

Precision autophagy: Will the next wave of selective autophagy markers and specific autophagy inhibitors feed clinical pipelines?

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Abbreviations: ATG, autophagy related; HCQ, hydroxychloroquine; HTT, Huntingtin; HD, Huntington disease; HIV, human immunodeficiency virus; LIR, LC3-interacting region; PDAC, pancreatic ductal adenocarcinoma; SIV, simian immunodeficiency virus; TRIM, tripartite motif-containing; VAS, Vancouver Autophagy Symposium; VHL, von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase

Research presented at the Vancouver Autophagy Symposium (VAS) 2014 suggests that autophagy's influence on health and disease depends on tight regulation and precision targeting of substrates. Discussions recognized a pressing need for robust biomarkers that accurately assess the clinical utility of modulating autophagy in disease contexts. Biomarker discovery could flow from investigations of context-dependent triggers, sensors, and adaptors that tailor the autophagy machinery to achieve target specificity. In his keynote address, Dr. Vojo Deretic (University of New Mexico) described the discovery of a cargo receptor family that utilizes peptide motif-based cargo recognition, a mechanism that may be more precise than generic substrate tagging. The keynote by Dr. Alec Kimmelman (Harvard Medical School) emphasized that unbiased screens for novel selective autophagy factors may accelerate the development of autophagy-based therapies. Using a quantitative proteomics screen for de novo identification of autophagosome substrates in pancreatic cancer, Kimmelman's group discovered a new type of selective autophagy that regulates bioavailable iron. Additional presentations revealed novel autophagy regulators and receptors in metabolic diseases, proteinopathies, and cancer,

and outlined the development of specific autophagy inhibitors and treatment regimens that combine autophagy modulation with anticancer therapies. VAS 2014 stimulated interdisciplinary discussions focused on the development of biomarkers, drugs, and preclinical models to facilitate clinical translation of key autophagy discoveries.

Precision Autophagy

Dr. Vojo Deretic investigates autophagy in infection, immunity, and inflammation, and recently coined the term "precision autophagy" in reference to the mechanism of substrate recognition of a family of antiviral proteins that his group found to both regulate autophagy and serve as selective autophagy cargo receptors.¹ A screen of the TRIM family for roles in autophagy revealed that several TRIM family members modulate autophagy and directly bind subsets of mammalian Atg8 family proteins, favoring GABARAP binding. Detailed functional characterization of TRIM5/TRIM5 α revealed an ability to regulate phagophore nucleation and to act as a cargo receptor that tailored the autophagy machinery to degrade human immunodeficiency virus (HIV)-1 capsid protein p24. In a rhesus macaque cell line, TRIM5 increases autophagy flux, colocalizes with active phospho-ULK1 (Ser317) and BECN1 in proximity to the omegasome marker ZFYVE1/DCP1, and pulls down complexed ULK1-BECN1 in co-immunoprecipitation experiments. These findings generalized to a wider panel of TRIM proteins in a SPRY domain-dependent manner. The SPRY domain of TRIM5 specifically binds the tertiary structure of target protein HIV p24 and not simian immunodeficiency virus p24, leading to

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a lysosomal elimination of HIV-1 viral particles; the elimination was dependent on both autophagy and TRIM5 binding of GABARAP, via LC3-interacting region (LIR) domains. In contrast to the less selective strategy of targeting ubiquitin- or LGALS/galectin-tagged substrates for degradation by autophagy, this characterization of TRIM function highlights the existence of a more *precise* autophagy—the exclusive recognition of motifs on targets by particular domains on cargo receptors—and suggests that a much larger pool of selective autophagy receptors awaits discovery.

Selective Autophagy Eats Iron

Dr. Alec Kimmelman studies the interplay of constitutively activated basal autophagy and dysregulated metabolism in pancreatic cancer. Building autophagy incompetent (conditional *Atg5* null allele) mouse models previously engineered to recapitulate the lethal human disease (conditional *KRAS*^{G12D/+}; *TRP53*^{-/+}), Kimmelman's group confirmed that autophagy is required for progression from benign pancreatic intraepithelial neoplasia to pancreatic ductal adenocarcinoma (PDAC)—affirming autophagy inhibition as a viable complementary treatment for PDAC.² Tumor progression also requires spontaneous *TRP53* loss of heterozygosity; however, *Trp53* genotype (*Trp53*^{-/+}, *Trp53*^{-/-} or *Trp53*^{R172H/+}) does not affect treatment response to pharmacological inhibition of autophagy in a panel of murine PDAC cell lines and in patient-derived xenografts. Dr. Kimmelman's research also focuses on identifying mechanisms of altered tumor metabolism that can be exploited for targeted therapies in PDAC, including recent work that identified a reliance on rewired glutamine metabolism that is selective for pancreatic cancer cells but is dispensable in non-transformed cells. Autophagy is required for PDAC growth and is an important metabolic regulator in RAS-driven cancers. Kimmelman's group applied quantitative proteomics and bioinformatic analyses to systematically identify cargo in pancreatic cancer autophagosomes, excluding proteins nonspecifically captured by bulk autophagy by filtering candidate enriched autophagosomal proteins against their relative abundance in the total proteome. Characterization of highly enriched candidate NCOA4 (nuclear receptor coactivator 4)—in cancer and normal-like cell lines of diverse tissue origin—revealed it to be a cargo receptor for FT/ferritin and a novel selective autophagy (ferritinophagy) that regulates bioavailable iron.³ In response to low iron, NCOA4 colocalizes with FT/ferritin in MAP1LC3B-positive puncta, accumulates along with its target following pharmacological autophagy inhibition, and is necessary to direct FT/ferritin to the lysosome in an ATG5-dependent manner. NCOA4 knockdown increases ferritin levels and decreases bioavailable iron as evidenced by an increase in IREB2 (iron-responsive element binding protein 2). The discovery of ferritinophagy catalogs a new trigger-cargo receptor-target combination, expanding the repertoire of metabolic stimuli for which selective autophagy governs homeostasis.

Biomarker Buffet: Novel Autophagy Receptors and Regulators in Disease

Autophagy exerts a profound influence on the health of diverse tissues and cell types, in a myriad of contexts of development, homeostasis, and disease. The multiplicity of autophagy's roles is achieved through the retooling of its machinery to respond to specific triggers and to capture precise targets. Defining the agents of autophagic specificity is key for the development of novel targeted therapies and measures that will stratify potential patients by autophagy status. A panel discussion at the symposium agreed that to accurately determine the clinical utility of modulating autophagy, there is a pressing need for as-yet undiscovered biomarkers that measure autophagy pathway activity in vivo with respect to detection, screening, diagnosis, and treatment of various diseases. A number of short talks and poster presentations focused on the identification and characterization of autophagy receptors and regulators in novel disease contexts.

Autophagy receptors simultaneously bind specific targets and the core autophagy machinery; CLU/clusterin is a receptor that binds multiple autophagy-related proteins. Dr. Fan Zhang of Dr. Martin Gleave's group (Vancouver Prostate Center) demonstrated that the increased efficacy gained from CLU inhibition in combination with chemotherapy in castrate-resistant prostate cancer—currently in phase III trials—is in part due to a role as an autophagy receptor that increases autophagosome biogenesis in response to treatment stress.⁴ CLU-LC3B binding was mapped to LIR domain 341YNEL, and its simultaneous colocalization and direct interaction with ATG3 promotes LC3B lipidation and autolysosome maturation in mouse models of prostate cancer. Pharmacological inhibition of CLU in combination with AZD5363 or paclitaxel significantly delays mouse tumor growth and reduces LC3-II protein levels, suggesting concomitant autophagy suppression. Junyan Shi from Dr. Honglin Luo's group (Center for Heart Lung Innovation, St. Paul's Hospital) described a mechanism of viral pathogenesis that alters selective autophagy receptors themselves. Coxsackievirus type B3 proteases cleave SQSTM1/p62 and its functional homolog, NBR1, creating cleavage fragments with dominant-negative effects against their respective native proteins.⁵ The C-terminal truncated proteins result in accumulated endogenous SQSTM1, suggesting they contribute to noxious aggregate accumulation and viral pathogenesis by crippling ubiquitin-mediated selective autophagy.

Defective selective autophagy underpins multiple proteinopathies. Drs. Dagmar Ehrnhoefer and Dale Martin of Dr. Michael Hayden's group (Center for Molecular Medicine and Therapeutics) reported that dysregulated autophagy and increased pathogenesis in Huntington disease (HD) results from a gain-of-function mutant HTT/huntingtin and a loss of wild-type HTT function, mediated by disease-related HTT proteolysis and changes to autophagy-regulating, posttranslational modifications of mutant and wild-type HTT.⁶⁻⁸ Dr. Martin identified a novel autophagy-inducing domain (HTT553-586) released by caspase cleavage at D552 and D586, that is dependent on myristoylation at G553 to localize HTT to the endoplasmic reticulum and promote autophagosome formation. Myristoylation of HTT is

reduced in the presence of the HD mutation, suggesting a possible mechanism by which autophagy is defective in HD patient lymphoblasts. In addition, Dr. Ehrnhoefer demonstrated that the ablation of CASP6, which is the primary caspase responsible for cleavage at D586, leads to altered levels of autophagy proteins and reduced mutant HTT levels in a mouse model of HD, suggesting a possible protective effect.⁶

The list of autophagy pathway regulators and interactors continues to increase rapidly, providing ever-new quarry for the hunt for biomarkers in disease contexts. Chandra Lebovitz from Dr. Sharon Gorski's group (BC Cancer Agency and Simon Fraser University) defined a set of 211 autophagy-associated genes curated from the literature to query The Cancer Genome Atlas public reservoir of cancer patient raw sequence data and to comprehensively catalog significant DNA and RNA alterations of autophagy-associated genes, in patient tumors across multiple cancer types. Using mouse models of diet-induced insulin resistance, Dr. Gary Sweeney (York University) discovered that adiponectin mediates its insulin sensitizing effects in skeletal muscle via AMPK-induced autophagy⁹—a novel mechanism of autophagy regulation that his laboratory is pursuing in parallel to explain adiponectin's protective effects in mouse models of heart failure, a common complication associated with diabetes. The breadth of regulators and contexts in which autophagy modulation was demonstrated highlights autophagy's impact as a key mediator of health and disease, and underscores its potential to be a measurable indicator of normal biological processes, pathogenic processes, and pharmacological responses of patients to therapeutic interventions.

Exotic Flavors of Combination Autophagy Inhibition in Cancer

Chloroquine and its derivatives are inhibitors of autophagy that target the lysosome, but may not accumulate to sufficient levels in all tumors to adequately inhibit autophagy in vivo. Wieslawa Dragowska of Dr. Marcel Bally's group (BC Cancer Agency) reported that pharmacological or genetic inhibition of treatment-induced protective autophagy further decreases viability of triple threat (PIK3CA-mutated, ERBB2/HER2-overexpressing, Lapatinib-resistant) breast cancer cells in culture, whereas hydroxychloroquine (HCQ) fails to reach meaningful inhibition of drug-induced autophagy in mouse tumors. Dr. Jagbir Singh of the same group is leading the development of intravenously injected, lipid-based nanoparticle formulations of HCQ with the goal to achieve high plasma and tumor tissue drug levels over extended periods of time. Specific inhibitors of autophagy would be a boon for use in preclinical settings, and potentially in clinical trials. Dr. Damien Bosc of Dr. Robert Young's group (Simon Fraser University) presented the development of an ATG4B inhibitor—a key enzyme responsible for activation of pro-LC3 to LC3-I and for LC3-II deconjugation during autophagosome maturation. Drs Jianghong An and Steven Jones (BC Cancer Agency) performed extensive in silico screening against the NCI and Chembridge small molecule libraries, and in collaboration with Dr. Sharon

Gorski's group, hits were evaluated for inhibition of ATG4B activity (using a fluorescence-based, cell-free assay developed by the Young group^{10,11}) and autophagy flux in cancer cells. A structure-activity relationship study for analogs of a lead quinoline-based compound produced an ATG4B inhibitor with an excellent pharmacokinetic profile in mice, which has been confirmed to inhibit autophagy flux in vitro and reduce growth of cancer cells. Lys05 is a previously published lysosomal inhibitor that exhibits more potent in vivo autophagy inhibition than HCQ.¹² Dr. Lindsay DeVorkin of Dr. Julian Lum's group (BC Cancer Agency) tested Lys05 autophagy inhibition in a panel of clear cell ovarian cancer cell lines in combination with Sunitinib—a multi-targeted receptor tyrosine kinase inhibitor that achieved only a modest response rate of 8.3% in a recent phase II trial—and found that combination treatment impairs cell recovery, reduces cell viability and increases apoptotic cell death. Dr. Spencer Gibson (Cancer-Care Manitoba's Research Institute of Oncology and Hematology) is employing similar combination treatment strategies to disable cell survival benefits conferred by cancer-specific mechanisms of induced autophagy: increased apoptotic cell death is achieved in various cancer lines by inhibiting hypoxia-induced autophagy in combination with EGFR inhibitors. Dr. Sandra Turcotte (Atlantic Cancer Research Institute) described a small molecule STF-62247 that alters both autophagy and lysosomal activity, and specifically kills renal cells lacking VHL (von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase), a tumor suppressor gene frequently lost in renal cell carcinoma. Although the reagents for successful in vivo inhibition of autophagy are still under development, combination strategies that pair autophagy and/or lysosomal inhibition with standard therapy to potentiate treatment efficacy continue to gain credibility across cancer types, particularly in treatment-refractory disease.

Conclusions

Our ability to modulate autophagy with clinical utility will likely depend on the depth of our understanding of its context dependence, and of the selective nature of its regulation and substrate targeting. Increasing numbers of studies identify perturbed selective autophagy of proteins, aggregates, and organelles as pathogenic mechanisms in metabolic and neurodegenerative diseases, as well as in cancer and immunity. The discovery of precision autophagy begs the question of whether there are cooperating layers of increasingly selective autophagy overseeing the well being of cells, analogous to innate and adaptive mechanisms of immune surveillance. Research presented at VAS 2014 suggests that continued elucidation of autophagy receptors and adaptors in novel contexts of health and disease—including the unique triggers and regulators that kick them into action and the recognition mechanisms that tether them to cargo and the autophagy machinery—will identify *precision* biomarkers, and pinpoint novel therapeutic targets that refine our ability to measure and modulate autophagy for the benefit of patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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