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Synthetic autophagy receptor

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ABSTRACT

Macroautophagy/autophagy receptors target their substrates to phagophores for subsequent sequestration within autophagosomes. During phagophore membrane expansion in mammalian cells, autophagy receptors simultaneously interact with the ubiquitinated substrates and the LC3/GABARAP proteins on the expanding membrane. In this punctum, we summarize and discuss our recent research progress on synthetic autophagy receptors (AceTACs). The series of AceTACs were designed by engineering the essential interacting domains and motifs of SQSTM1/p62 (sequestosome 1), a major mammalian autophagy receptor. Particularly, we replaced the ubiquitin-associated domain of SQSTM1 with a target-specific antibody, redirecting the bifunctional interactions of wild-type SQSTM1 and directing the degradation target into the autophagy process. We successfully demonstrated the targeted degradation of aggregation-prone proteins using the AceTAC degraders. Moreover, we presented a model system with a guideline to induce targeted degradation of organelles through the autophagy machinery.

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Antibody-fusion protein; autophagy receptor; targeted organelle degradation; targeted protein degradation; proximity-based therapeutics

The field of proximity-based therapeutics features a broad interest in developing targeted degradation technologies. Major development in the field has been focused on utilizing the ubiquitin – proteasome system (UPS) for targeted protein degradation. Bifunctional molecules are designed to enhance the interaction between the degradation target and an E3 ubiquitin ligase, inducing the ubiquitination of the target and its subsequent proteolysis through the proteasome. However, as the UPS degradation system does not process aggregation-prone and large cellular components, there is a technical gap in the targeted degradation methodologies to address the large-sized aggregates and organelles. Such a technology could fill a therapeutic gap for neurodegenerative diseases. Among the intracellular degradation systems, the autophagy – lysosomal pathway (ALP) contributes to the clearance of unwanted protein aggregates, organelles, and pathogens. Employing the ALP for targeted degradation methods is therefore a promising avenue for selectively removing large intracellular structures [1].

Macroautophagy (hereafter denoted as autophagy) programs the degradation of protein aggregates and organelles (*i.e.*, substrates). Autophagy receptors facilitate the delivery of ubiquitinated substrates to the forming autophagosomes. Particularly, the ubiquitin-associated domain of the autophagy receptor interacts with the ubiquitin chains on the substrate, while the LC3-interacting region (LIR) of the autophagy receptor binds with the LC3/GABARAP proteins on the expanding phagophore membrane. After phagophore closure and autophagosome maturation, the engulfed substrates are delivered to a proteolytic environment upon fusion of the autophagosome with the lysosome. We were inspired by the protein-protein interaction of autophagy receptors and sought to develop proximity-based therapeutics for targeted intracellular degradation.

What is the design principle of synthetic autophagy receptors?

The synthetic autophagy receptors (AceTACs) were designed from the full-length structure of SQSTM1. Using the HTT (huntingtin) protein with polyglutamine expansion (HTT-103Q) as the target for degradation, we concluded that the design principle for AceTACs was the co-presence of a target-specific antibody and the LIR motif of TP53INP2. In detail, the antibody construct in the AceTACs was a previously reported single-domain antibody (*i.e.*, nanobody) against an ALFA-epitope tag, enabling the binding between the AceTACs and the target that contains the ALFA-tag. The TP53INP2-LIR motif was initially designed to bind tightly to the six primary LC3/GABARAP proteins, with a surprising observation that its presence also enhanced the autophagic activity. Using this design principle, we obtained efficient targeted

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degradation of aggregation-prone proteins, including HTT-103Q, SNCA/α-synuclein, and mutants of TARDBP/TDP-43, FUS, and MAPT/tau.

How to apply synthetic autophagy receptors for targeted organelle degradation?

We adapted the bifunctional interaction feature of synthetic autophagy receptors for targeted degradation of organelles. Apart from the AceTAC degrader, an outer membrane protein anchor was required in the degradation system. In this work, the membrane anchor construct was based on a SNAP-tag protein, with an N-terminal targeting signal for organelle localization and a C-terminal ALFA-tag for the interaction with the AceTAC. Using the AceTAC degradation system with varied localization signal on the SNAP-tag anchor, we successfully reduced the overall fluorescence intensity of organelles, including mitochondria, peroxisomes, and endoplasmic reticulum. Additionally, by regulating the expression of the membrane anchor construct for the mitochondrial surface, we found that an increased level of membrane anchor led to an increased mitochondrial reduction. This result demonstrated the tunability of our targeted organelle degradation system, indicating the possibility of quantitative control over the total organelle content.

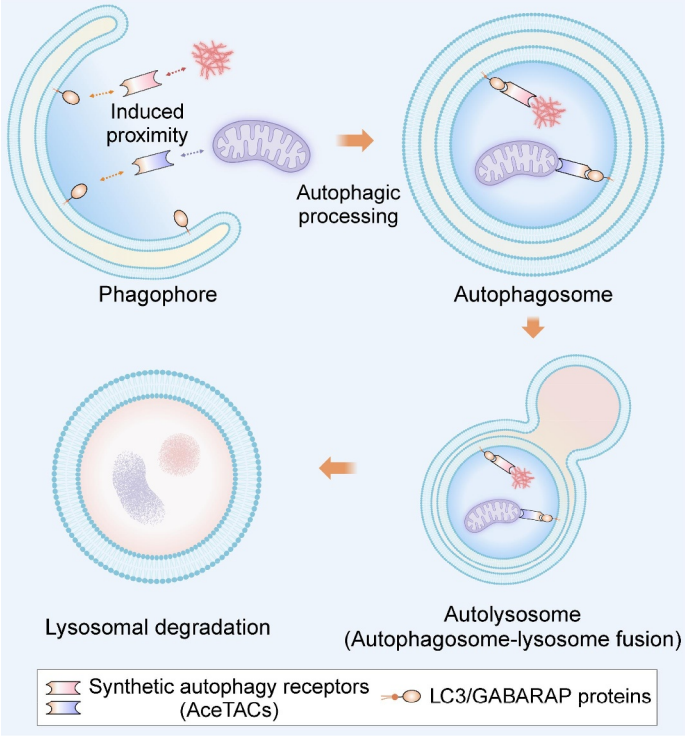

Figure 1. Workflow of the synthetic autophagy receptors (AceTACs) for targeted intracellular degradation.

What is the mechanism of action for synthetic autophagy receptors?

We confirmed that the synthetic autophagy receptors mainly operated through the autophagy pathway (Figure 1). From immunofluorescence results, the AceTAC induces puncta formation upon interacting with the targets, meanwhile colocalizing with two autophagy markers (*i.e.*, endogenous SQSTM1 and LC3B). When autophagy flux is inhibited via the knockdown of ATG5 or ATG7, the targeted degradation of HTT-103Q by AceTACs is also suppressed. The contribution of the autophagy pathway for AceTACs was further confirmed by their enhanced degradation efficacy of HTT-103Q in autophagy-inducing conditions (*e,g.*, torin-1 treatment). Based on existing knowledge that the substate degradation occurs after autophagosome-lysosome fusion, it would be helpful to further elucidate the colocalization between the AceTAC-target and lysosomal markers, clarifying the downstream processes leading to AceTAC-induced targeted degradation.

Does LC3B-overexpression enhance the targeted degradation efficiency?

LC3B is the most commonly utilized LC3 isoform in autophagy-related research. The lipidation of LC3B facilitates LC3B and its binding partners (such as autophagy receptors) to anchor on the phagophore membrane. Apart from the reported data, we assessed the effect of LC3B overexpression on the efficacy of AceTAC degraders. The co-expression of AceTAC degrader and LC3B was introduced by a T2A polycistronic construct, where similar levels of AceTAC and LC3B are presented in mammalian cells. From the comparison, we do not observe significant improvement in the AceTAC-induced HTT-103Q degradation upon the overexpression of LC3B. However, this result does not rule out the participation of LC3B during the functional process of synthetic autophagy receptors, as we do observe the colocalization of endogenous LC3B within the AceTAC-bound puncta in cells. Meanwhile, after our initial report on AceTAC degraders, a very recent study reported the fusion between LC3B and antibody (ATNC) as a degrader platform, further supporting the feasibility of the degrader design through inducing the proximity between the target and the phagophore membrane.

Overall, we think that AceTAC technology has broad implications in the development of autophagy-focused proximity-based therapeutics. Deeper understanding of several aspects will certainly accelerate the development and translation of synthetic autophagy receptors for therapeutic strategies. For example, the ubiquitination state of targets during AceTAC-induced degradation was not assessed. We hypothesized that the AceTAC can result in the degradation of target irrespective of its ubiquitination state. Addressing this question could shed light on the principles for utilizing the intracellular degradation systems, such as selecting the degradation pathway (*e.g.*,

UPS or ALP) depending on the target properties. Moreover, the autophagy-inducing function of the TP53INP2-LIR motif suggests that the LIR motif possesses functions beyond binding with the LC3/GABARAP proteins. Revisiting the cellular functions of existing LIR motifs may help generate the next generation of synthetic autophagy receptors.

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Disclosure statement

We have filed a US provisional patent on this technology (No. 63/584,617).

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