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Review

Complex regulation of autophagy in cancer – Integrated approaches to discover the networks that hold a double-edged sword

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ABSTRACT

Autophagy, a highly regulated self-degradation process of eukaryotic cells, is a context-dependent tumor-suppressing mechanism that can also promote tumor cell survival upon stress and treatment resistance. Because of this ambiguity, autophagy is considered as a double-edged sword in oncology, making anti-cancer therapeutic approaches highly challenging. In this review, we present how systems-level knowledge on autophagy regulation can help to develop new strategies and efficiently select novel anti-cancer drug targets. We focus on the protein interactors and transcriptional/post-transcriptional regulators of autophagy as the protein and regulatory networks significantly influence the activity of core autophagy proteins during tumor progression. We list several network resources to identify interactors and regulators of autophagy proteins. As *in silico* analysis of such networks often necessitates experimental validation, we briefly summarize tractable model organisms to examine the role of autophagy in cancer. We also discuss fluorescence techniques for high-throughput monitoring of autophagy in humans. Finally, the challenges of pharmacological modulation of autophagy are reviewed. We suggest network-based concepts to overcome these difficulties. We point out that a context-dependent modulation of autophagy would be favored in anti-cancer therapy, where autophagy is stimulated in normal cells, while inhibited only in stressed cancer cells. To achieve this goal, we introduce the concept of regulo-network drugs targeting specific transcription factors or miRNA families identified with network analysis. The effect of regulo-network drugs propagates indirectly through transcriptional or post-transcriptional regulation of autophagy proteins, and, as a multi-directional intervention tool, they can both activate and inhibit specific proteins in the same time. The future identification and validation of such regulo-network drug targets may serve as novel intervention points, where autophagy can be effectively modulated in cancer therapy.

1. Introduction

Most mutations affecting the integrity of signaling pathways and cellular processes display either pro- or anti-oncogenic effects. Autophagy (cellular self-degradation) is usually considered as a tumor-suppressing mechanism, though it can also enable tumor cell survival upon stress, and may promote metastasis formation. Thus, it is not obvious which therapeutic approaches can modulate autophagy in the desired way. Here, we show that systems-level knowledge is needed to select efficient anti-cancer drug targets that affect autophagy. Multi-target drugs and combination therapies may become more effective than previous autophagy-related monotarget approaches.

Macroautophagy involves the sequestration of cytosolic material into double membrane vesicles termed autophagosomes for delivery to the lysosome, where the cargo is degraded by acidic hydrolase enzymes [1]. Autophagy is a key response mechanism to numerous extracellular and intracellular stresses [2]. These include, for example, nutrient and growth factor deprivation and hypoxia. Under starvation, the enhanced autophagic activity provides the cells with metabolic intermediates to meet their bioenergetic

Abbreviations: ERK, extracellular signal regulated protein kinase; FoxO1/3, forkhead family transcription factor; GSK3, glycogen synthase kinase-3; HIF, hypoxia-inducible factor; IGF, insulin-like growth factor; IRE1, inositol-requiring protein 1; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa beta; NRF2, nuclear factor erythroid 2-related factor 2; PKA, protein kinase A; p53, TP53 tumor suppressor protein; RAS, small GTPase protein; SREBP, sterol regulatory element-binding protein; TFEB, transcription factor EB; TGF-β, transforming growth factor beta; WNT, wingless and int-like protein.

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demands [2]. Autophagy is the only cellular catabolic process that can eliminate damaged or reactive oxygen species (ROS)-overproducing mitochondria, and thereby limit general oxidative damage [2].

Autophagy is regulated by conserved upstream signaling pathways integrated by the mammalian kinase target of the immunosuppressant rapamycin (mTOR) [1]. Available nutrient or growth factors activate the insulin/IGF-1 – TSC – TOR signaling axis, which inhibits autophagy, and stimulates cell growth and proliferation. Nutrient or growth factor limitation, hypoxia and other cellular stressors are known to deactivate this signaling system, leading to autophagy induction and suppression of cell growth and proliferation [3]. Several other pathways (including RAS/PKA, RAS/ERK, IRE1/JNK, TGF-β, WNT/GSK3, HIF) and transcription factors (TFs), such as NRF2, FoxO and p53 have also been described to effect autophagy [4,5]. Interestingly these signaling pathways are also important in cell growth, proliferation, angiogenesis, immunity, cell survival and cell death [6], functions whose alteration are listed among the hallmarks of cancer [7]. Thus, these data show that the control of autophagy is affected during tumorigenesis.

Numerous studies examined the role of autophagy in cancer, but the results are quite ambiguous. On the one hand, autophagy has tumor suppressing functions by (a) suppressing chromosomal instability and therefore preventing the accumulation of oncogenic mutations; (b) restricting oxidative stress, which is also an oncogenic stimulus; (c) promoting oncogene-induced senescence, and (d) reducing intratumoral necrosis and local inflammation [2,8,9]. On the other hand, enhanced autophagy represents a prominent mechanism used by tumor cells to escape from hypoxic, metabolic, detachment-induced and therapeutic stress as well as to develop metastasis and dormant tumor cells [2,8,9]. During tumorigenesis, autophagy is frequently switched on and off, resulting in highly regulated anti- and pro-tumorigenic effects. Therefore, autophagy can be considered as a double-edged sword during tumorigenesis [10]. As autophagy is switched on and off during tumorigenesis, we can assume that it is not the autophagic machinery itself but the protein–protein interaction and regulatory networks that continuously is changing during tumor progression. These networks can context-dependently control the mechanism of autophagy (Fig. 1).

In the following sections, we briefly review protein–protein and regulatory network resources to examine autophagy on the systems-level. Then, we summarize frequently used *in vivo* genetic models, forward and reverse genetics-based methods, as well as fluorescence techniques to experimentally study autophagy and validate the systems-level predictions of network analysis. Finally, we present the challenges and possibilities of network pharmacological approaches to modulate autophagy in cancer.

2. Interactors of autophagy proteins

Currently, several databases describing protein–protein interactions (PPI) exist, but only few of them contain sufficient information on autophagy-related proteins. We benchmarked six well-known, general PPI resources and two autophagy-specific network databases to analyze the presence of a core set of 38 autophagy components (listed in Table 1). With this comparison we pointed out the number of autophagy components and their interactions in various resources (Table 2).

We selected major PPI resources where the experimental/literature source of the given interaction is listed allowing the users to check and examine the details of the interactions. We examined three PPI databases that contain manually curated interaction data: (1) the Human Protein Reference Database (HPRD) [11]; (2) the IntAct resource [12]; and the Molecular INTeraction database (MINT) [13]. From these databases, IntAct represents the highest number of core autophagy components (36 of the 38) and interactions (2702). We also examined two PPI resources that contain more interactions gained from high-throughput screens: (1) the Search Tool for the Retrieval of Interacting Genes (STRING) [14] and (2) the Biological General Repository for Interaction Datasets (BioGRID) resource [15]. In STRING, there were interaction data (335 PPIs) for 37 of the 38 autophagy core proteins but BioGRID contained more interactions (641 PPIs for 36 proteins). In addition, we examined the Interologous Interaction Database (I2D) containing the mostly predicted PPIs [16]. I2D has 10,182 PPIs for 37 autophagy core proteins. Note that most of these PPIs are inferred based on orthology, and the original experimental evidences were coming from mainly high-throughput screens. Despite the fact of the potential high number of false positive PPIs, I2D could serve as an efficient pool of possible autophagy-related interactions. Further filtering and experimental validation could point out true positive interactions in given experimental contexts.

We also examined interaction databases focusing specifically on autophagy. To our knowledge, there are only two such databases: the Autophagy Database (ADB) and the Autophagy Regulatory Network (ARN). ADB contains a lot of different information on the components of the autophagic process [17]. ADB includes 28 proteins from the 38 core autophagy components

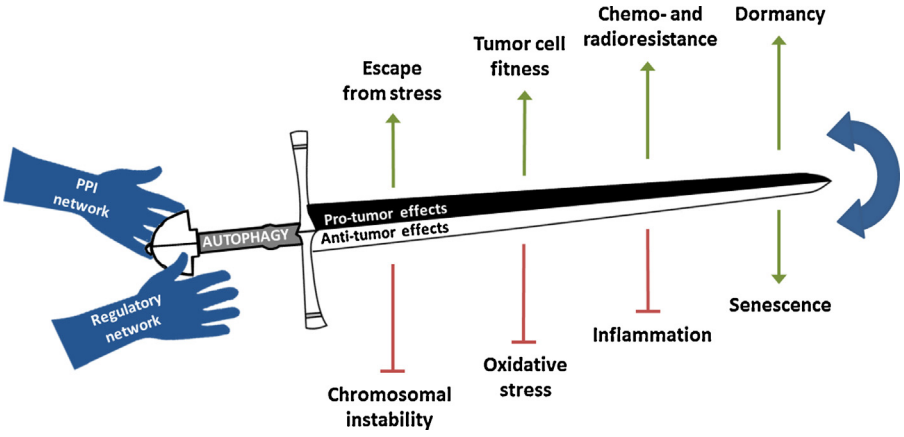

Fig. 1. Autophagy as a double-edged sword in cancer biology. Autophagy has both pro-, and anti-tumor effects. Autophagic activity is precisely regulated and continuously switched on and off during the phases of tumorigenesis. This mechanism is carried out by the protein–protein interaction (PPI) network containing protein interactors (e.g., enzymes and adaptors) of autophagy proteins as well as by regulatory networks of transcription factors and miRNAs. Coordinated action of these networks controls the activity of autophagy in cancer. For a detailed network view of this connection, see Fig. 2.

Table 1
List of core autophagy proteins and their roles in the phases of autophagy. Note, that some proteins have multiple functions. The list was made by manual curation of the literature.

			Phases of autophagy						
			Induction	Cargo recognition and packaging	Atg protein cycling	Vesicle nucleation	Vesicle expansion and completion	Transport of autophagosome	Fusion with lysosome
Gene name	**Protein name**	**UniProt ID**							
ULK1	Serine/threonine-protein kinase ULK1	O75385	■		■				
ULK2	Serine/threonine-protein kinase ULK2	Q8IYT8	■						
RB1CC1	RB1-inducible coiled-coil protein 1	Q8TDY2	■						
ATG13	Autophagy-related protein 13	O75143	■		■				
ATG101	Autophagy-related protein 101	Q9BSB4	■						
p62/SQSTM1	Sequestosome-1	Q13501		■					
ATG2A	Autophagy-related protein 2 homolog A	Q2TAZ0			■				
ATG2B	Autophagy-related protein 2 homolog B	Q96BY7			■				
WIPI1	WD repeat domain phosphoinositide-interacting protein 1	Q5MNZ9			■				
WIPI2	WD repeat domain phosphoinositide-interacting protein 2	Q9Y4P8			■				
BECN1	Beclin-1	Q14457				■			
ATG14	Beclin 1-associated autophagy-related key regulator	Q6ZNE5				■			
PIK3R4	Phosphoinositide 3-kinase regulatory subunit 4	Q99570				■			
PIK3C3	Phosphatidylinositol 3-kinase catalytic subunit type 3	Q8NEB9				■			
UVRAG	UV radiation resistance-associated gene protein	Q9P2Y5				■			
AMBRA1	Activating molecule in BECN1-regulated autophagy protein 1	Q9C0C7				■			
ATG3	Ubiquitin-like-conjugating enzyme ATG3	Q9NT62					■		
ATG4A	Cysteine protease ATG4A	Q8WYN0					■		
ATG4B	Cysteine protease ATG4B	Q9Y4P1					■		
ATG4C	Cysteine protease ATG4C	Q96DT6					■		
ATG4D	Cysteine protease ATG4D	Q86TL0					■		
ATG5	Autophagy protein 5	Q9H1Y0					■		
ATG7	Ubiquitin-like modifier-activating enzyme ATG7	O95352					■		
MAP1LC3A	Microtubule-associated proteins 1A/1B light chain 3A	Q9H492					■		
MAP1LC3B	Microtubule-associated proteins 1A/1B light chain 3B	Q9GZQ8					■		
MAP1LC3C	Microtubule-associated proteins 1A/1B light chain 3C	Q9BXW4					■		
MAP1LC3B2	Microtubule-associated proteins 1A/1B light chain 3 beta 2	A6NCE7					■		
GABARAP	Gamma-aminobutyric acid receptor-associated protein	O95166					■		
GABARAPL1	Gamma-aminobutyric acid receptor-associated protein-like 1	Q9H0R8					■		
GABARAPL2	Gamma-aminobutyric acid receptor-associated protein-like 2	P60520					■		
GABARAPL3	Gamma-aminobutyric acid receptor-associated protein-like 3	Q9BY60					■		
ATG9A	Autophagy-related protein 9A	Q7Z3C6	■				■		
ATG10	Ubiquitin-like-conjugating enzyme ATG10	Q9H0Y0					■		
ATG12	Ubiquitin-like protein ATG12	O94817					■		
ATG16L1	Autophagy-related protein 16-1	Q676U5					■		
ATG16L2	Autophagy-related protein 16-2	Q8NAA4					■		
FYCO1	FYVE and coiled-coil domain-containing protein 1	Q9BQS8						■	
TECPR1	Tectonin beta-propeller repeat-containing protein 1	Q7Z6L1							■

Table 2
Protein–protein interaction resources in autophagy research. A brief benchmark listing the URL addresses, as well as the strengths and the weaknesses of the resources in terms of autophagy research. We indicate the total number of proteins and interactions in each resource as well as their autophagy-specific numbers.

Database	Webpage	Strengths	Weaknesses	Number of proteins (core autophagy proteins) in human	Number of interactions (for core autophagy proteins) in human	Reference
HPRD	http://www.hprd.org/index_html	- many information on proteins	- limited number of autophagy proteins - limited number of interactions - suspended in 2010	30,047 (20)	41,327 (87)	[11]
IntAct	http://www.ebi.ac.uk/intact	- manually curated		64,888 (36)	202,883 (2702)	[12]
MINT	http://mint.bio.uniroma2.it	- manually curated	- limited number of autophagy proteins	8735 (24)	26,830 (160)	[13]
STRING	http://string-db.org	- many interaction for each protein	- high number of high-throughput and predicted interactions	no data available (37)	no data available (335)	[14]
BioGRID	http://thebiogrid.org	- many interaction for each protein	- high number of high-throughput interactions	16,746 (36)	182,911 (641)	[15]
I2D	http://ophid.utoronto.ca	- high number of interactions for autophagy core proteins	- high ratio of high-throughput and predicted interactions	no data available (37)	173,338 (10,182)	[16]
Autophagy Database	http://tp-apg.genes.nig.ac.jp/autophagy/overview.html	- autophagy specific - all known autophagy (related) proteins - many information on proteins	- limited number of interactions - sources of interactions are not given	426 (28)	369 (179)	[17]
Autophagy Regulatory Network	http://arn.elte.hu	- autophagy specific - all known autophagy (related) and autophagy regulating proteins - sources of interactions are given	- does not contain orthology-based predictions	3907 (38)	43,048 (236)	Turei et al. *in prep.*

with 179 interactions but the sources of the interactions are not indicated. ARN (http://arn.elte.hu) is a complex source of information on human autophagy proteins and the regulation of the autophagy machinery (publication is under preparation by Turei D, Foldvari-Nagy L, Fazekas D, Csermely P, Korcsmaros T and Vellai T). ARN contains the 38 autophagy core proteins with 236 interactions. For all interactions, the exact sources are listed. In addition to the core autophagy network, ARN also contains 482 first neighbors of the core network, termed as known or predicted autophagy regulators. These 482 regulators have 760 interactions with the core network. We note that ARN contains manually curated, imported and predicted interactions of autophagy components. Thus, it also encompasses PPIs from MINT, IntAct and HPRD. The predicted PPIs in ARN are not based on orthology (as in I2D), but coming from domain-motif and domain-domain interaction predictions (described in Ref. [18]).

3. Regulators of autophagy proteins

Our present knowledge on the transcriptional regulation of autophagy is far from complete. Several transcriptional regulators are predicted for each gene involved in the autophagy process by bioinformatics algorithms that search for promoter binding sites for specific transcription factors (TFs) [19]. However, only a few TFs have been identified and validated by detailed experimental studies (e.g., TFEB, FoxO1/3, SREBP, NRF2, NF-κB [20–22]). Transcriptional regulation of autophagy has been found important during normal response to starvation, oxidative stress, lipid depletion, as well as its failure has been connected to several related human diseases [20,22].

TFs that are capable of regulating the expression of core autophagy proteins can be examined using different databases containing regulatory information (i.e., TF-gene connections). The Human Transcriptional Regulation Interactions database (HTRIdb) contains curated information on experimentally verified human transcriptional regulatory connections [23]. In HTRIdb, there are 13 TFs known to regulate 11 autophagy genes (from the list of 38 core genes) with altogether 34 connections indicating an overlapping target gene set among the TFs. The recently published ENCODE project [24] found a more specific pattern for 10 TFs to regulate only 2 autophagy genes with 11 TF-gene connections. In addition, the JASPAR server allows the prediction of TF regulation using the binding profiles of 129 TFs [25]. JASPAR predicts TF binding sites for almost all of the 38 core autophagy genes: it contains 31 TFs that may bind to 35 genes with 325 connections. Note, that many of these connections are false positives or highly context specific. However, similarly to the previously presented PPI predictions, these potential connections could also serve as a pool of possible autophagy-related regulatory mechanisms that should be examined and confirmed experimentally.

The ARN resource contains all the listed regulatory information for human autophagy proteins and data from many more regulatory resources (unpublished data by Turei et al.). Altogether ARN contains 60 known and predicted TFs for all the 38 autophagy genes with 378 TF-gene connections. Of note, we found only a few TFs present in multiple resources indicating the importance of different approaches to discover TFs capable to regulate autophagy.

In addition to transcriptional regulators, several miRNAs are known to down-regulate the activity of mRNAs coding autophagy proteins. A recent review enumerates more than 16 miRNAs that post-transcriptionally regulate the accumulation of autophagy proteins [26]. These miRNAs are able to detain specific steps of the mechanism of autophagy (e.g., miR-376b acts on ATG4 and Beclin-1, while miR-630 acts on Atg12 and UVRAG). Most of these miRNAs affect the early stage of autophagic vesicule formation, possibly because to prevent the accumulation of autophagosomes [26]. Cellular context and environmental conditions (e.g., starvation) affects the expression of these miRNAs and could modify their inhibitory effect. Several studies recently pointed out the therapeutic potential of miRNAs to block autophagy in cancerous cells. For example, miR-30a, miR-34a, miR-101, miR-204 and miR-375 known to negatively regulate autophagy were found to be able to repress tumorigenesis [26]. Interestingly, the autophagy-inhibitor miR-101 was found to be progressively lost through the transition from clinically localized disease to metastatic prostate cancer [27]. It is tempting to speculate that the balance between miRNAs down-regulating autophagy genes and miRNAs down-regulating autophagy inhibitor proteins and repressor TFs could determine autophagic activity during tumorigenesis. If so, then miRNAs may control the switching mechanism of autophagy in the different phases of tumorigenesis.

For the systems-level identification of miRNAs capable of down-regulating autophagy, several miRNA prediction resources exist. For example, two widely-known resources, miRanda and TargetScan, list 823 and 771 highly confident miRNAs that could bind to the mRNA of 38 and 35 autophagy genes, respectively [28,29]. In addition, a recent web resource, doRiNA, contains 423 miRNAs for 32 autophagy genes [30]. The ARN resource integrates these three and six other resources, and lists 1329 miRNAs that may regulate the autophagic machinery. As we emphasized before, experimental screenings and focused tests should be carried out to validate the role of these miRNAs in autophagy control.

4. Experimental models to examine autophagy in cancer

The use of genetic model organisms has already been instrumental in deciphering how autophagy works and contributes to cellular homeostasis during development and adulthood [31]. Despite of a powerful genetic toolkit [32], which can be applied to modify and examine almost any gene within the genome, the nematode *Caenorhabditis elegans* is still only of limited use in most cancer studies. Though most signal transduction mechanisms are well conserved in this animal, and the origin of most germline tumors are well documented, no somatic tumor can be observed [33]. This also means that only inferences can be made about the cell biological role of oncogenes and tumor suppressors, and specific properties of somatic tumors cannot be examined in this model.

It is worth noting however, that due to its powerful genetics, *C. elegans* had an important role in uncovering the identity and function of Metazoan autophagic genes [34]. Influential genetic screens were conducted to this aim, and the alleles identified this way could be further tested for a possible role in germline tumor progression or using other established cancer paradigms [33].

Another popular invertebrate model organism, the fruit fly *Drosophila melanogaster*, has already proven indispensable in uncovering some general aspects of both autophagy [35] and tumor growth and metastasis [36]. For example, mutants with disrupted apico-basal polarity (*lgl, dlg, scrib*) [37] form metastatic tumors in the larval brain. Interestingly, mutations in the human orthologs of these tumor-suppressor genes have also been linked to cancer progression [38], showing the relevance of the findings.

In this model organism, simple overexpression of the oncogenic Ras^{V12} construct in larval neural epithelium results in a benign overgrowth phenotype. However, compounded with further mutations, Ras^{V12} overexpression can also lead to aggressive, metastatic tumors. Screens to identify enhancing mutations, amongst other candidates, have identified several components of the lysosomal

complex, and also showed that chloroquine, a potent inhibitor of lysosomal degradation, can promote metastasis [39]. Although indispensable to decipher general rules in tumor growth and metastasis, the use of invertebrate model organisms is still limited. In the absence of organs homologous to the vertebrate pancreas, liver or breast, the tumors affecting these organs are impossible to model.

In contrast, the growing popularity of the zebrafish (*Danio rerio*), suggests that it can offer a new tool to model several human cancer types. Previous studies also suggest that it is ideally suited to assess the possible role of autophagy in the etiology of different cancers. The ease to maintain large stocks and expose them to different chemical compounds has made zebrafish a popular model of drug screens from the beginning [40]. But it is the combination of a diverse pool of cancer models and a plethora of small molecule screens that make zebrafish an ideal model to be used for the development of effective therapies. Forward and reverse genetic screens have already identified several mutations causing elevated cancer incidence in zebrafish (many of them in the homologues of human oncogenes or tumor suppressors), and novel transgenic approaches have also created useful disease models (for detailed references see [41] and [42]). Furthermore, attempts to grow malignant human tissue on the yolk-sac of zebrafish larvae have also yielded significant success, providing a high-throughput model to study metastasis, invasiveness, and tumor-driven angiogenesis [43]. The relevance of these studies was further enhanced by the fact that xenografted embryos can be easily exposed to existing chemical libraries, thus they provide a practical resource for early-phase drug trials. Although neglected by autophagy researchers for a long time, recently the role of autophagy in fish development and cellular homeostasis has also been addressed [44,45]. These studies, unsurprisingly, revealed that zebrafish larvae, when exposed to the usual set of autophagy modulators, show the expected changes in the level of autophagic activity. This observation raises the prospect that the relevant zebrafish models for many malignant tumor could be used to assess whether modulation of autophagy can have an effect on tumor growth and metastasis. Indeed, rapamycin treatment was already shown to suppress the phenotype of the *lkb1* mutation, which could provide a cue for the effective treatment of the Peutz–Jeghers syndrome in humans [46].

The combination of existing mouse cancer and autophagy mutants will also yield important information about the biology of tumors. Classical transgenesis (i.e., overexpression of human oncogenes) and reverse genetic approaches have already created mouse mutants for the most relevant human oncogenic diseases [47]. Recently several transposon- and retrovirus-based forward genetic screens have also contributed to our understanding of the genetic networks involved in malignant transformations (for a detailed review see Ref. [47]). These disease lines could be crossed with existing autophagy mutants to yield important new insights into which cancer types are modulated by autophagic activity. For autophagy mutants that are adult viable, this will be relatively straightforward, whereas for mutations that are embryonic or neonatal lethal [48], tissue specific knock-out systems could be used.

Indeed, the use of mouse mutants has been instrumental in discovering the link between autophagy and cancer progression. For example, heterozygous *Beclin-1* mutants showed elevated levels of spontaneous tumor formation, indicating that this gene is a haploinsufficient tumor-suppressor [49]. Similar phenotypes were observed in *Bif* mutant animals [50], also defective in autophagy, while mutations in *Atg4C* increased the susceptibility to develop fibrosarcomas [51]. Other murine models have also demonstrated a link between autophagy and a variety of cancer types, such as pancreatic [52] or liver tumors [53].

5. Fluorescence techniques for autophagy detection in human cells and tissues

In the last decade, *in silico* predictions and *in vivo* experiments performed with model organisms pointed out several novel autophagy components, their protein interactors and regulators. To validate the predicted and inferred components in human cells and tissues, it is important to apply reliable methods for monitoring the process of autophagy in humans. These attempts also help us to understand the consequences of autophagy modulation in cancer therapy and prevention. Here, we present a summary of fluorescent techniques used in autophagy detection in human cells.

Upon induction of autophagy, LC3B conjugates with phosphatidylehanolamine (PE) and associates to the autophagosome formation. Therefore this form of LC3B (LC3B II, hereafter referred as LC3) is a suitable marker for monitoring autophagy as it correlates with increased levels of autophagic vesicles [54]. However, due to the basal level of autophagy in the cell, the normal level of LC3 is not a sufficient evidence for autophagy. It is important to distinguish physiological level of LC3 and show the positive (e.g., rapamycin-treated, starvation-induced) and the negative (e.g., inhibitor-treated) controls in any applied assays [54,55]. LC3 and its GFP-tagged form (GFP-LC3B) have been widely used to monitor autophagy through indirect immunofluorescence or direct fluorescence microscopy, measured as an increase in punctate LC3 or GFP-LC3. However, it is worth to note that while the intracellular number of LC3 punctuate increases during autophagy induction, the total amount of LC3 does not refers to the complete process of autophagy [56]. An efficient alternative to monitor the whole process (i.e., the autophagic flux) is based on the dissection of the autophagosome maturation process. An mRFP GFP tandem fluorescent tagged LC3 shows a double mRFP, GFP (yellow) signal before the fusion with lysosomes, and exhibits only mRFP (red) signal subsequently [57]. Thus, the yellow-red transition could indicate the level of the autophagic flux. A similar set up allowed the high-throughput detection of the autophagic flux [58]. Recently, a FRET-based high-throughput assay was also developed to determine the protease activity of ATG4 on LC3 upon autophagosome formation [59].

In addition to LC3, other proteins can also be used as autophagic markers in certain studies. For example, the autophagy receptor p62, WIPI-1 and ATG9 accumulate at LC3-positive membrane structures when autophagy is induced and autophagosome is formed [60–62]. The early step of autophagy can be monitored via the detection of ATG5, ATG12 and ATG16L1: these proteins show diffuse distribution in cytoplasm under physiological circumstances, but upon autophagy induction there is a marked increase in the proportion of cells with punctate ATG5-, ATG12- and ATG16L1-associated to phagophore formation [63].

Immunohistochemistry detection of major autophagy-related proteins (LC3 and Beclin 1) has been reported as a prognostic factor in various human cancer types, including lymphoma [64], breast carcinoma [65], glioma [66], non-small cell lung carcinomas [67]. However, a recent study that examined immunohistochemical assessment of autophagy-related marker proteins (such as LC3, ATG5, cathepsin D, Beclin 1 and p62) concluded that immunohistochemical detection of these proteins is not recommendable for monitoring autophagy particularly in clinical samples, due to lack of differential gene expression or doubtful specificity [68]. Accordingly, autophagy detection currently faces several other problems of tissue fixation and sampling tumor tissue [54]. Therefore, immunohistochemical detection of autophagy markers from clinical samples needs to be further explored and established in order to successfully use them in clinical trials.

6. Pharmacology of autophagy modulation

Anti-cancer therapies, such as hormonal agents, chemotherapy and irradiation, frequently induce autophagy, in many cases as a pro-survival response potentially contributing to treatment resistance [3]. However, in particular genetic backgrounds and microenvironment (i.e., disabled apoptotic system during starvation or oxidative stress), autophagy activation can also lead to cell death, thus, enhancing treatment efficiency [3]. Therefore, context-specific autophagy modulations can be promising novel therapeutic attempts to extend the currently available anti-cancer treatments [3]. Cytotoxic, targeted, and radiation therapies that amplify stress and indirectly stimulate autophagy, could be improved by including both direct autophagy stimulators and inhibitors [9]. For a comprehensive list of ongoing and preclinical studies on direct autophagy modulation in cancer treatment, see reference [3]. Enhanced stimulation of autophagy can induce cell death, while the indirect, stress-activated autophagy is well-regulated and supports cancer cell survival. Alternatively, autophagy inhibitors can repress autophagy-mediated stress-survival [9]. For example, combining autophagy inhibitors, such as hydroxychloroquine with inducers of metabolic stress (like angiogenesis inhibitors or 2-deoxyglucose) can block survival to metabolic stress [9]. In addition, autophagy inhibitors sensitize cancer cells to therapy and avoid chemoprevention in certain cases, thus inclusion of such inhibitors in the treatment regime may significantly improve therapeutic efficacy [3]. On the other hand, anti-cancer approaches that modulate autophagy could have opposite effects on normal cells: autophagy inhibitors can induce tumorigenesis and autophagy stimulators may be useful for cancer prevention by enhancing damage mitigation and senescence [9,69].

In conclusion, a context-dependent modulation of autophagy would be favored in anti-cancer therapy: stimulated in normal cells, while inhibited only in stressed cancer cells. Maintaining the basal, anti-tumorigenic role of autophagy in normal cells is essential during therapy in order to avoid tumor formation at healthy tissues. On the other hand, inhibiting the stress-inducible, pro-tumorigenic autophagy, which is generally responsible for metastasis formation and chemotherapy resistance could block tumorigenesis and increase the effectiveness of certain therapies. As direct autophagy inducers and inhibitors can only be used in a combined fashion that also requires precise diagnosis on the tumor stage, one may consider alternative targets and strategies to modulate autophagy in anti-cancer treatments. Network analysis of the regulation of autophagy may point out such context-specific intervention points [70].

7. Regulo-network drug targets in autophagy modulation

Targeting protein interactors and regulators of autophagy core proteins would be a promising approach to identify novel autophagy modulating mechanisms. In Fig. 2, we visualize a section of the autophagy regulatory network and highlight the most central interactors and regulators as well as those components that were found to be mutated in different cancer types or already used as drug targets (based on [71] and [72]). Interestingly, there are high number of drug targets and low number of driver mutations among the interactors and regulators. Further analysis on this network could help us to identify proper anti-cancer drugs to modulate the process of autophagy. Accordingly, mTOR, the upstream regulator of autophagy was expected to be a promising therapeutic target. mTOR participates in two protein complexes, mTORC1 and mTORC2, from which mTORC1 has a direct autophagy-regulating role [73]. mTORC1 suppresses autophagy via phosphorylating and thus inactivating ULK1, which is a member of the autophagy induction complex [74]. Rapamycin and rapalogs, the earliest known inhibitors affecting mTOR signaling, selectively affect mTORC1 as allosteric inhibitors of mTOR. Rapamycin and rapalogs passed the clinical trials and now are used as immunosupressants and anti-cancer agents under the trade names Sirolimus®, Temsirolimus® and Everolimus®. After the first test the limitations of these therapies manifested: rapalogs could only reach cytostasis (i.e., inhibition of cell growth and division) instead of apoptosis. Therefore, the aim to develop new inhibitors with other mechanisms of action has emerged. Except for special cases, such as renal cancer, mTOR could not meet the expectations because of its multi-pathway position and highly dense interaction network [75]. (Nevertheless, rapamycin and rapalogs remained essential elements in multi-target therapies and numerous studies are going on to utilize these compounds in combination therapies or as anti-aging agents.)

To identify more efficient and context-dependent targets to modulate autophagy, network analysis of the autophagy regulatory network is a desirable strategy. A systems-level approach would be to combine data from the listed PPI, TF and miRNA resources, and also directly from specific experiments of model organisms and high-throughput (e.g., proteomic) screens. A structured workflow starting with a network analysis that *in silico* predicts key autophagy regulators, and followed by tissue- and cancer- (type and stage) specific filtering with data from high-throughput screens could point out promising context-dependent intervention points. For example, the network topology of the autophagy regulatory network can be examined with modularization methods, such as ModuLand, which identifies major network components and provides an importance-ranked list of possible key regulators [76]. This analysis can be combined with dynamical network perturbation methods to fine-tune the module-based predictions of key regulators by selecting proteins that can optimally distribute the signals in the network [77]. Identification of such regulators is an essential step in drug target prioritization. However, very often these regulators are not favorable drug targets because of their poorly-known structure, cellular localization and multi-functional roles.

To point out efficient drug therapies that modulate autophagy, novel, network-based concepts can be utilized [78]. One such strategy is called polypharmacology or multi-target drug targeting, where the therapeutic aim is accomplished by simultaneous attacks on many proteins, wherein the targeting efficiency on each protein may only be partial [79,80]. Thus, a short list of promising autophagy regulators targeted simultaneously with low efficiency may serve as a good solution. In addition, drug targets can be selected based on the recently proposed allo-network drug concept, where drug effects can propagate across several proteins via specific, inter-protein allosteric pathways activating or inhibiting the final target [81].

Analogously, here we suggest promising drug targets among the key components of the autophagy regulatory network, whose effect propagates through transcriptional or post-transcriptional control of autophagy proteins. Such regulo-network drug targets can have multi-target effects as some TFs and miRNAs regulate not only one but a set of autophagy proteins. Furthermore, in most of the cases (as we have earlier seen in the regulators of autophagy section), these regulators are not exclusive allowing only a partial attack on the expression of the indirectly targeted autophagy components. Regulo-network drug targets could also serve as multi-directional intervention tools that can activate and inhibit specific proteins in the same time. The rationale behind this could be twofold: (1) Many TFs can both repress and stimulate the expression of their specific target genes. This is a highly regulated process, usually determined by co-factors or nucleic acid differences in the binding sites; (2) some TFs have only stimulatory effect on their targets that could contain both autophagy proteins as well as miRNAs down-regulating other autophagy

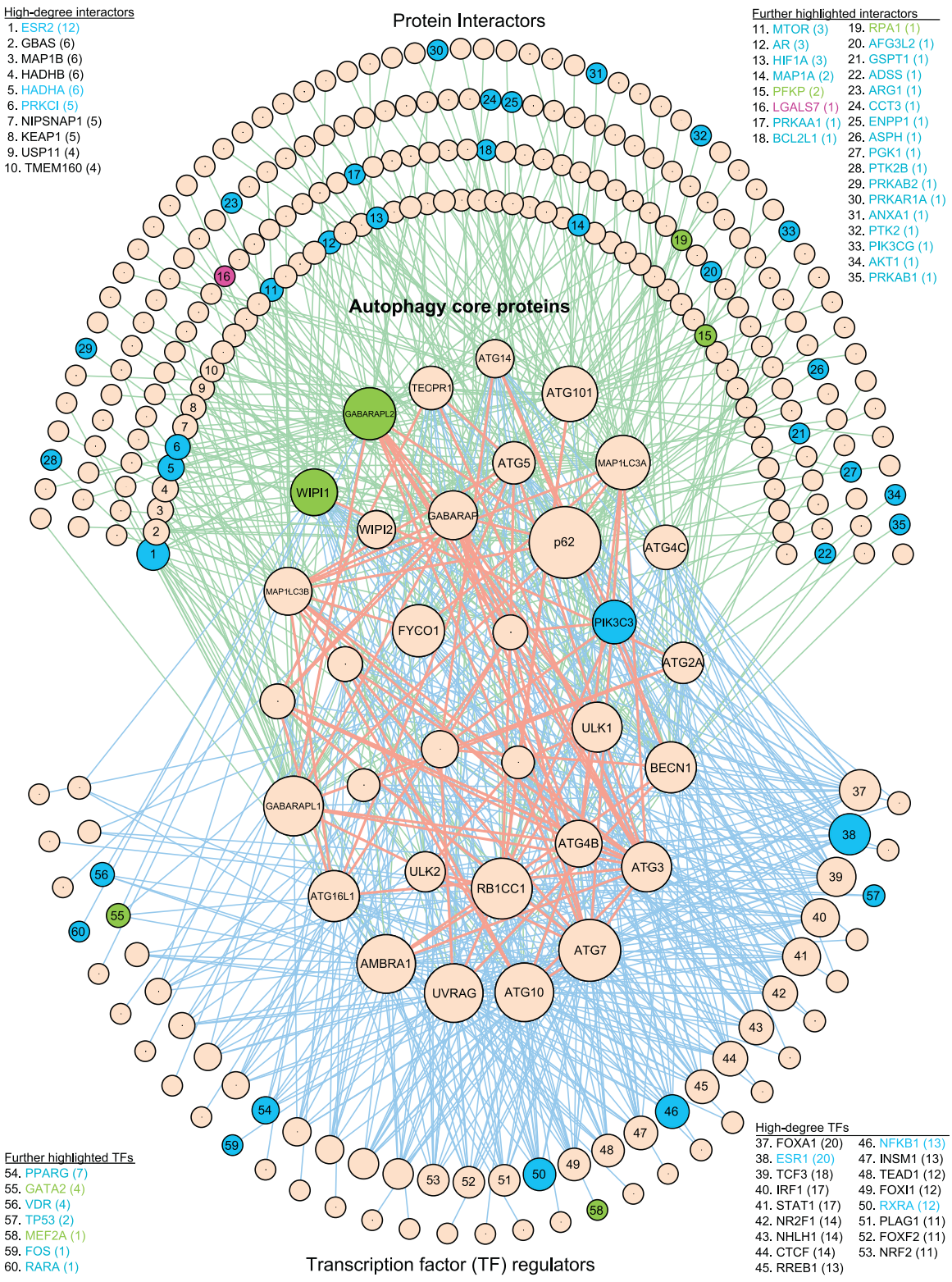

Fig. 2. Interconnected network of autophagy regulation. The first neighbor interactors and transcription factor (TF) regulators of the core autophagy proteins are visualized. Interactors and regulators are sorted according to their degree (i.e., number of autophagy protein partners). Top 10 interactors and regulators of autophagy proteins are listed as well as those that were found mutated in different types of cancer (green circles), known as drug targets (blue circles), or both (purple circles). The size of a circle is proportional with the number of its connections (i.e., degree). The degree of the highlighted interactors and regulators are shown in parenthesis after the name of the protein. Protein–protein interactions between interactors and autophagy proteins are shown with green edges, interactions between autophagy core proteins with red edges, while transcriptional connections of TFs and autophagy proteins are colored with blue edges. For clarity, interactions between protein interactors and regulatory connection of TFs among each other and with the protein interactors are not shown. Also, miRNA regulators of autophagy proteins and five autophagy components having no interactions (ATG9A, ATG4D, ATG4A, GABARAPL3, ATG16L2) are not listed in the figure. Note that the TFs ESR1 and RXRA are also protein interactors but for clarity, only their regulatory connections are shown. See Table 1 for details on the autophagy core proteins. Source of the network data: http://arn.elte.hu. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

components. As an example, consider a pharmacological modulation of a regulo-network drug target that can simultaneously inhibit a stress pathway-related autophagy inductor protein (e.g., ULK1) and activate a basal autophagy component (e.g., ATG5). With this approach we may block autophagy activation during chemotherapy in cancer cells, but enhance the basal, anti-tumorigenic autophagy process in normal cells. We may identify regulo-network drug targets with network modeling approaches, such as CellNetOptimizer [82]. With such approaches, we can build predictive logic models of the autophagy regulatory network by training the network with cell-type and context-specific experimental data. This modeling may point out those regulators (i.e., potential regulo-network drug targets) that have the desired and most specific regulatory effect on autophagy components. Finally, experimental approaches in model organisms and human cells should be carried out to validate that regulo-network targets could serve as more efficient autophagy modulating points than previous targets.

8. Conclusion

Coordinated movement of hands requires the development of complex (neuronal) circuits. The same stands for the modulation of autophagy during anti-cancer treatment. In this case, however, the protein and regulatory networks that move the double-edged sword of autophagy are highly context-dependent. Thus, we first have to explore and understand the network topology and dynamics that govern autophagy. For this purpose, we have listed several protein–protein and regulatory interaction resources and benchmarked the presence of autophagy core proteins in them. *In silico* analysis of these networks often requires experimental validation in model organism. In case of success, detailed monitoring of the autophagic process (flux) could be measured in human cells and tissues using multiple and combined fluorescent-tagged proteins. We have briefly summarized the advantages and disadvantages of the most popular model organisms to experimentally examine autophagy in cancer, as well as listed major fluorescence techniques for the high-throughput monitoring of autophagy in humans. Finally, we have reviewed the challenges of pharmacological modulation of autophagy, and suggested network-based concepts to overcome these difficulties. We suggested the introduction of regulo-network drugs, whose effect propagates through transcriptional or post-transcriptional regulation of autophagy proteins. These regulo-network drug targets positively or negatively regulate a specific subset of autophagy components often with low or medium efficiency and having a summed effect that is favorable. We hope that the future identification and use of such regulo-network drug targets will serve as intelligent intervention points to control the networks that hold the double-edged sword of autophagy.

Conflict of interest

Authors declare no conflict of interest.

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