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MEETING REPORT

Puncta intended: connecting the dots between autophagy and cell stress networks

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ABSTRACT

Proteome profiling and global protein-interaction approaches have significantly improved our knowledge of the protein interactomes of autophagy and other cellular stress-response pathways. New discoveries regarding protein complexes, interaction partners, interaction domains, and biological roles of players that are part of these pathways are emerging. The fourth Vancouver Autophagy Symposium showcased research that expands our understanding of the protein interaction networks and molecular mechanisms underlying autophagy and other cellular stress responses in the context of distinct stressors. In the keynote presentation, Dr. Wade Harper described his team's recent discovery of a novel reticulophagy receptor for selective autophagic degradation of the endoplasmic reticulum, and discussed molecular mechanisms involved in ribophagy and non-autophagic ribosomal turnover. In other presentations, both omic and targeted approaches were used to reveal molecular players of other cellular stress responses including amyloid body and stress granule formation, anastasis, and extracellular vesicle biogenesis. Additional topics included the roles of autophagy in disease pathogenesis, autophagy regulatory mechanisms, and crosstalk between autophagy and cellular metabolism in anti-tumor immunity. The relationship between autophagy and other cell stress responses remains a relatively unexplored area in the field, with future investigations required to understand how the various processes are coordinated and connected in cells and tissues.

Abbreviations: A-bodies: amyloid bodies; ACM: amyloid-converting motif; AMFR/gp78: autocrine motility factor receptor; ATG: autophagy-related; ATG4B: autophagy related 4B cysteine peptidase; CALCOCO2/NDP52: calcium binding and coiled-coil domain 2; CAR T: chimeric antigen receptor T; CASP3: caspase 3; CCPG1: cell cycle progression 1; CAR: chimeric antigen receptor; CML: chronic myeloid leukemia; CCOCs: clear cell ovarian cancers; CVB3: coxsackievirus B3; CRISPR-Cas9: clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9; DDXs: DEAD-box helicases; EIF2S1/EIF-2alpha: eukaryotic translation initiation factor 2 subunit alpha; EIF2AK3: eukaryotic translation initiation factor 2 alpha kinase 3; ER: endoplasmic reticulum; EV: extracellular vesicle; FAO: fatty acid oxidation; GABARAP: GABA type A receptor-associated protein; ILK: integrin linked kinase; ISR: integrated stress response; MTOR: mechanistic target of rapamycin kinase; MPECs: memory precursory effector T cells; MAVS: mitochondrial antiviral signaling protein; NBR1: NBR1 autophagy cargo receptor; PI4KB/PI4KIIIβ: phosphatidylinositol 4-kinase beta; PLEKHM1: pleckstrin homology and RUN domain containing M1; RB1CC1: RB1 inducible coiled-coil 1; RTN3: reticulon 3; rIGSRNAs: ribosomal intergenic noncoding RNAs; RPL29: ribosomal protein L29; RPS3: ribosomal protein S3; *S. cerevisiae: Saccharomyces cerevisiae*; sEV: small extracellular vesicles; *S. pombe: Schizosaccharomyces pombe*; SQSTM1: sequestosome 1; SF3B1: splicing factor 3b subunit 1; SILAC-MS: stable isotope labeling with amino acids in cell culture-mass spectrometry; SNAP29: synaptosome associated protein 29; TEX264: testis expressed 264, ER-phagy receptor; TNBC: triple-negative breast cancer; ULK1: unc-51 like autophagy activating kinase 1; VAS: Vancouver Autophagy Symposium

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-Omes by -omics: exploring the proteomes of selective autophagy with proteomics

Although initially characterized as a nonselective cellular degradation mechanism, accumulating evidence in recent years has highlighted that the process of macroautophagy (hereafter referred to as autophagy) also plays a crucial role in the maintenance of intracellular homeostasis through the selective degradation of components within the cell [1]. Selectivity is conferred by various autophagy receptors that bridge the interaction between a specific cytoplasmic cargo and the nascent phagophore during the process of autophagosome formation [1]. To help uncover molecular mechanisms underlying the selective degradation of cellular proteins and organelles in various contexts, Dr. Wade Harper (Harvard Medical School, USA) leverages quantitative proteomics. At the fourth Vancouver Autophagy Symposium (VAS), Dr. Harper described published findings from his group that

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uncovered a new endoplasmic reticulum (ER) reticulophagy receptor, TEX264, and its role in ER remodeling under conditions of nutrient stress [2]. TEX264 was discovered through a quantitative proteomics screen of changes in response to nutrient deprivation or MTOR inhibition in cells proficient or deficient in ATG7 or RB1CC1. TEX264 mediates reticulophagy independently of other reticulophagy receptors, such as CCPG1 and the long isoform (full length) of RTN3. Global proteome analyses revealed that at least half of starvation-induced reticulophagy flux is dependent on TEX264. Mechanistically, his team showed that TEX264 is tethered to the ER and interacts with Atg8-family proteins in *trans* via a synapse formed by the juxtaposition of the ER membrane and the phagophore membrane of autophagic structures. Fusion of these membranes eventually results in autophagosome formation and the degradation of ER components by the autophagy machinery [2].

Dr. Harper also described interesting new findings regarding an autophagy-independent reduction in ribosome protein abundance under conditions of nutrient stress. Using a novel quantitative reporter system known as Ribo-Halo to track ribosomes, Dr. Harper and his team observed a 25% reduction in the ribosomal proteins RPS3 and RPL29, and a 25% reduction in cellular volume following MTOR inhibition. Autophagy inhibition had no effect on the reduction of these ribosomal proteins in the absence or presence of MTOR inhibition. Work by Dr. Harper's team also showed that nutrient-dependent changes in ribosomal protein abundance was driven by a complex interplay of translational suppression of ribosomal proteins, degradation by both non-autophagic and to a lesser extent autophagic pathways, and by effects on dilution as a result of inhibition of cell division. Dr. Harper highlighted these new findings as a disconnect from his team's previous investigations into autophagy-mediated ribosome degradation [3].

His team previously established Ribo-Keima, a new quantitative reporter system to monitor ribophagy, and observed that MTOR inhibition results in an increase in ribosome turnover and ribophagic flux. This increase is dependent on the essential autophagy protein PIK3C3/VPS34 (phosphatidylinositol 3-kinase catalytic subunit type 3) [3], but the new results indicate that the overall contribution of ribophagic flux to ribosome homeostasis is small relative to the other pathways that are affected. Dr. Harper stressed the importance of utilizing more than a single assay when studying autophagy, and the need for a better understanding of the molecular mechanisms underlying non-autophagic ribosomal turnover and how it functions distinctly from ribophagy.

A higher-resolution view of autophagy protein complexes

There are many unanswered questions regarding how the different corresponding Atg proteins coordinate the different steps of autophagy. At the fourth VAS, Dr. Calvin Yip presented recent progress on elucidating the biochemical and structural properties of the Atg1 autophagy initiation complex from yeast and a late-acting metazoan-specific autophagy factor. The well-studied budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) Atg1 autophagy initiation complex consists of five subunits (Atg1, Atg13, Atg17, Atg29 and Atg31). Dr. Yip showed previous structural findings from his group that the Atg17-Atg31-Atg29 subassembly of the *S. cerevisiae* Atg1 complex forms an S-shaped architecture, and the implications of this subunit arrangement in the regulation of early vesicle tethering and Atg1 kinase activation [4]. Interestingly, the orthologous mammalian ULK1 complex has a different subunit composition compared to the *S. cerevisiae* Atg1 complex. Notably, a novel protein, ATG101, is present in place of Atg29 and Atg31. Dr. Yip next presented data on their studies on the fission yeast *Schizosaccharomyces pombe* (*S. pombe*) Atg1 complex which has the same subunit composition as the ULK1 complex, and consists of four subunits (Atg1, Atg13, Atg17 and Atg101). Dr. Yip's team found that, although the interactions amongst the core subunits (Atg1, Atg13, Atg17) are preserved, Atg101 does not bind Atg17 and instead serves a unique function. Furthermore, their structural finding that *S. pombe* Atg17 adopts a rod-shaped architecture suggests that early vesicle tethering can be mediated by protein factors lacking inherent curvature. In the last part of his talk, Dr. Yip shared unpublished work on a mammalian autophagy factor that has been suggested to participate in the autophagosome-lysosome fusion process. His group performed a systematic biochemical affinity isolation that revealed this protein is capable of interacting with the mammalian Atg8-family proteins, but preferentially binds to the three GABA type A receptor-associated protein members, GABARAP, GABARAPL1 and GABARAPL2. His group also obtained preliminary electron microscopy data showing that this protein has an extended architecture reminiscent of vesicle tethering factors. Ongoing studies by Dr. Yip and his team continue to significantly boost our knowledge in this understudied area of the molecular structures and functions of autophagy factors.

Dot by dot: cellular stress triggers various physiological responses

The autophagy process is frequently upregulated in conjunction with various response pathways under conditions of stress [5,6]. Changes in the interactome of proteins that are part of these pathways, and the crosstalk between them, have been longstanding interests in the field of autophagy and stress response processes. At the fourth VAS, multiple oral and poster presentations reported investigations in this area including 1) the formation of amyloid bodies, otherwise known as A-bodies, 2) the formation of stress granules, 3) the induction of anastasis, and 4) the crosstalk between autophagy and extracellular vesicle (EV) biogenesis.

"A-body" of stress aggregates

Dr. Timothy Audas and his team investigate a cellular stress response that involves the assembly of amyloid bodies (A-bodies). A-bodies are membrane-less subnuclear foci that form reversibly, and contain heterogenous populations of amyloid-like and insoluble proteins [7]. These structures are distinct from amyloids that are commonly observed in

neurodegenerative diseases, which are β-sheet-rich protein aggregates that form in an irreversible fashion [7]. Using various normal and cancer cell line models, Dr. Audas and colleagues showed that, upon exposure to external stressors such as heat, hypoxia, or acidosis, folded soluble nuclear and cytosolic proteins enter the nucleolus to form insoluble amyloid aggregates in a rapid fashion [7]. They identified a unique motif on A-body targeted proteins, known as the amyloid-converting motif (ACM), which targets proteins for conversion from their native-fold into an amyloid-like state [7].

Dr. Audas and colleagues also showed that proteins within these A-bodies are released and resolubilized when stress stimuli are terminated, a process regulated by heat shock chaperones [7]. Interestingly, A-body formation is mediated by interactions with ribosomal intergenic noncoding RNAs (rIGSRNAs), and cells enter a dormant state when these rIGSRNAs-A-bodies form [7]. Using stable isotope labeling with amino acids in cell culture-mass spectrometry (SILAC-MS), they identified various proteins that are involved in cell cycle progression and DNA synthesis within A-bodies that formed under conditions of acidosis. This event coincides with a reduction in cellular proliferation and DNA synthesis, suggesting that the sequestration of proteins that are involved in crucial cellular processes into A-bodies functions as a protective mechanism [7]. Together, his work showed that proteins that bear ACMs can be targeted to A-bodies under conditions of stress, and this allows storage of large amounts of proteins as a cell enters a state of dormancy under duress. These findings are significant as they highlight how amyloid formation may have physiological functions aside from the generation of toxic protein aggregates in neurodegenerative diseases. Dr. Audas emphasized that our understanding of the biological roles of A-bodies, along with the molecular mechanisms underlying their assembly, is still limited.

To help address these questions, Dr. Audas and his team utilized their SILAC-MS data to evaluate the heterogeneity of A-body proteins that form in response to distinct stressors [8]. Of note, Dr. Audas presented new findings made regarding proteins known as DEAD-box helicases (DDXs). DDXs are a large and highly conserved family of RNA helicases, which are important in many facets of RNA regulation and metabolism [9]. His group found that closely related RNA helicases can be differentially targeted to A-bodies, depending on the stressor. Using site-directed mutagenesis, they showed that altering key amino acids within the DDX ACM is sufficient to impair A-body sequestration under some, but not all, conditions, highlighting the cell's ability to tailor its response to divergent cellular stressors. Further investigations to uncover mechanisms of A-body formation and their functional relevance are currently underway in the Audas lab.

Integrating stress into granules

Medulloblastoma accounts for approximately 20% of all childhood brain tumors [10]. Medulloblastoma patients with tumors that present with an amplification of *MYC* fall under the group 3 subtype and have one of the highest mortalities [11]. Aberrant activation of EIF2AK3/PERK and hyper-assembly of stress granules have been associated with the tumorigenesis and progression of medulloblastomas [12,13]. Sofya Langman, a trainee from Dr. Poul Sorensen's group, studies the crosstalk between the integrated stress response (ISR) pathway and stress granule formation in *MYC*-amplified medulloblastoma. Sofya discovered that the genetic knockdown of EIF2AK3 in group 3 medulloblastoma cells promoted cell death under hypoxia-inducing conditions. Similarly, pharmacological inhibition of ISR, using the small molecule integrated stress response inhibitor ISRIB, promoted cell death under hypoxia. Sofya also showed that pharmacological inhibition of ISR mitigated stress granule formation in the presence of sodium arsenite. Overall, her research demonstrated that the inhibition of the ISR pathway sensitizes group 3 cells to hypoxic stress and presents a new potential therapeutic avenue for the treatment of group 3 medulloblastoma patients.

Resuscitation from extreme stress

The crosstalk between apoptosis and stress response pathways following exposure to cellular insults often dictates a cell's fate. If cells fail to withstand stress, cell death pathways are activated. However, in some contexts, the activation of a cell death pathway does not inevitably result in apoptosis, but cells may still come back to life via a process known as anastasis [14]. Anastasis is a reversal of end-stage apoptosis following CASP3 activation, and occurs only in the presence of initiator caspases [14]. Using a cell line model of triple-negative breast cancer (TNBC), Jennifer Nagel, a trainee from Dr. Shoukat Dedhar's group, demonstrated that not all cells that activate CASP3 following cisplatin treatment undergo cell death. In fact, Jennifer discovered that not only do a subpopulation of cells survive, but that they also expressed a truncated CASP3 isoform that was previously observed by others [15]. She found that the expression of this isoform could be inhibited using an SF3B1 subunit splice inhibitor, and is currently working on deciphering the underlying molecular mechanisms. Jennifer's research also focuses on exploring the link between anastasis and chemoresistance in TNBC. Her studies showed that epithelial-mesenchymal transition/EMT-associated proteins, like SNAI/Snail and ILK, were upregulated in TNBC cells that have undergone anastasis. Jennifer discovered that pharmacological inhibition of ILK in anastatic treatment-resistant TNBC cells resensitized them to cisplatin or paclitaxel. Her research supports a potential role for anastasis as a novel mechanism for resistance in TNBC and provides mechanistic insight into the role of anastasis in tumor cell biology.

To eat, or not to eat

The contributions of the autophagy machinery to the composition and function of extracellular vesicles are of special interest to the field of cancer biology [16]. Dr. Morgana Xu, a postdoctoral fellow from Dr. Sharon Gorski's group, investigates the crosstalk between autophagy and small extracellular vesicle (sEV) biogenesis in TNBC cells. To understand the role of autophagy in anthracycline resistance, Dr. Xu and colleagues previously showed that treatment-resistant TNBC

cells have a higher basal level of autophagy compared to treatment-sensitive cells [17]. Treatment with the autophagy inhibitor chloroquine (CQ) sensitizes resistant TNBC cells to the anthracycline epirubicin [17]. Using TMT-based quantitative proteomic profiling, Dr. Xu discovered that various Atg8-family proteins and autophagy receptor proteins are enriched in TNBC-derived sEVs following CQ treatment. Dr. Xu showed that Atg8-family proteins localize in the lumen of sEVs, and this localization is dependent on the lipidation state of the Atg8-family proteins. These findings indicate a role for autophagy proteins (in particular, Atg8-family proteins) in sEV biogenesis. Further studies regarding the functional relevance of these observations are warranted, and Dr. Xu reported initial findings of the impact of CQ on the biological function of sEVs. Dr. Xu's work has important new implications for potential cell non-autonomous effects of CQ treatment in cancer.

Connecting the dots – autophagy and its roles in diseases

Significant work over the past two decades has provided experimental and clinical support for autophagy's role in the initiation and progression of various diseases [18–21]. However, the role of autophagy is often Janus-faced depending on the context. Improving our current understanding of the molecular mechanisms underlying autophagy in the pathogenesis of different diseases will help identify and clarify contexts where autophagy modulation will be beneficial [18–21]. Here we summarize select talks presented at the VAS that describe new findings regarding roles and regulatory mechanisms of autophagy in diseases such as viral infections, cardiac diseases and cancer.

Autophagy in viral infections and cardiac diseases

Dr. Honglin Luo and her group study the interplay between enteroviral infections and autophagy in cardiac diseases. Using coxsackievirus B3 (CVB3) as a model, her group previously showed that viral infections are associated with a block in autophagic flux. Cells infected with CVB3 present with an accumulation of autophagosomes, poly-ubiquitinated proteins, and protein aggregates. In addition, autophagy adaptor proteins, such as SQSTM1/p62 and NBR1, are cleaved by viral proteinases [22,23], resulting in a disruption of selective autophagy. Building on these studies, Dr. Luo and her team discovered that the CVB3 viral proteinase 3C targets two other proteins involved in the autophagy process, SNAP29 and PLEKHM1 [24]. Cleavage of SNAP29 and PLEKHM1 consequently prevents autophagosome-endolysosome fusion and facilitates viral replication [24]. Dr. Luo's team also showed that CVB3 targets CALCOCO2 for cleavage by viral proteinase 3C, generating a stable pro-viral fragment that retains the activity of the full-length protein [25]. This pro-viral CALCOCO2 fragment promotes autophagy-mediated degradation of MAVS, thereby suppressing antiviral type I interferon signaling and promoting viral propagation. Together, these findings suggest a model whereby CVB3 appropriates the autophagy machinery to evade host cell immunity and facilitate viral propagation.

The Luo lab is currently investigating the mechanism by which CVB3 initiates the autophagy pathway. Using a combination of gene-silencing and editing approaches, they determined that CVB3 bypasses well-established canonical factors such as the ULK1/2 and BECN1-PIK3C3/VPS34 complexes to initiate autophagy. Because the viral protein 3A was previously shown to recruit PI4KB/PI4KIIIβ to organelles that function as hubs for viral replication [26], the Luo group knocked down the expression of PI4KB and discovered that CVB3-induced autophagy was reduced. In parallel, the Luo group is investigating the association between CVB3-induced EIF2S1/eIF2alpha phosphorylation and the initiation of non-canonical autophagy.

Cancer and autophagy

Yueyang Shen, a trainee from Dr. Xiaoyan Jiang's team, is currently investigating the utility of the small molecule inhibitor LV-320, which inhibits ATG4B [27], in chronic myeloid leukemia (CML). His studies expand on previous findings by the Jiang lab that uncovered a pro-tumorigenic role of ATG4B in CML progression and drug resistance [28]. Shen presented recent findings demonstrating that LV-320 treatment resensitized CML stem and progenitor cells, derived from drug-nonresponder patients, to the tyrosine kinase inhibitor imatinib. Interestingly, LV-320 treatment *in vivo* was most effective in preventing leukemia progression when mice were fed with a caloric restriction diet, suggesting that ATG4B is critical for leukemic cell survival upon nutrient deprivation and autophagy induction. These findings provide pre-clinical support for LV-320 as a potential therapeutic drug for ATG4B inhibition in CML.

Parsa Alan, a trainee from Dr. Ivan Nabi's group, investigates the role of AMFR/glycoprotein 78/gp78 in damage-induced, PRKN-independent mitophagy [29]. AMFR is an E3 ligase that is involved in ER-associated degradation/ERAD [30]. Using a fibrosarcoma cell line model that expresses elevated levels of AMFR [31], the Nabi lab knocked out the expression of this protein using CRISPR-Cas9 gene editing and showed that this resulted in a defect in mitophagy. Alterations in mitochondrial morphology and an impairment in mitochondrial metabolism were also observed, although tumor volumes were variable *in vivo* [31]. Further studies to determine if AMFR plays a similar role in regulating mitophagy in other cancers may provide promise for investigating the therapeutic potential of targeting AMFR and/or mitophagy in a cancer context.

Crosstalk within the triad: autophagy, metabolism and the immune system

Dr. Julian Lum and his team investigate the functional links between autophagy and cellular metabolism. At this year's meeting, Dr. Lum presented new findings from his team regarding the role of autophagy as a metabolic switch in immune cells. T cells often undergo metabolic reprogramming, where they switch between catabolic and anabolic

phases depending on their biosynthetic and energy needs at different stages of development [32]. For example, regulatory T cells/(Tregs) and memory T cells typically produce energy through oxidative phosphorylation and fatty acid oxidation (FAO). In contrast, effector T cells rely on glycolysis and fatty acid synthesis to maintain their basic cellular functions [32]. Studies by Dr. Lum and his team uncovered a pivotal role of the autophagy process in regulating the glycolytic metabolism of T cells [33]. They showed previously that *atg5* knockout mice that lack functional autophagy contain reduced levels of effector T cells, and are unable to generate memory T cells [34]. Consequently, these mice present with increased levels of memory precursor effector T cells (MPECs) that have higher levels of glycolysis and reduced levels of FAO. Dr. Lum and his team then showed that autophagy is required for the liberation of fatty acids to facilitate FAO, and the increased reliance on glycolysis in these MPECs is a direct result of impaired autophagy and reduced biosynthetic substrates for FAO. Dr. Lum and his team are currently working on elucidating the mechanisms underlying the role of autophagy in facilitating the metabolic switch between different catabolic pathways in T cells.

The Lum lab is also presently investigating the role of T cell immunity in the tumorigenesis of clear cell ovarian cancers (CCOCs). Hypoxia is a known hallmark of CCOC [35], but it is not known how this hypoxic nature affects the immune cells in the tumor microenvironment. Dr. Lum described early findings from his team that showed that, under conditions of hypoxia, T cells in the tumor microenvironment of CCOCs upregulate autophagy for survival [36]. Indeed, they showed that autophagy inhibition was associated with a reduction in T cell levels. Recent findings in Dr. Lum's lab have revealed an unexpected role for autophagy in suppressing antitumor immune responses. Genetic ablation of *ATG5* in T cells led to an increase in their metabolic activity and ability to kill tumor cells. This discovery presents a novel therapeutic opportunity to improve the efficacy of chimeric antigen receptor (CAR) T cell immunotherapy in solid tumors. CAR T cells are genetically engineered immune cells that recognize tumor-associated antigens in a manner much more robust than T cells that bear native T cell surface receptors [37]. To this end, the Lum lab have devised a CRISPR-Cas9 gene-editing strategy to knock out the function of autophagy in CAR T cells as a novel immunotherapy strategy to treat ovarian cancer.

Conclusions

Omics approaches have contributed significantly to our understanding of the regulation of autophagy and its extended protein-interaction network. At the same time, new EM imaging technologies are revealing the precise architecture of individual protein complexes within this vast network. Given the immense contributions of autophagy to the pathogenesis of various diseases, such knowledge is important to guide the development of effective therapeutic targets and combination strategies. Research presented at the fourth VAS underscored the significance of proteomic approaches, in particular, that shed new insights into proteins that are part of various stress response machineries and also potential therapeutic targets for disease treatment. Although autophagy is a major protective mechanism that is activated when cells are under stress, it is important to recognize that other cellular processes, such as protective amyloidogenesis or stress granule formation, can also be activated. Little is known about the temporal, spatial and molecular coordination between autophagy and these other cellular stress response pathways. Similarly, it is not well understood how intracellular recycling by autophagy is coordinated with the extracellular release of materials though EVs. Future research in these areas is required to better define the players and their roles within pathways, the connections and coordination between these "dots" in response to distinct stressors, and ultimately to pinpoint potential new therapeutic strategies for disease treatment and management.

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