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ABSTRACT

CRISPR/Cas9 is the latest tool introduced in the field of genome engineering and is so far the best genome-editing tool as compared to its precedents such as, meganucleases, zinc finger nucleases (ZFNs) and transcription activator-like effectors (TALENs). The simple design and assembly of the CRISPR/Cas9 system makes genome editing easy to perform as it uses small guide RNAs that correspond to their DNA targets for high efficiency editing. This has helped open the doors for multiplexible genome targeting in many species that were intractable using old genetic perturbation techniques. Currently, The CRISPR system is revolutionizing the way biological researches are conducted and paves a bright future not only in research but also in medicine and biotechnology. In this review, we evaluated the history, types and structure, the mechanism of action of CRISPR/Cas System. In particular, we focused on the application of this powerful tool in autophagy research.

INTRODUCTION

Genome engineering technology has come a long way since its humble beginning in the 1970s and since then, it has undergone rapid development which saw better, more efficient and robust tools for use in genetic perturbations. Genome engineering is essentially the process of modifying the genetic configuration of an organism in a targeted and specific manner, and encompasses the strategies or techniques to carry out the modification process as well. Such a breakthrough in biology has permitted researchers to expand our knowledge of what is known about gene function and the capacity to alter DNA also allows researchers to model human diseases in animal models, making it possible to exploit this for gene therapy and drug development (1).

To date, there are currently four major classes of genome editing technologies namely meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effectors (TALENs) and the most recent addition, the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) (CRISPR/Cas) as well as the CRISPR-Cpf1 (CRISPR from *Prevotella* and *Francisella 1*) systems (2, 3). These four technologies manipulate genetic material by inducing site-specific DNA double-strand breaks (DSBs) that result in genome editing either via homologous recombination (HR)-mediated recombination events or non-homologous end joining (NHEJ) (4). Although all of them are collectively classified under the same category of programmable nucleases, the mechanism of each of these genome editing technologies differ from one another.

In general, meganucleases, ZFNs and TALENs nucleases target specific DNA sequences through protein-DNA interactions(5). Meganucleases, also known as homing endonucleases, are nature's

highly specific nucleases whereby its nuclease and DNA-binding domains are combined into one single domain. In contrast, ZFNs and TALENs are artificially engineered nucleases with a DNA binding domain fused to a non-specific nuclease domain of FokI. In this sense, meganucleases are not as efficient as ZFNs and TALENs because they are limited in their capacity to bind to new DNA sequences with high specificity. ZFNs and TALENs would seemingly be better alternatives but these two tools are not without drawbacks. The complication of context-dependent binding preference between individual finger domains of ZFNs make designing of programmable ZFNs difficult even though solutions have been drawn up to address this limitation (6) as extensive screening process is necessary. On the other hand, TALENs exhibit lesser context-dependent binding preference and their modular assembly makes it possible to target any DNA sequence (7). Furthermore, assembly of DNA encoding the repetitive domains of TALENs requires unconventional molecular biology cloning methods which can be costly in terms of time and labor (8). Now, genome engineering technology has seen widely used with the advent of the CRISPR system that has shown promising results in addressing the issues pertinent to modular DNA-binding protein construction. The CRISPR system has been employed in a variety of studies for its ease of customization to target any desired DNA sequences in a genome simply via customized sgRNA (4).

Autophagy is an evolutionarily conserved pathway for degradation of cytoplasmic proteins and organelles via lysosome. Proteins coded by the autophagy-related genes (Atgs) are the core molecular machinery in control of autophagy. The ability of precise genome editing of autophagy-related-genes (Atgs) plays a critical role to study the underlying mechanisms of this complex process(9). The first knockout Atgs is *Beclin1* via embryonic stem (ES) cell-based gene-targeting technique in mice, which showed significant phenotypes (10, 11). After that,

many Atgs such as *ATG4B, ATG5, ATG7* have been modified in cells and mice using Cre-Lox recombinase and/or ES cell-based gene-targeting approaches to study the role of those Atgs in autophagy regulation and related biological functions (12-16). Recently, the CRISPR system have been developed and the convenience of design, construction, and delivery of sgRNAs offered an excellent possibility of rapid genome editing in autophagy study via targeting Atgs using CRISPR system. Here, we reviewed the very recent study of the novel genome editing tool CRISPR in knockout of autophagy genes and reported our partial date in order to elaborate the important role of CRISPR in autophagy research.

THE CRISPR STORY

History of CRISPR

CRISPR systems have created a profound and lasting effect ever since it was established as the latest genome editing tool along with past technologies like meganucleases, ZFNs and TALENs. CRISPR clustered repeats was first discovered in 1987 while Nakata and team were working on the IAP enzyme in *E. coli* and a set of 29-nt repeats downstream of the *iap* gene was found (17). In 2002, Jansen and Mojica collectively described the genomic loci of microbials which consists of an interspaced repeat array with the term CRISPR (18). The research on CRISPR was at its crux in 2005 when further analyse on spacer sequences that separate each direct repeats resulted in the conclusion that they were of extra chromosomal and phage-related nature (19).

By 2010, the functional mechanism of the natural Type II CRISPR system was better understood to construct an RNA-guided DNA endonuclease for genome editing. Cas9 is the sole enzyme within the *cas* gene array to exert nucleolytic activity on DNA (20). Together with this data, a non-coding trans-activating crRNA (tracrRNA) which hybridizes with crRNA to facilitate RNA-

guided targeting of Cas9 has been to be the key component in crRNA biogenesis and processing in Type II CRISPR system (21). Later in 2012, it was shown that crRNA-guided cleavage by purified Cas9 was possible (22) and that a single guide RNA (sgRNA) could be designed by joining a crRNA containing the guide sequence to a tracrRNA (23) which aids DNA cleavage by Cas9. Currently, multiple guide RNAs could also be designed to target multiple genes at once for genome editing with high efficiency (24). Many open-source distributors and online user forums have helped to advance the Cas9 technology as well.

Types of CRISPR/Cas system

The CRISPR adaptive immune system and CRISPR-associated (Cas) systems which originated from bacterial and archaeal hosts (25) primarily function as an immune system that cleaves exogenous DNA (26) or RNA (27) via an RNA-guided nuclease. Therefore, this serves to protect the bacterial and archaeal hosts from invading viruses or plasmids. CRISPR systems have been classified into three major types **(Table 1)** based on their genetic content, structural and functional differences whereby the key differences among the three is established by the *Cas* genes and encode proteins (28). However, among the three, the Type II CRISPR system is the best characterized (29) which comprises of the nuclease Cas9, the crRNA array and an ancillary trans-activating crRNA (tracrRNA).

The structure of Cas9

It was found that *S. pyogenes* Cas9 (SpCas9 for short) has two lobes; recognition (REC) lobe and nuclease (NUC) lobe (30). The variable, α-helical REC lobe is composed of three regions as follows: a long α helix referred to as the bridge helix, REC1 domain and REC2 domain (31). The

NUC lobe similarly has three domains but they are the RuvC, HNH and PAM-interacting (PI) domains. Within both of these two lobes, two clefts that bind to gRNAs and target DNA sequences by the REC and NUC lobes respectively were identified. Between the REC and NUC lobes is a positively charged groove, which is formed as a result of RuvC domain interfacing with PI domain, where the negatively charged sgRNA:target DNA heteroduplexdocks.

As aforementioned, Cas9 must first undergo conformational change to activate its catalytic function. Based on single-particle electron microscopy reconstructions, conformation of Cas9 in the apo (unbound) state do not permit binding and cleaving of target DNA (32). It is only upon association of crRNA-tracrRNA duplex with Cas9 that it induces the two lobes to rearrange its structure into a channel for the target sequence to dock (33). Hence, the presence of the crRNA-tracrRNA duplex determines if Cas9 is activated or not. Additionally, the HNH and RuvC domains can be mutated for functions other than for carrying out strand-specific cleavage (29). By substituting aspartate with alanine (D10A) in the RuvC domain, the mutant Cas9 now nicks DNA to yield single-stranded breaks and the favored homology-directed repair (HDR) potentially reduces the frequency of undesirable indels from off-target DSBs (29).

With a thorough and deeper understanding of the mechanism for the Type II CRISPR/Cas system, attempts to redesign its structure to facilitate genome engineering purpose have been successful. The result was the construction of a chimeric RNA with crRNA and tracrRNA-derived sequences which was subsequently named as guide RNA (gRNA) (23). For highly specific DNA targeting, the crRNA or gRNA can be redesigned to target any DNA sequences and guide Cas9 to result in sequence-specific DSB.

Mechanism of action

The Type II CRISPR/Cas system derived from the SpCas9 has been studied the most extensively and as such, its mechanism of action is one of the most established (34). One crRNA unit consists of a partial direct repeat and a 20-nt guide sequence that is responsible for guiding Cas9 to a complementary 20-bp DNA target via Watson-Crick base pairing. The crRNA and tracrRNA fuse together to form a two-RNA structure which binds to either strand beside a PAM sequence. The target DNA precedes a 5'-NGG PAM (23) that is important for target recognition of Cas9 nuclease. This double-stranded (ds) DNA endonuclease targets specific sites for cleavage via crRNA and tracrRNA to stimulate a DSB.

Through single-molecule imaging, Cas9-gRNA complex was observed to strongly interact with target sequence containing a PAM (35) as compared to non-target sequences or complementary sequences lacking PAMs whereby binding was observed to be transient. Following PAM recognition, the Cas9-gRNA must unwind the double helix and initiate strand separation for complementary base pairing to occur between the target DNA and the crRNA guide sequence. It has been postulated (35) that PAM binding could either cause a destabilization of the DNA duplex along the length of the target sequence leading to random nucleation of the RNA-DNA heteroduplex or cause a local melting of the duplex. The latter involves the RNA-DNA heteroduplex nucleating at the 3' end that is adjacent to PAM before nucleating towards the 5' end.

In addition to SpCas9, more than 20 additional Cas9 homologs derived from a variety of bacterial species have been isolated. The PAM sequences also shows the big variation which can

provide more option to us when no suitable SpCas9 PAM available in the gene you interested (36).

A Cas9 homolog: Cpf1

Cpf1, a putative new class 2 nuclease was recently annotated from Feng Zhang's lab. Cpf1 is classified as a novel, type V CRISPR system. Cpf1 contains a RuvC-like endonuclease domain which is similar to Cas9, but without HNH endonuclease domain, indicating that Cpf1 may shows different function (37, 38).

Cpf1 cleaves DNA requires only one RNA rather than the two (tracrRNA and crRNA) which is more convenient than Cas9. In addition, Cpf1's preferred PAM is 5'-TTN, differing from that of Cas9 (3'-NGG) in both genomic location and GC-content. In terms of cleavage pattern, Cpf1 can cause 5 nucleotide 5' overhang which is also different to Cas9 that created blunt double stranded cleavage (39). Since both Cpf1 and its guide RNAs are smaller than those in the SpCas9 system, they will also be easier to deliver in low-capacity vectors and shows high efficiency. Hence, the introduction of Cpf1-driven systems has added another option to the CRISPR toolbox and the application of Cpf1 to genome editing shows the potential advantages over Cas9 system.

APPLICATIONS AND DELIVERY SYSTEM

Native Cas9-mediated genome editing

As stated earlier, the native Cas9-mediated genome editing is executed through two steps. Firstly, Cas9 induces a DSB at a targeted site on the genomic DNA which is guided by a 20-nt guide sequence in the crRNA. Secondly, the DSBs then undergo either the error-prone NHEJ or the high-fidelity HDR pathway. For the native Cas9 system to work, the basic components required includes the Cas9 nuclease, tracrRNA and the customizable crRNA which should all be expressed in the foreign host. With the ease of customization of the 20-nt guide sequences, double deletion and/or multiplexed editing were made possible in *E. coli*(40) and human (41) genomes in one step.

When the Type II CRISPR/Cas system was further simplified to include just the Cas9 nuclease and custom gRNAs, it opened up an even broader selection of cell types and organisms for genome editing. Studies to date have successfully engineered and edited the genomes of humans, mice, fruit flies, zebrafish, yeast, thale cress, tobacco, wheat and rice plants (42). This simplified version of the Type II CRISPR/Cas system was observed to be capable of disrupting five genes in a single genome simultaneously (43). Indeed, the Type II CRISPR/Cas system is an excellent platform for genomic studies with broad applications in a variety of hosts.

Cas9 nickase-mediated genome editing

By mutating the RuvC or HNH domain, the gRNA-guided Cas9 which originally induces a DSB at the target site now has the acquired nickase ability. This mutated complex, gRNA-guided Cas9n, with nicking function is useful for successful genome editing at target sites specifically through the generation of DSBs and NHEJ-induced mutations when used as a pair (42). This

double nicking strategy targets the opposite strand of a target site to initiate HDR that is higher in efficiency and faster in rate as compared to the native Cas9-mediated HDR and single Cas9n-mediated HDR respectively (44).

Off-target cleavages were surprisingly reduced by 50 to 1500 times in human cells via this paired nicking mechanism without comprising the efficiency of on-target cleavages (44). One other advantage brought about by paired nicking is that it generates accurate overhangs as predicted. Together with NHEJ-mediated ligation, double-stranded repair templates with complementary overhangs have demonstrated success in HDR-independent fragment integration at target sites (42). Furthermore, about 6kb worth of genomic fragments in HEK293FT cells were deleted when paired double nicks are induced at two sites using four customized gRNAs. As such, it could be concluded that Cas9n is able to induce highly accurate genome editing.

Inactivated Cas9-based transcriptional control

A completely inactive dCas9 coupled with a custom gRNA is able to exert transcriptional control without changing the target sequence. Such inactivated Cas9-based transcriptional control is termed as CRISPR interference (CRISPRi) and identifies target sequences via complementary base pairing. Once the target sequence is identified, CRISPRi inhibits the initiation of transcription and elongation (42) which were successfully observed in *E. coli* and human cells (45). Since the gRNAs are customizable, it makes it plausible to exploit this system for regulating several genes at any one time.

Besides inhibiting the initiation of transcription and elongation, genes could also be silenced and this effect is made reversible with anhydrotetracycline-inducible promoter to initiate dCas9 and gRNA expression (45). The efficiency of this system to repress transcription was found to attain

repression about 1000 fold (45) which proves its usefulness in gene expression regulation at the transcriptional level.

Gene therapy

Besides its use in genetic studies in many species, Cas9 can be utilized to model the causal roles of specific genetic variations in human induced pluripotent stem cells (iPSCs) with specific mutations introduced or rectified (46). Human iPSCs are useful as they are a renewable source of cells for human biology and disease research and are also a potential candidate for gene or cell therapy development (47). In one recent study conducted by Smith et al., whole-genome sequencing analysis was performed and it was found that CRISPR/Cas9 demonstrated high specificity genome editing in human iPSCs. However, it remains to be evaluated in this study if the higher off-target rates observed in cancer cell lines are accounted by gRNAs and Cas9 overexpression and/or due to aggravated faulty repair systems in these cells.

Currently, ongoing studies are being conducted on the therapeutic potentials of the CRISPR/Cas9 system and results have been promising (48). Non-genetic or genetic disorders, which is largely due to point mutations, substitutions, deletions and insertions (49), or complex diseases could be rectified with engineered endonuclease Cas9. In the search for a form of cure for AIDS, it was understood that individuals who are homozygous for Δ32 deletion in CCR5 (CCR5Δ32) have resistance to CCR5-tropic HIV-1 infections (50). Therefore, it is highly possible that through specific deletions executed by the engineered endonuclease Cas9, this novel technology could be a gateway for an eventual cure for AIDS (51). Moreover, besides a feasible strategy to circumvent HIV infection, deletion of PCSK9 (51)or angiopoietin(52) have

demonstrated convincing results as a potential means against stain-resistant hypercholesterolemia or hyperlipidemia.

The different delivery systems of CRISPR

The CRISPR system can be delivered into a wide range of cell types and organisms **(Table 2)** as mentioned above through various delivery techniques. Electroporation(53), nucleofection and Lipofectamine-mediated transfection (54)of non-replicating plasmid DNA have been utilized to transiently express Cas9 and gRNAs in mammalian cells in vitro(55-57). These methods are traditionally used to deliver RNA-guided nucleases but in terms of efficiency, it is not as efficient as lentiviral vectors which confer very high gene delivery efficiency of about 95-100%.

On the other hand, it is also possible to directly introduce RNAs and plasmid DNA by microinjection into zebrafish, fruit flies, mice and rats embryos(55). Gonads of roundworms were similarly subjected to the same direct injection method in addition to the direct injection of purified Cas9 protein complexed with gRNA(58)in a separate study. Not only in animals, Cas9 was also successfully delivered into many plant species such as wheat, rice, sorghum, tobacco and thale cress via delivery methods like PEG-mediated transformation of protoplasts, Agrobacterium-mediated transfer in embryos and leaf tissue and/or bombardment of callus cells with plasmid DNA(59).

Off-target effects

The issue of off-targeting exists because CRISPR/Cas9 tolerates mismatches up to 5-bp within the protospacer region (54). When the gRNA binds to a site within the genome that is not completely complementary, the Cas9 nuclease is misguided to stimulate a DSB at the off-target site instead. The repercussion of off-target cleavage would be a mis-interpretation of phenotypic effects in gene knockout experiments which could also result in undesirable toxicities (60). Off-targeting is positively correlated with Cas9 concentration whereby off-target activity becomes more significant as concentration of Cas9 increases (61). However, this problem could be easily solved by adjusting Cas9 concentration and hence, the Cas9-sgRNA complex levels. As evident in the study by Hsu et al., specificity increased significantly as equimolar amounts of Cas9 and sgRNA transfected into 293FT cells were reduced from 400 ng to 10 ng of Cas9-sgRNA plasmid (61). Besides regulating Cas9-sgRNA complex levels, decreasing the amount of transfected DNA was another method to increase specificity (61). Although effective, decreasing the amount of transfected DNA would have an effect on on-target cleavage.

Facing such an issue, two independently discovered CRISPR variants: eSpCas9 and SpCas9-HF1 have been development to improve the on-target specificity respectively (62). Slaymaker et al believed that, if they decreased the positive charge in the HNH/RuvC groove would theoretically decrease off-target cutting (62). Hence, a variety of alanine substitutions throughout the groove in 32 separate Cas9 mutants had created to decrease the electropositivity of the HNH/RuvC groove. Two of the mutants, SpCas9(K855A) and eSpCas9, revealed that these mutants do not cause off-target effects at unanticipated sites. Meanwhile, Kleinstiver et al. reasoned that the weaken sequence independent interactions between Cas9 and DNA also could diminish off-target cutting (63). SpCas9-HF1 (mutation Q926A) generated fewer off-target cuts when

compared to WT SpCas9 across a variety of genomic sites. Accordingly, with the enhanced specificity or other mutation combination, eSpCas9 and SpCas9-HF1 should enable researchers to make precise edits in mammalian cells with decrease worries about off target effects in future.

CRISPR AS A POWERFUL TOOL IN AUTOPHAGY STUDY

Current application of CRISPR in autophagy

Although many chemical inhibitors of autophagy had been widely used in autophagy study, those can block a given pathway but have limitations for specific and efficient inhibition. For example, some inhibitors such as chloroquine, bafilomycin A1, and 3-methyladenine (3-MA), can target many components or many aspects of the autophagic pathway, suggesting that these inhibitors are not exclusive (64). Chloroquine and bafilomycin A1 can block autophagy by impairing lysosomal function, while 3-MA is an inhibitor of phosphoinositide 3-kinase (PI3K) (65, 66). In addition, these inhibitors also play an important role in other pathways involved in other different physiology function (67, 68).

To enhance the specification of the inhibition of autophagy, siRNA and shRNA had been used to inhibit autophagy by genetic silencing of ATG genes. Compared with those pharmacological inhibitors, these strategies show more specific function in autophagy inhibition. While the knockdown effects caused by gene silence are often incomplete so as to the autophagy inhibition. Hence we need new genome-engineering strategies, such as CRISPR/Cas9 to achieve complete gene deletion and autophagy inhibition.

With the development of the excited CRISPR technique, researchers had applied this tool to autophagy research field. Since autophagy is an evolutionarily conserved pathway and proteins coded by the autophagy-related genes (Atgs) are the core molecular machinery in control of autophagy (9). The ability of precise genome editing of Atgs plays a critical role to study the

underlying mechanisms of this complex process (Figure 1). Currently, most works for genome editing of the Atgs were focued on the gene knockout as well as knockin (69). The effects of several Atg genes knockout have been well studied from the formation of autophagosomes to autolysosomal biogenesis (70, 71). Using CRISPR/Cas9 to delete of the canonical autophagy-essential genes *ATG5, ATG7, ATG16L* and *ULK1* have also been reported in *in vitro* or *in vivo* models (72-76). In addition, A genome-wide CRISPR screen in MTOR signaling and the entire macroautophagy machinery as key regulators of *SQSTM1* suggesting that the pooled CRISPR screening as a powerful method to map the cellular pathways that regulate the fate of an individual target protein (77) (Table 3). Table 3 summarized the recent studies of the CRISPR/Cas9 application for Atgs target. Next, we present our own data on *ATG7* knockout and investigate the effect of *ATG7* deletion on autophagy.

ATG7 knockout blocks basal and starvation-induced autophagy level

Here, two construct systems pSpCas9 (BB)-2A-GFP (PX458) (Addgene plasmid #48138) and pSpCas9(BB)-2A-Puro (PX459) (Addgene plasmid #48139) were used to investigate the effect of ATG7 knockout on autophagy. Sanger sequencing results show that in instances where indels, do occur, they were mostly found within the target sequences. Overall, indel rates for the two target sites of ATG7 (ATG7-1 and ATG7-2) in both PX458 and PX459 transfection systems were 87.0% ,75.0% and 85.0%, 84.7% respectively (Data not shown).

ATG7 protein levels were significantly lower in knockout mutants as compared to the controls, non-transfected cells and cells transfected with empty vectors. There is a general trend of high SQSTM1 protein levels among knockout mutants as observed. It was also noted that mutants had

lower LC3-II protein levels than the controls suggesting that the ATG7 knockout effectively blocks the basal level of autophagy in the two Cas9 systems (Figure 2A).

Under starvation condition, *ATG7* mutants generally had relatively higher levels of SQSTM1 and lower levels of LC3-II than the control cells transfected with empty vectors regardless EBBS (Earle's Balanced Salt Solution) and/or CQ treatment. As expected in PX458 transfection systems (Figure 2B), when controls in both transfection systems were administered EBSS and CQ simultaneously, levels of LC3-II in mutant were significantly reduced compare to control. Thus, we believe that the disruption of ATG7 genes induced by CRISPR-Cas9 either at single or multiple target sites could effectively reduce the basal and starvation induced autophagy level. In addition, *ATG7-1* and *ATG7-2* knockout mutants exhibited significantly greater cell death under starvation conditions (EBSS treatment groups) as compared to mutants that were not starved (DMEM treatment groups) suggesting that ATG7 knockout promotes cell death (data not shown).

CONCLUSION

Clearly, the understanding of the CRISPR/Cas9 system has been evolving over the past 3 decades and so far, the system has proved itself to be full of potential in the field of genome engineering. The customizable 20-nt guide sequence of the Cas9-gRNA complex confers flexibility to the CRISPR/Cas9 system to recognize any desired target sequences. The simplification of the system through the construction of a chimeric RNA to give rise to the gRNA has allowed greater targeting efficiency and multiplexible genome targeting. Although primarily found to provide immunity against exogenous genetic elements, the CRISPR/Cas9 system was found to be involved in various other applications as well as in autophagy study. Since this system is still evolving, we believe that in the future we will be able to understand the mechanism behind CRISPR systems better and be well equipped to operate this technology more cost effectively. The CRISPR/Cas9 system has aided in many genomic studies in many areas where traditional tools were unable to achieve. This emerging trend of genome editing is set to potentially help to study the underlying mechanisms in autophagy research in detail via genome modification. Since CRISPR-Cas9 system is an appealing approach to disrupt specific genomic regions with the easily designed gRNA. This rapid and convenient technique might facilitate understanding of molecular mechanisms of a wide range of biological function and regulation involving in autophagy process.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

FIGURE LEGENDS

Figure 1. Schematic diagram of the mammalian autophagy core machinery and CRISPR/Cas9 targeted genes The Atg proteins form several important functional groups in control of autophagosome formation. (i) The ULK1 complex, consisting of the serine/threonine kinase ULK1, ATG13, focal adhesion kinase family interacting protein of 200 kDa (FIP200) and ATG101, controls the induction or initiation of autophagy for the formation of phagophore and is negatively regulated by mechanistic target of rapamycin (mTOR). (ii) The Beclin 1-class III PI3K complex controls the nucleation step of autophagosome formation. (iii) The two ubiquitin-like conjugation systems (the ATG12-ATG5 system and the LC3 system) mediate the elongation stage, leading to formation of a complete autophagosome. And (iv) The ATG9 retrieval process functionally involves a protein complex of WIPI1/2 and ATG2. The CRISPR targeted genes (Table 3) in the autophagy core machinery in this review was indicated by an asterisk (*).

Figure 2.The effect of knocking out ATG7 on autophagy. (A) The effect of knocking out ATG7 on basal level of autophagy in the two Cas9 systems. (B) The effect of starvation and/or CQ treatment on autophagic flux in ATG7 knockout cells in PX458 systems. Immunoblotting of ATG7, LC3 and SQSTM1 using lysates from HEK293FT cells transfected with respective vectors inserted with two target sites, either ATG7-1 or ATG7-2. β-ACTIN served as a loading control. Cells were subjected to EBSS starvation and/or 50 μM of CQ treatment for two hours.

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435 **Table 1.** 3 Major types of CRISPR systems

Types	I					II				III	
Subtypes	A	B	C	D	E	F	A	B	C	A	B
Organism	Bacteria					Bacteria				Archaea & Hyperthermophiles	
Target	DNA					DNA				DNA	RNA
Genetic	*cas1,cas2,cas3*,cas5,cas6,cas7*					*cas1,cas2, cas9**				*cas1,cas2,cas6,cas10**	
Signature genes	*cas8a*	*cas8b*	*cas8c*	*cas10d*	*cse1,cse2*	*csy1*	*csn 2*	*cas4*	-	*csm2*	*cmr5*
References	(28)					(28)				(78)	

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449 **Table 2.** Application of Type II CRISPR/Cas system in various cell types and organisms

Application	Cell types/Organisms	References
Genome editing	Mouse	(79)
	Human HUES62, HEK293T, 293FT, K562 & iPS cells	(79-82)
	Rice protoplast and callus cells	(83-86)
	Streptococcus pneumoniae	(40)
	Escherichia coli	(40)
	Zebrafish embryos	(82, 87)
	Drosophila preblastoderm embryos	(88)
	Caenorhabditis elegans germ line	(89, 90)
	Xenopus tropicalis embryos	(91)
	Saccharomyces cerevisiae	(92)
	Arabidopsis protoplast and seedlings	(40, 93)
	Wheat protoplast	(86)
	Tobacco protoplast and leaf	(84, 93, 94)
	Sorghum immature embryos	(84)
	C. elegans germ line	(95)
	Mouse zygotes	(96)
Transcriptional control	*E. coli*	(97)
	S. pneumoniae	(97)
	Human HEK293, 293T cells	(80, 98)
	S. cerevisiae	(98)

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455 **Table 3 The genes targeted by CRISPR/Cas9 involved in autophagy core machinery**

Target Genes	Effects on autophagy	Delivery systems	Refs
ULK1,ATG101	Suppression of induction	Transfected into U937, MEFs	(72, 99)
ATG5	Resistance to gossypol in ATG5 knockout cells is associated with increased cytoprotective autophagy, independent of ATG5.	Transfected into A375P cells, IPEC-J2.	(74, 100)
ATG3,ATG7, ATG13	Suppressed translation and ULK1 degradation can restrict autophagy under prolonged starvation; Suppression of Vesicle Elongation	lentiCRISPR v1 vector; Transfection in K562;	(73, 75, 101-103)
Atg8/LC3	Knockout of LC3/GABARAPs was failure to drive autophagosome-lysosome fusion	Transfected into HeLa cells	(71)
ATG9	Suppression of retrieval process	injecting an expression plasmid	(104)
ATG16L1	Suppression of Vesicle Elongation	Transfected into HAP1 cells	(105)
SQSTM1/MTOR	Pooled CRISPR screening to map MTOR signalling and the entire macroautophagy machinery	lentiviral delivery into H4 cells	(77)
VPS34/ATG14	Suppression of vesicle nucleation	Transfected into HEK293	(106, 107)
SMPD1	Induces a severe autophagy defect characterized by altered trafficking of ATG9A	Transfected into MCF7	(108)

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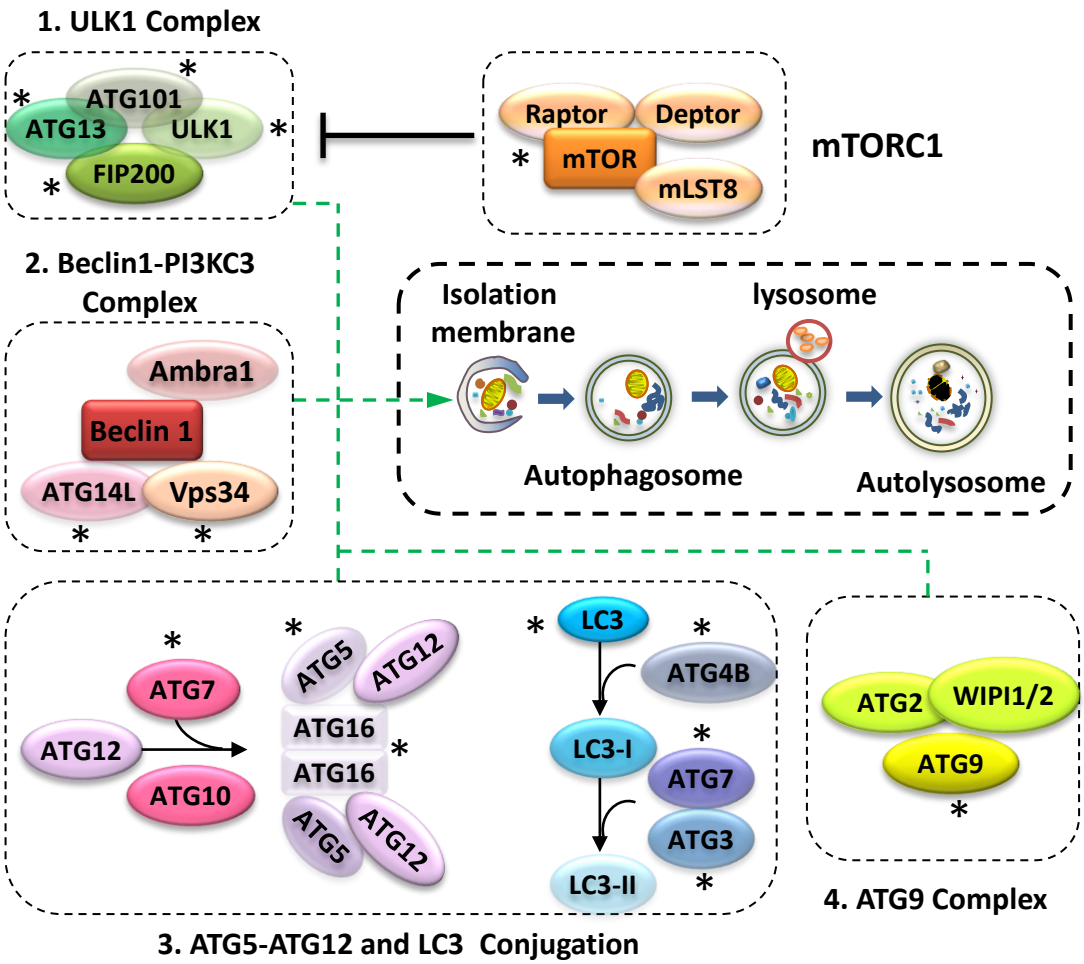
1. ULK1 Complex
ATG101
ATG13
ULK1
FIP200
Raptor
Deptor
mTOR
mLST8
mTORC1
2. Beclin1-PI3KC3 Complex
Ambra1
Beclin 1
ATG14L
Vps34
Isolation membrane
lysosome
Autophagosome
Autolysosome
ATG7
ATG12
ATG10
ATG5
ATG12
ATG16
ATG16
ATG5
ATG12
LC3
ATG4B
LC3-I
ATG7
ATG3
LC3-II
ATG2
WIPI1/2
ATG9
4. ATG9 Complex
3. ATG5-ATG12 and LC3 Conjugation

Figure 1

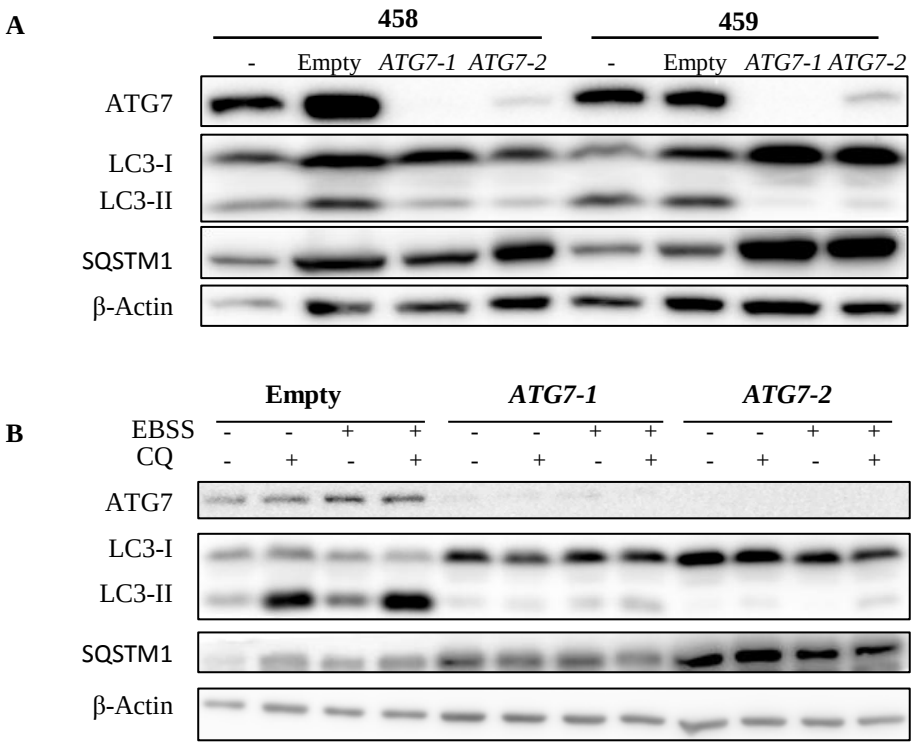
A
458
459
-
Empty
ATG7-1
ATG7-2
-
Empty
ATG7-1
ATG7-2
ATG7
LC3-I
LC3-II
SQSTM1
β-Actin
B
Empty
ATG7-1
ATG7-2
EBSS
CQ
ATG7
LC3-I
LC3-II
SQSTM1
β-Actin

Figure 2