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High-throughput screening approaches to identify regulators of mammalian autophagy

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ABSTRACT

This article discusses the issues to consider in the development and implementation of high-throughput screens (HTSs) using both siRNA libraries and small molecule compound collections, in order to discover autophagy regulators in mammalian cells. We discuss how to develop the screen, focusing on the key parameters to establish in order to perform a successful screen. As our understanding of autophagy increases and its impact on human disease is elucidated, this technology can be further exploited to uncover novel genes, which may one day become new therapeutic targets.

1. Introduction

1.1. Overview

Macroautophagy is a highly conserved membrane-mediated pathway by which cells sequester cytoplasmic material and target it to the lysosome for degradation. This degradation and turnover of material is called autophagic flux and is required for functional autophagy. Macroautophagy continuously operates at basal levels to remove damaged organelles and cytoplasmic material in a non-selective manner (*note:* macroautophagy is commonly called autophagy). In addition, organelles (for example mitochondria) or pathogens can be selectively removed in an autophagic process called mitophagy and xenophagy, respectively. Stress, nutrient deprivation, and infection all increase autophagy and autophagic flux. Autophagy has been implicated in many human diseases, and although its role can be complex [1], targeting autophagy is seen as a possible new avenue for therapeutic intervention [2].

The autophagy pathway starts with the formation of a double-membrane cisternae, called a phagophore, which originates from the endoplasmic reticulum. The growth and expansion of the phagophore requires membranes from other compartments such as the Golgi complex, recycling endosomes, and the plasma membrane [3]. Once expansion is complete, closure of the double-membrane vesicle leads to an autophagosome that can then fuse with endosomes and lysosomes to become a degradative autolysosome.

Autophagy requires 18 core Atg (autophagy related) proteins which act in a concerted hierarchy to drive membrane formation, and mediate both non-selective and selective degradation [4]. Except for the Atg8 family of proteins, other Atg proteins act transiently and do not become a part of the autophagosome. Upon induction of autophagy, Atg8, and its mammalian family members (LC3A, B, C, GABARAP, GABARAPL1 and GABARAPL2/GATE-16) are conjugated to phosphatidylethanolamine (PE) and bound to both surfaces of the phagophore, and eventually retained inside the

Abbreviations: Atg, autophagy related; BafA, Bafilomycin A1; CrispR, clustered regularly interspaced short palindromic repeats; EBSS, Earle's balanced salt solution; HTS, high-throughput screen; LIR, LC3-interacting region; MAD, median absolute deviation; PE, phosphatidylethanolamine; RP, rank product; SCPO, spot count per object; siRNA, small interfering RNA; shRNA, short hairpin RNA; SMERS, small-molecule enhancers of rapamycin-induced TOR inhibition; SMIRS, small-molecule inhibitors of rapamycin-induced TOR inhibition; STAPO, spot total area per object; STIPO, spot total intensity per object; UBA, ubiquitin-associated.

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closed autophagosome. Thus, the Atg8 family members are the only Atg proteins that can serve as markers of the forming and closed autophagosome. The Atg8 family members are thought to themselves be crucial for phagophore membrane expansion and closure [5], and in addition they recruit proteins containing LIR motifs (LC3-interacting regions). Many LIR motif-containing proteins are known to be cargo receptors as they can bind and recruit other proteins into the autophagosome. One well-characterised cargo receptor is SQSTM1/p62, which binds ubiquitinated cargo via a UBA domain [6].

1.2. Autophagy initiation versus flux

Atg8 family members or cargo receptors can be tagged on their N-terminus with GFP or a tandem tag of mRFP-GFP (see Fig. 1) to visualise autophagosome formation either by live cell imaging or after chemical fixation [7,8]. Using the tandem tag, GFP is quenched in the acidic autolysosome whilst RFP remains fluorescent and identifies the protein resident in the autolysosome thus providing an advantage over GFP alone. The number of yellow (a merge of red and green) puncta indicates the total number of autophagosomes that have formed, whilst red puncta indicate those that have matured and are autolysosomes. Perturbation of autophagosome formation will alter the number of yellow spots: no increase in yellow puncta indicates inhibition of formation, an increase in yellow puncta indicates normal formation and maturation, and an accumulation of yellow and a decrease of red indicates an inhibition of maturation. Furthermore, the ratio of the two fluorophores can be determined and provide a flux measurement (rate of initiation versus consumption). If a GFP-tagged marker is used it is still possible to measure formation using lysosomal protease inhibitors such as leupeptin, or Bafilomycin A1 (BafA). Leupeptin inhibits lysosomal proteases, whilst BafA treatment causes neutralisation of the acidic pH of lysosomes and accumulation of autophagosomes formed during the treatment. In most normal cells basal autophagy levels are very low, and treatment of cells in fed conditions with either leupeptin or BafA can reveal the level of basal autophagy in these cells (see [9] for details and references).

1.3. Brief review of some recent HTS for autophagy regulators

Because fluorescent puncta containing GFP-LC3, mRFP-GFP-LC3, or a similarly tagged cargo receptor protein such as p62 can be easily counted, scientists have accessible tools for quantification of autophagy. These tools have provided the opportunity to screen for novel regulators of autophagy using siRNA technologies under a variety of experimental conditions, which can mimic stress and disease, or after treatment with small molecules to assess their effect on autophagy. Several genome-wide siRNA screens have been performed under either basal conditions [10] or after induction using amino acid starvation [11]. Additionally, a more complex experimental set-up using viral infection and induction of mitophagy was used to screen for regulators of selective autophagy [12]. These screens were done in three different cell lines (H4, HEK293, and HeLa respectively) all stably expressing GFP-LC3, but had different secondary screens. The novel regulators identified in each screen were different which is expected for the selective autophagy screen but are perhaps surprising for non-selective (macro)autophagy screens done by McKnight et al., 2012 and Lipinski et al., 2010 for which it would be anticipated that basal and starvation-induced autophagy screens should reveal some common regulators [13]. These screens were performed in academic labs and the reader is also encouraged to consult the following review for more information about screening in this setting [14].

In addition to siRNAs, small molecules and compounds can be used to uncover targets and discover pathways. This has been exploited in assays using the BIOMOL compound catalogue of 480 compounds, screening for an increase in autophagy using GFP-LC3, followed by validation using long-lived protein degradation and removal of expanded polyglutamine aggregates [15]. Using a fixed concentration and time, a screen using 3584 drugs on normal cells revealed several inhibitors of the mTOR pathway that stimulated autophagy [16]. Using about 1400 cytotoxic drugs from the National Cancer Institute, GFP-LC3 puncta were measured in cells also stained with Hoechst and the vital dye propidium iodide [17]. This screen addressed the relationship between drugs and their effect on autophagy, apoptosis and necrosis. All of these chemical screens were image-based screens. Using a different approach, Sarkar and colleagues used a compound library (50,729) to screen for inhibitors (SMIRS) or enhancers (SMERS) of cytostatic effects in rapamycin-treated yeast, then performed an in-depth analysis of 72 in mammalian cells [18].

1.4. Aim

This article discusses the issues to consider in the development and performance of high-throughput screens using both siRNA libraries and small molecule compound collections in order to discover novel autophagy regulators in mammalian cells. We will focus on the key parameters to establish in order to perform a successful screen. Finally, based on our experience ([11] and Joachim et al., unpublished data) we outline below our protocols for performing an siRNA screens under amino acid starvation using GFP-LC3 puncta as a reporter, and a chemical screen under basal conditions using antibody labelling for endogenous p62 puncta. Whilst the experimental manipulation of screens using of GFP-puncta is straightforward, the GFP signal reflects the LC3 bound to autophagosome membranes, most of which are neutral pH, closed non-degradative autophagosomes. If the siRNA or chemical treatment alters the pH of the late endosome or lysosome this will also increase the number of GFP-puncta detected but not necessarily the number of autophagosomes. This may be an issue with chemical screens, which may contain compounds that alter the pH of the lysosome, and the use of endogenous p62 staining overcomes this issue.

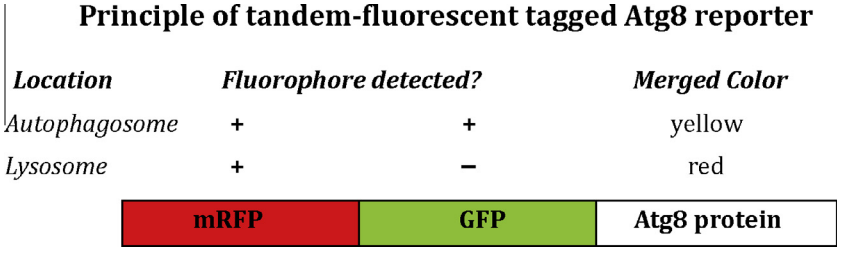

Fig. 1. mRFP-GFP-LC3 tandem fluorescent tag scheme. The principle of the tandem fluorescent tag appended to LC3, or any of the Atg8 family members. The yellow signal provides a value for the amount of the protein in non-acidic compartments, including autophagosomes, whilst the red signal provides a value for the amount of the protein in acidic compartments, including autolysosomes.

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