obscure. Apparently, shifting LC3 from nucleus to cytoplasm following autophagy is a reversible action. Reports show that acetylation prevents LC3-Atg7 interaction in cytoplasm, leading to transferring LC3 back to the nucleus [27].

C. Nuclear Transport Pathway:

The nuclear transport of molecules, either import or export, is controlled by Nuclear Pore Complex (NPC), which is a well-known nuclear transporting pathway. Normally, macromolecules require to link with other proteins to enter or exit the nucleus, such as importins or exportins. On the other hand, cargos need to have a small peptide motif called Nuclear Localization Signals (NLS) or Nuclear Exporting Signals (NES) [41]. The involvement of nuclear transport pathways in the nucleocytoplasmic trafficking of LC3 is controversial. There are two different observations, in which one excludes the possibility of Nuclear Transport Protein pathway involvement in cytoplasmic shuttling of LC3. Treatment of COS-7 and HeLa cell lines with Leptomycin B, an Anti-fungal antibiotic which is known to suppress nuclear export protein [42], for 3 hours, did not show nuclear enrichment of EGFP-LC3 [27]. On the opposite side, the other observation, highly suggests the possibility of nuclear transport pathway participation in LC3 translocation. Treatment of glioblastoma and lung K cell lines with Leptomycin B for a longer period, 24 hours, resulted in nuclear accumulation of LC3 type A and C, not B [31]. In the light of current literature, although the first observation has of limited use, because EGFP-LC3 itself lacks any importing or exporting signals [26], both studies vary in terms of used methodologies. Firstly, the incubation time of cells treated with Leptomycin B varies in each study, 3 hours in the first instance and 24 house in the other. Secondly, the used cell lines were different. Thus, the type of cell lines seems to make a difference. As a note of caution, it has been claimed that treatment of cells could alter other cellular functions [18], therefore, inventing other techniques to confirm the probability of involving nuclear transport pathway in exporting and importing of LC3, rather than using Leptomycin B, would be of great importance in future implications.

Reviewing recent published papers Drake et al. [26], Huang et al. [27] and Koukourakis et al. [31] reveal that a number of factors contribute in studying and findings of nucleocytoplasmic distribution as well as function of LC3s. These factors force themselves as potential variables in studying autophagy; 1) The type of used cell lines. 2) Type and family member of demonstrated LC3 (LC3-I, LC3-II and LC3A, LC3B, LC3C). 3) Techniques used to monitor LC3 (Exogenous/Antibody-based or Exogenous/EGFP-LC3). 4) The nutrient condition of grown cells. 5) The status of autophagy, upregulated or downregulated.

IV. CONCLUSION

In short, it is apparent that LC3 presents in unequal ratio within both nucleus and cytoplasm. Induction of autophagy is the main phenomena behind shuttling of LC3 from nucleus to cytoplasm. The function of translocated LC3 is to participate in autophagosome biogenesis. In this regard, the term 'Dispersal' can best describe LC3 presence at subcellular level, because the translocated LC3 returns to the nucleus after autophagy ends. It is also highly likely that multiple pathways participate in the LC3 shuttling from nucleus to cytoplasm, due to the fact that LC3 has 3 family members, LC3A and LC3C that localize in the nucleus and LC3B in the nucleaolar region, the nucleocytoplasmic trafficking pathways of these LC3 members seem to be distinct, not the same. This is a potential area for further research along with exploring the functions of each of these LC3 members. What is more, determining LC3 localization within different cell lines both in normal and upregulated cells could broaden our understanding to better target and control autophagy, which could ultimately have of future implications.

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