SYSTEMS AND METHODS FOR SELECTIVE ISOLATION AND CHARACTERIZATION OF ORGANELLES USING PROGRAMMABLE TARGETING MODULES

TECHNICAL FIELD

[0001] The present disclosure relates to the field of cellular and molecular biology and, more specifically, to systems and methods for the selective isolation, enrichment, and purification of organelles, in particular intracellular organelles and vesicular compartments, in particular from eukaryotic cells.

BACKGROUND

[0002] Eukaryotic cells comprise a wide variety of intracellular organelles and vesicular compartments that carry out essential biological functions, including, for example, autophagosomes, endosomes, lysosomes, mitochondria, peroxisomes, Golgi-derived vesicles, and secretory vesicles. Among these, autophagosomes are double-membrane vesicles generated during autophagy, a highly conserved cellular pathway involved in the sequestration, degradation, and recycling of intracellular components.

[0003] These compartments are central to the maintenance of cellular homeostasis and are involved in diverse processes including metabolism, immunity, stress responses, and disease. Autophagy, in particular, plays an important role in cellular adaptation to stress, pathogen defense, development, and numerous pathological conditions, including cancer, neurodegenerative diseases, and infections. Autophagy may have context-dependent effects in disease, being protective in certain conditions, such as neurodegenerative disorders including Alzheimer's disease and Parkinson's disease, while supporting cell survival in certain cancers. Despite its importance, currently available diagnostic markers of autophagy remain insufficiently specific.

[0004] A major limitation in the study and practical exploitation of autophagy and other organelle-associated processes resides in the lack of reliable methods for selectively isolating intact organelles, particularly in a stimulus-specific or condition-specific context. Autophagosomes are highly transient and dynamic structures, which makes their selective purification, enrichment, and subsequent analysis particularly challenging.

[0005] Methods for isolating and purifying organelles have been reported in the art, including, without limitation, approaches based on antibody-mediated isolation and density gradient centrifugation.

[0006] **US10912947 B2** discloses methods for the magnetic enrichment of intracellular organelles, including lysosomes, by means of LAMP1-labeled nanoparticles.

[0007] **WO2019222708 A2** discloses a method for the isolation of extracellular vesicles (EVs), based on the selective binding of at least one capture reagent and one binding reagent to a plurality of surface markers present on said EVs.

[0008] **WO2021015298 A1** discloses a method for the isolation of mitochondria from cells, wherein the method is carried out in the presence of a surfactant.

[0009] However, existing methods for isolating organelles remain associated with significant drawbacks, including, without limitation: (i) density gradient centrifugation, which frequently produces heterogeneous fractions and exhibits limited specificity; (ii) antibody-based capture approaches, which rely on antibodies that may compromise organelle integrity and/or may fail to discriminate between organelle populations associated with different cellular conditions; (iii) chemical probe and labeling approaches, which may allow detection of organelles but do not permit selective purification thereof; and (iv) biochemical fractionation methods, which lack sufficient precision and reproducibility.

[0010] These limitations prevent or substantially hinder the selective isolation of organelles formed under defined physiological or pathological conditions, thereby impeding detailed molecular characterization of their molecular composition and contents.

[0011] Accordingly, there remains a need for a universal, programmable, and genetically encoded method that enables the selective isolation of intact intracellular organelles, in particular autophagosomes, under defined cellular, physiological, or pathological conditions.

[0012] The present invention addresses the above-mentioned needs.

SUMMARY

[0013] The present invention relates to systems and methods for the selective isolation, purification, and characterization of organelles, in particular organelles in eukaryotic cells, in particular autophagosomes, using programmable heterodimerization modules designated ORBIT-A and ORBIT-B. ORBIT-A is fused to an organelle-associated protein, such as members of the ATG8 family, thereby labeling said organelles. ORBIT-B is localized to a defined capture surface, organelle, or substrate, enabling selective recruitment, clustering, and isolation of intact organelles, such as intact autophagosomes.

[0014] The invention enables stimulus-specific purification of organelles induced by defined biological conditions, including immune activation, metabolic stress, infection, or pharmacological treatment. The isolated organelles can be analyzed using proteomic, lipidomic, metabolomic, or nucleic acid profiling methods. The invention further enables identification of stress-specific cargo, biomarkers, and therapeutic targets, and has broad applications in biotechnology, agriculture, diagnostics, and medicine.

[0015] According to a first main embodiment, the invention relates to an *in vitro* method for isolating and/or purifying an organelle, comprising a step of:

a) providing a cell, culture medium or extract thereof, the cell, medium, or extract comprising **(i)** a target organelle, **(ii)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(iii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3** ;

b) recovering from the cell, medium or extract thereof, a protein complex formed by the first and second polypeptide **SEQ ID NO 1** and **SEQ ID NO 3,** thereby recovering said target organelle.

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle, of which at least one is the target organelle.

[0016] According to some embodiments, the target organelle is selected from the group consisting of: vesicle, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, vacuoles, chloroplast.

[0017] According to some embodiments, the target organelle is a vesicle selected from the group consisting of: lysosome, endosome, peroxisome, liposome, extracellular vesicle, exosome, phagosome.

[0018] According to some embodiments, at least one of the first or second polypeptide comprises a surface marker polypeptide sequence expressed at the surface of an endosome or an autophagosome.

[0019] According to some embodiments, said surface marker of an endosome or a autophagosome is selected from the group consisting of: ATG8, LC3, GABARAP, LAMP1, and LAMP2.

[0020] According to some embodiments, at least one of the first or second polypeptide comprises a surface marker of a mitochondria.

[0021] According to some embodiments,

- one of the polypeptides comprises a surface marker polypeptide sequence expressed at the surface of a mitochondria; and
- one of the polypeptides comprises a surface marker polypeptide sequence expressed at the surface of an endosome or a phagosome;

said surface marker polypeptide sequences being the same or different, preferably different.

[0022] According to some embodiments, the surface marker of a mitochondria is selected from the group consisting of: VDAC1, TOMM70, TOMM20, TOM22, OMP25, FIS1.

[0023] According to some embodiments, the cell or extract is a plant cell, or an extract thereof.

[0024] According to some embodiments, the cell or extract comprises **(i)** a target organelle, **(ii)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 2,** and **(iii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 4.**

[0025] According to some embodiments, the first and second polypeptide each comprise at least one distinct organic fluorophore, a pH-sensitive fluorophore, an enzyme activated fluorophore, a redox-sensitive fluorophore, a fluorogenic probe, a nonfluorescent protein and/or electron dense particle.

[0026] According to a second main embodiment, the invention relates to a composition comprising **(i)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0027] According to a third main embodiment, the invention relates to an expression vector **(i)** coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0028] According to a fourth main embodiment, the invention relates to a cell comprising:

- **(i)** an heterologous nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and

(ii) an heterologous nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:**

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0029] According to a fifth main embodiment, the invention relates to a kit comprising **(i)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker expressed at the surface of an organelle.

DEFINITIONS

[0030] Unless otherwise defined, the following terms as used herein have the meanings set forth below.

[0031] As used herein, the term **"organelle"** is intended to refer to any compartment, in particular any intracellular compartment, such as any membrane-bound compartment that may be produced in, present in, or associated with a biological cell, such as a eukaryotic cell. Unless otherwise specified, the term is not restricted to any particular type of compartment or vesicle. Thus, the term may designate, without limitation, compartments selected from the group consisting of vesicles, nuclei, endoplasmic reticulum, Golgi apparatus, mitochondria, vacuoles, chloroplasts, and the like. Examples of vesicular compartments include, without limitation, lysosomes, endosomes, such as early endosomes and late endosomes, multivesicular bodies, peroxisomes, liposomes, extracellular vesicles, exosomes, phagosomes, autophagosomes, and the like. As used herein, the term "**surface of an organelle**" is intended to refer to the surface of the organelle which is in contact with the cytoplasm (for intracellular compartments), or the extracellular medium (for compartments susceptible to be secreted or produced from a cell membrane (e.g. the plasma membrane).

[0032] As used herein, the singular forms "**a**", "**an**", and "**the**" include plural references unless indicated otherwise. Terms of degree such as "**substantially**", "**about**" and "**approximately**", as used herein, mean a reasonable amount of deviation of the modified term such that the end result is not significantly changed. These terms of degree should be construed as including a deviation of at least ±5% of the modified term if this deviation would not negate the meaning of the word it modifies.

[0033] As used herein, the term "**at least one**" includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 50, 75, 100, 250, 500, 750, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, 10^{15} or more. "**A plurality of**" means two or more than two; for example 2, 3, 4, 5, 6, 7, 8, 9, 10, 25, 50, 75, 100, 250, 500, 750, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, 10^{15} or more.

[0034] As used herein, the term "**comprising**" means "**consisting at least in part of**". When interpreting each statement in this specification that includes the term "comprising", features other than that or those prefaced by the term may also be present. Related terms such as "comprise" and "comprises" are to be interpreted in the same manner. Accordingly, the term "comprising" may refer more specifically to "**consisting of**". Related terms such as "consists of" are to be interpreted in the same manner.

[0035] As used herein, **"identity"** as in **"polypeptide sequence identity" or "nucleic acid sequence identity",** when used in a relationship between the sequences of two or more polypeptides or of two or more nucleic acid molecules, refers to the degree of sequence relatedness between polypeptides or nucleic acid molecules, as determined by the number of matches between strings of two or more amino acid or nucleotide residues. "Identity" measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms"). Identity of related polypeptides can be readily calculated by known methods. Preferred methods for determining identity are designed to give the largest match between the sequences tested. Methods of determining identity are described in publicly available computer programs. Preferred computer program methods for determining identity between two sequences include the GCG program package, including GAP (Devereux et al. "*A comprehensive set of sequence analysis*

programs for the VAX." Nucleic acids research, 1984, volume 12,1 Pt 1, pages 387-95), BLASTP, BLASTN, and FASTA (Altschul, et al. "*Basic local alignment search tool.*" Journal of molecular biology, 1990 volume 215, 3, pages 403-10). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources. The well-known Smith Waterman algorithm may also be used to determine identity. In one embodiment, the term identity is measured over the entire length of the sequence to which it refers. Accordingly, an 80% sequence identity with a given reference may encompass 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94% 95%, 96%, 97%, 98%, 99% or 100% sequence identity with said reference.

[0036] As used herein, "**nucleic acid**" refers to a polymer of nucleotides covalently linked by phosphodiester bonds, such as deoxyribonucleic acids (DNA) or ribonucleic acids (RNA), in either single- or double-stranded form. As disclosed herein, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. As disclosed herein, a nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues. Unless otherwise specified, as disclosed herein, a nucleotide sequence encoding an amino acid sequence includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. As disclosed herein, a nucleotide sequence that encodes a protein or a RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain at least one intron.

[0037] As used herein, the terms **"polypeptide," "protein,"** and **"peptide"** are used interchangeably and denote a polymer comprising amino acid residues and having any length. The polymer may be linear or branched, may comprise one or more modified amino acids, and may be interrupted by or linked to one or more non-amino acid moieties.

The terms further encompass amino acid polymers modified by, for example, disulfide bond formation, glycosylation, amidation, acetylation, phosphorylation, pegylation, or any other chemical or biochemical modification, including conjugation with a labeling moiety. As used herein, the term **"amino acid"** includes natural, non-natural, and synthetic amino acids, including glycine, D- and L-isomers thereof, amino acid analogs, and peptidomimetics.

[0038] As used herein, "**recombinant protein**" or "**recombinant peptide**" or "**recombinant polypeptide**" refers to a protein, peptide or polypeptide generated from recombinant DNA, i.e. from DNA artificially inserted in a host cell.

[0039] As used herein, a "**vector**" refers to any vehicle carrying a foreign nucleic sequence into a cell, for replication and/or expression. As disclosed herein, the term encompasses a replication vector, an expression vector, a donor vector. Vectors generally include at least one – or more – promoter region (e.g. inducible or non-inducible promoter region) and at least one – or more – terminator and/or regulatory region, although not necessarily.

[0040] As used herein, the term **"expression vector"** is intended to encompass any vector capable of permitting expression of a nucleic acid sequence in a given expression system. Such expression may include transcription of the nucleic acid sequence and, in certain embodiments, both transcription and translation of the corresponding nucleic acid sequence. An expression vector generally comprises one or more promoter regions, which may be inducible or non-inducible, and may further comprise one or more terminator regions and/or additional regulatory elements, although such elements are not necessarily required in all embodiments. In some embodiments, the expression vector may form part of an expression system comprising one or a plurality of vectors, for example, a dual-vector expression system.

[0041] As used herein, the term **"operably linked"** is intended to mean that, within an expression vector, a nucleotide sequence of interest is linked to one or more regulatory elements in a manner that allows or facilitates expression of said nucleotide sequence. Such expression may occur, for example, in an in vitro transcription/translation system

or in a host cell following introduction of the vector into said host cell. In some embodiments, suitable vectors include viral vectors, such as lentiviral vectors and adeno-associated viral vectors, and such vectors may be selected or configured to target particular cell types.

[0042] As used herein, the term "**regulatory element**" is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g., transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY : METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g., liver, pancreas), or particular cell types (e.g., lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector comprises one or more pol III promoter (e.g., 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (e.g., 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g., 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, Cell, 41 :521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter. Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE ; CMV enhancers ; the R-U5′ segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988) ; SV40 enhancer ; and the intron sequence between exons 2 and 3 of rabbit β-globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the

design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., clustered regularly interspersed short palindromic repeats (CRISPR) transcripts, proteins, enzymes, mutant forms thereof, fusion proteins thereof, etc.).

[0043] As used herein, "**promoter**" refers to a nucleic acid sequence which is required for expression of a gene product operably linked to said promoter. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter may, for example, be one which expresses the gene product in a tissue specific manner. Unless stated otherwise, the term may refer to inducible promoters or constitutive promoters. Unless stated otherwise, the term may refer to mammalian promoters or non-mammalian promoters.

[0044] As used herein, the term "**pharmaceutical composition**" refers to a combination of ingredients that facilitates administration of one or more agents of interest to a subject. A pharmaceutical composition generally comprises one or more agents of interest in admixture with one or more pharmaceutically acceptable excipients (e.g. carriers or diluents). Pharmaceutically-acceptable "carriers" and "diluents" are known in the art and generally refer to a pharmaceutically-acceptable materials, compositions, or vehicles, including liquid or solid fillers, diluents, excipients, solvents, binders, or encapsulating materials.

[0045] As used herein, the expression "**pharmaceutically acceptable excipient**" refers to an inert vehicle or carrier used as a solvent or diluent in which the pharmaceutically active agent is formulated and/or administered, and which does not produce an adverse, allergic or other reaction when administered to an animal, preferably a human. This includes all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic agents, absorption retardants and the like. For human administration, preparations must meet standards of sterility, general safety and purity as required by regulatory agencies, such as the FDA or EMA.

[0046] As used herein, the terms "**subject**" and "**patient**" and "**individual**" may be used interchangeably. The terms "**treat**," "**treating**" and "**treatment**" include the eradication, removal, amelioration, modification, reduction, management, or control of a pathological condition, in particular of a tumor, tumor cells or cancer, the minimization, prevention or delay of metastasis, or the prolongation of survival of the subject.

[0047] As used herein, the term "**prevent**", "**preventing**" and "**prevention**" may include a reduction of all or part of a risk (or likelihood) of occurrence or re-occurrence of a given disorder. Unless specified otherwise, the term may thus encompass both subjects/patients/populations which are at risk of developing a given disorder, yet which never had such disorder, and those which are at risk of developing a disorder which they already had in the past.

[0048] As used herein, the term "**effective amount**" or "**therapeutically effective amount**" is intended to mean that amount of a therapeutic component, or components that will elicit a desired biological or medical response in a cell, tissue, tumor, system, or subject, which result is generally sought by a researcher, veterinarian, doctor or other clinician or technician.

[0049] The present disclosure will be better understood, and further specific features and advantages will become apparent, from the following description of, non-limiting illustrative embodiments, made with reference to the accompanying drawings.

DETAILED DESCRIPTION OF THE INVENTION

[0050] The inventors have experimentally demonstrated that a defined polypeptides comprising a coiled-coil heterodimer sequence, in association with one or more surface markers expressed at the surface of organelles, enables the selective isolation and recovery of said organelles. Coiled-coil heterodimers suitable for protein detection and immunolabeling are described in **Doh et al**., *"VIPER is a genetically encoded peptide tag for fluorescence and electron microscopy"* (PNAS, 2018, Vol. 115, No. 51), and in **US11360100 B2**.

[0051] Synthetic biology further reports examples of coiled-coil (CC) dimer-forming modules for regulating biological processes and/or for subcellular targeting; as disclosed for example in **Plaper et al.** ("*The art of designed coiled-coils for the regulation of mammalian cells*"; Cell Chemical Biology 31, 2024).

[0052] However, to the knowledge of the inventors, such platforms have neither been proposed nor experimentally validated in the context of methods for isolating and/or purifying organelles, particularly intracellular organelles such as autophagosomes.

[0053] Unlike prior technologies, such as VIPER, which rely on exogenous delivery of a probe peptide for visualization only, the present invention makes use of a co-expression strategy to form stable intracellular capture modules. Such modules permit the isolation and recovery of intact organelles, including vesicles, while preserving the contents thereof for downstream analyses, including proteomics, lipidomics, metabolomics, and transcriptomics. The present invention thus provides a programmable, genetically encoded platform based on a heterodimerization system comprising complementary targeting modules referred herein as ORBIT (Organellar Rerouting for Biochemical Isolation via Tethering) designated ORBIT-A and ORBIT-B, enabling selective tethering, capture, and purification of organelles in intact form. The invention is particularly suited for the isolation and purification of autophagosomes, but can also be applied to other organelles, such as intracellular organelles, including vesicles and/or diverse membrane-bound compartments.

[0054] The inventors further demonstrate that such intracellular capture modules can be generated by providing a first targeting module directed to a reference organelle and a second targeting module directed to a target organelle, thereby enabling association between said reference organelle and said target organelle. For example, the reference organelle may comprise or consist of mitochondria, or a mitochondria-containing fraction, and the target organelle may comprise or consist of autophagosomes, or an autophagosome-containing fraction.

[0055] This programmable platform, referred to herein as **ORBIT**, comprises at least two separate ORBIT modules, for example **ORBIT-A** and **ORBIT-B**, which may respectively comprise the sequences of **SEQ ID NO: 1** and **SEQ ID NO: 3**, or

heterodimerizable variants or derivatives thereof, provided that said modules retain the capacity to specifically heterodimerize. In general, one module, for example the **ORBIT-B** module, such as **CoilR** or a heterodimerizable derivative thereof, is associated, directly or indirectly, with a target intracellular organelle or vesicle population, such as autophagosomes, whereas the other module, for example the **ORBIT-A** module, such as **CoilE** or a cognate heterodimerizable derivative thereof, is associated with a capture surface, advantageously a reference organelle, such as mitochondria, thereby enabling capture of said target organelle or vesicle population.

[0056] In certain embodiments, each coiled-coil consensus sequence is associated with a marker expressed at the surface of an organelle, wherein at least one of said organelles is a target organelle, for example a subcellular organelle such as an autophagosome. Selective markers for subcellular organelles, including early endosomes, lysosomes, late endosomes, recycling endosomes, mitochondria, and autophagic vesicles, are described, for example, in **WO2010045270 A2**.

[0057] In advantageous embodiments, the marker expressed at the surface of an organelle is a heterologous form of an endogenous functional protein, thereby reducing or avoiding disruption of the function of said organelle, for example the physiological function of mitochondria.Interaction (heterodimerization) of the coiled-coil consensus sequences **SEQ ID NO 1** and **SEQ ID NO 3** (i.e. between said ORBIT-A and ORBIT-B modules) results in selective tethering of the target organelles to the capture surface.

[0058] Optionally, this programmable platform can be further combined with detectable or label moities (e.g. a fluorescent small molecule, a fluorescent protein, a quantum dot, a gold nanoparticle, a sensor, a photoxidizer, biotin, streptavidin, radionuclide, spin label, lanthanide chelator, or any other appropriate label), allowing both capture and visualization simultaneously. One or more linker and cleavage sequences may be further introduced for improving the stability and/or expression of the heterologous constructs.

[0059] As a result, the target organelles are spatially concentrated, which facilitates their selective isolation and recovery in an intact and structurally preserved form.

[0060] The invention thus enables the enrichment, isolation, and purification of organelles in an intact form, while preserving the structural, membrane, and molecular

integrity thereof, as well as the composition of their molecular cargo. Efficient purification may be achieved by any suitable means, including, without limitation, magnetic separation, affinity purification, differential centrifugation, and mechanical recovery.

[0061] More particularly, the invention enables the purification of organelles formed under defined physiological or pathological conditions, including, without limitation, conditions associated with stress, infection, pharmacological treatment, or development. The purified organelles may then be subjected to molecular analysis in order to identify proteins, lipids, nucleic acids, metabolites, and/or other components associated with the target organelles.

[0062] The invention further encompasses nucleic acid constructs, expression vectors, recombinant cells or cell lines, and kits configured for the controlled expression of the ORBIT modules. The invention is broadly applicable to a variety of intracellular compartments, including, without limitation, autophagosomes, endosomes, lysosomes, mitochondria, peroxisomes, Golgi-derived vesicles, and secretory vesicles, in plant, fungal, and animal cells, and may be implemented in applications relating to research, biotechnology, agriculture, diagnostics, and medicine.

[0063] The invention has applications in a wide range of fields, including basic research, biotechnology, agriculture, and medicine. In plant cells, the invention enables the identification of stress-specific cargoes and regulators involved in pathogen defense, thereby providing means and strategies for developing crops with enhanced resilience. In certain embodiments relating to plant biotechnology, autophagy cargoes associated with fungal infection, including infection by *Magnaporthe* or *Blumeria*, may be identified in order to reveal novel resistance factors. In other embodiments, including yeast-based bioethanol production, stress-specific autophagy cargoes may be identified as markers or determinants associated with improved fermentation yields.

[0064] In human and animal systems, the invention permits the identification of disease-associated biomarkers, the development of improved methods for monitoring therapeutic responses, and the implementation of diagnostic applications.

[0065] Examples of fluorescent moieties suitable for incorporation into an ORBIT-A module and/or an ORBIT-B module include fluorescent dyes and fluorescent proteins, particularly fluorescent proteins characterized by a barrel structure that permits absorption of light and emission at a corresponding wavelength. Exemplary fluorescent proteins include, without limitation, green fluorescent protein (GFP), modified GFPs, GFP derivatives, and other fluorescent proteins such as CFP, YFP, BFP, mCherry, mEos, mEmerald, ECFP, and circularly permuted fluorescent proteins, including cpVenus, as well as combinations thereof.

[0066] Examples of labeling moieties, or **"labels,"** include any moiety and/or polypeptide sequence capable of enabling a machine, detector, sensor, device, column, or a human observer, optionally aided by microscopy or another detection technique, to distinguish a labeled composition from an unlabeled composition. Exemplary labels include, without limitation, radioactive isotopes or chelates thereof; dyes, including fluorescent and non-fluorescent dyes; sensors; stains; enzymes; non-radioactive metals, including lanthanides and gold; magnetic moieties; protein tags; antibody epitopes; and combinations thereof. A label may be attached to a biomolecule by covalent or non-covalent association, including, for example, hydrogen bonding, Van der Waals forces, or other intermolecular interactions. In some embodiments, the label is attached, whether covalently or otherwise, to the N-terminus, the C-terminus, or one or more amino acid residues of a polypeptide, for example to the N-terminus or C-terminus of an ORBIT-A or ORBIT-B module of the invention.

[0067] According to a **first main embodiment**, the invention thus relates to an *in vitro* method for isolating and/or purifying an organelle, comprising a step of:

a) providing a cell, culture medium or extract thereof, the cell, medium or extract comprising **(i)** a target organelle, **(ii)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(iii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3** ;
b) recovering from the cell, medium or extract thereof, a protein complex formed by the first and second polypeptide **SEQ ID NO 1** and **SEQ ID NO 3,** thereby recovering the target organelle.

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle, of which at least one is the target organelle.

[0068] According to particular embodiments of the *in vitro* method, the target organelle is an intracellular organelle, in particular an intracellular organelle of an eukaryotic cell.

[0069] According to particular embodiments of the *in vitro* method, the target organelle is an extracellular organelle, in particular an extracellular organelle of an eukaryotic cell, such as an extracellular vesicle and/or an exosome. When the target organelle is an extracellular organelle, it is preferably recovered from a cell culture medium.

[0070] According to particular embodiments of the *in vitro* method, the target organelle is selected from the group consisting of: vesicle, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, vacuoles, chloroplast.

[0071] According to particular embodiments of the *in vitro* method, the target organelle is a vesicle selected from the group consisting of lysosome, endosome, peroxisome, liposome, extracellular vesicle, exosome, phagosome.

[0072] According to particular embodiments, of the *in vitro* method, the target organelle is a phagosome or a vesicle derived from a phagosome, such as an autophagosome, a phagolysosome, a lysosome, or a combination thereof.

[0073] According to particular embodiments of the *in vitro* method, the target organelle is selected from the group consisting of early endosome, late endosome, recycling endosome, lysosome, and autophagosome.

[0074] According to particular embodiments of the *in vitro* method, at least one of the first and second polypeptides comprises a surface marker polypeptide sequence expressed at the surface of an endosome or an autophagosome.

[0075] According to particular embodiments of the *in vitro* method, the surface marker associated with an endosome or autophagosome is selected from the group consisting of **ATG8, LC3, GABARAP, LAMP1,** and **LAMP2**. In exemplary embodiments, the surface marker associated with the autophagosome is **ATG8** and/or **LC3**.According to

particular embodiments of the *in vitro* method, a surface marker of a mitochondria is VDAC1, TOMM70, TOMM20, TOM22, OMP25, FIS1.

[0076] According to a particular embodiment of the *in vitro* method, a surface marker of a mitochondria comprises a polypeptide sequence of a monoamine oxidase (MAO), such as a mammalian monoamine oxidase, or a fragment thereof, for example **SEQ ID NO 16.**

[0077] According to particular embodiments of the *in vitro* method, at least one of the first or second polypeptide comprises a surface marker of a mitochondria.

[0078] According to particular embodiments of the *in vitro* method, at least one of the first or second polypeptide comprises a surface marker of an organelle, in particular an intracellular organelle, distinct from a mitochondria.

[0079] According to particular embodiments of the *in vitro* method, at least one of the first or second polypeptide comprises a surface marker of an endosome or an autophagosome.

[0080] According to particular embodiments of the *in vitro* method, the first and second polypeptide comprise each a surface marker polypeptide that is different.

[0081] According to particular embodiments, the first and second polypeptide comprise each a surface marker polypeptide that is different, with at least one being a surface marker of a mitochondria.

[0082] According to particular embodiments, the first and second polypeptide comprise each a surface marker polypeptide that is different, with at least one being a surface marker of an endosome or an autophagosome.

[0083] According to particular embodiments of the *in vitro* method:

- one of the polypeptides comprises a surface marker polypeptide sequence expressed at the surface of a mitochondria; and
- one of the polypeptides comprises a surface marker polypeptide sequence expressed at the surface of an endosome or a phagosome;

said surface marker polypeptide sequences being the same or different.

[0084] According to particular embodiments of the *in vitro* method:

- one of the polypeptides comprises a surface marker polypeptide sequence expressed at the surface of a mitochondria; and
- one of the polypeptides comprises a surface marker polypeptide sequence expressed at the surface of an endosome or a phagosome;

said surface marker polypeptide sequences being different.

[0085] According to particular embodiments of the *in vitro* method for recovering an organelle from a cell, medium or extract thereof, said cell is an eukaryotic cell; in particular an eukaryotic plant cell or an animal cell, such as a human cell or a non-human cell. For example the animal cell can be an invertebrate or vertebrate cell (e.g., a mammalian cell, for example, a human cell). According to particular embodiments, the cell may have been previously obtained *ex vivo* or *in vivo*. According to particular embodiments, the cell is a gamete or a somatic cell, for example, a cancer cell, a stem cell, an immune cell, a neuronal cell, a muscle cell, or an adipocyte

[0086] According to particular embodiments, the cell is selected from a human cell, a yeast or a plant cell. According to preferred embodiments, the cell is a human cell. According to preferred embodiments, the cell is a yeast. According to preferred embodiments, the cell is a plant cell.

[0087] According to preferred embodiments, the invention relates to an *in vitro* method for isolating and/or purifying an organelle, comprising a step of:

a) providing a cell, or extract thereof, the cell or extract comprising **(i)** a target organelle, **(ii)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(iii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3** ;
b) recovering from the cell, or extract thereof, a protein complex formed by the first and second polypeptide **SEQ ID NO 1** and **SEQ ID NO 3,** thereby recovering the target organelle.

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle, of which at least one is the target organelle;

wherein the cell is an eukaryotic cell, such as an eukaryotic plant cell or an animal cell;

wherein the organelle is an intracellular organelle, such as a vesicle, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, vacuoles, chloroplast; and

when the target organelle is preferably a vesicle, such as an endosome or an autophagosome.

[0088] According to particular embodiments of the *in vitro* method, each of the first and second polypeptides comprises at least one distinct detectable label selected from the group consisting of an organic fluorophore, optionally a distinct organic fluorophore, a pH-sensitive fluorophore, an enzyme-activated fluorophore, a redox-sensitive fluorophore, a fluorogenic probe, a non-fluorescent protein, and an electron-dense particle.

[0089] According to a **second main embodiment**, the invention relates to a composition comprising **(i)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle. According to some embodiments, the composition is a pharmaceutical composition.

[0090] According to a **third main embodiment**, the invention relates to an expression vector **(i)** coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0091] According to particular embodiments, the expression vector encoding said first and second polypeptides comprises a first nucleic acid sequence encoding the first polypeptide and a second nucleic acid sequence encoding the second polypeptide, each

nucleic acid sequence being operably linked to at least one regulatory sequence, such as a promoter sequence.

[0092] According to particular embodiments, the first and second polypeptide comprise each a different surface marker polypeptide sequence expressed at the surface of an organelle.

[0093] In certain embodiments, the first and second nucleic acid sequences are operably linked to the same regulatory sequence, for example the same promoter sequence.

[0094] In certain embodiments, the first and second nucleic acid sequences are operably linked to a promoter sequence, of which at least one is an inducible promoter sequence. In certain embodiments, the first and second nucleic acid sequences are operably linked to a promoter sequence, of which at least one is a constitutive promoter sequence.

[0095] In certain embodiments, the first and second nucleic acid sequences are operably linked to a promoter sequence, of which one is an inducible promoter sequence and one is a constitutive promoter sequence.

[0096] In certain embodiments, the first and second nucleic acid sequences are operably linked to an inducible promoter sequence. In certain embodiments, the first and second nucleic acid sequences are operably linked to a constitutive promoter sequence.

[0097] Examples of inducible and constitutive promoter sequences are described in the experimental section.

[0098] According to a **fourth main embodiment**, the invention relates to a cell comprising:

- **(i)** an heterologous nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and
- **(ii)** an heterologous nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3;**

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0099] The cell may be an isolated cell and/or part of a composition or a pharmaceutical composition.

[0100] According to a **fifth main embodiment**, the invention relates to a kit comprising **(i)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0101] According to a preferred embodiment, the invention relates to a kit comprising **(i)** an expression vector coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** an expression vector coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle

ORBIT modules and ORBIT consensus regions for heterodimerization.

[0102] The invention relies on:

- **(i)** an heterologous nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and
- **(ii)** an heterologous nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3.**

[0103] The heterologous nucleic acid coding for said first polypeptide comprising a polypeptide consensus sequence **SEQ ID NO 1** may be referred herein as the **ORBIT-A** module. The heterologous nucleic acid coding for said second polypeptide comprising a polypeptide consensus sequence **SEQ ID NO 3** may be referred herein as the **ORBIT-B** module.

[0104] Each ORBIT module may, in particular, be characterized it that said first and second polypeptide comprises:

- at least one ORBIT-A or ORBIT-B consensus polypeptide sequence **SEQ ID NO 1** or **SEQ ID NO 3**, for example of **SEQ ID NO 2** or **SEQ ID NO 4;**

- at least one polypeptide sequence of a surface marker expressed at the surface of an organelle;

- optionally one or more linker polypeptide sequence(s) between said consensus polypeptide sequence and said polypeptide sequence of a surface marker;

- optionally one or more detectable polypeptide sequence(s);

- optionally on or more cleavable polypeptide sequence(s), such as a TEV polypeptide sequence.

[0105] According to some embodiments, the at least one ORBIT-A or ORBIT-B consensus polypeptide sequence is linked, via its N-terminal end, to at least one surface marker polypeptide sequence expressed at the surface of an organelle. According to other embodiments, the at least one ORBIT-A or ORBIT-B consensus polypeptide sequence is linked, via its C-terminal end, to at least one surface marker polypeptide sequence expressed at the surface of an organelle.

[0106] In some embodiments, the linker polypeptide sequence comprises or consists of 1 to 20 amino acid residues, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues, wherein said amino acid residues are selected from glycine and serine. In particular embodiments, the linker polypeptide sequence comprises or consists of a glycine-rich sequence, a serine-rich sequence, or a glycine/serine-rich sequence.

[0107] For example, a linker polypeptide sequence may comprised between at least one ORBIT-A or ORBIT-B consensus polypeptide sequence, and at least one polypeptide sequence of a surface marker expressed at the surface of an organelle.

[0108] The ORBIT modules, including ORBIT-A and ORBIT-B, are designed as complementary pairs respectively comprising the ORBIT-A and ORBIT-B consensus regions set forth in **SEQ ID NO: 1** and **SEQ ID NO: 3**. These consensus regions correspond to coiled-coil polypeptide sequences that, when combined as a pair, retain the capacity to specifically heterodimerize. Each consensus region is defined in further detail herebelow.

[0109] The ORBIT-A consensus sequence corresponds to **SEQ ID NO 1**; wherein

$X_1X_2X_3\mathbf{R}X_5X_6X_7X_8\mathbf{R}X_{10}\mathbf{R}X_{12}X_{13}X_{14}X_{15}\mathbf{R}X_{17}\mathbf{E}X_{19}X_{20}\mathbf{E}X_{22}\mathbf{E}X_{24}\mathbf{E}X_{26}X_{27}\mathbf{R}X_{29}\mathbf{E}X_{31}\mathbf{E}X_{33}X_{34}X_{35}X_{36}\mathbf{E}X_{38}\mathbf{R}X_{40}X_{41}X_{42}X_{43}$

wherein X_1 to X_{43} are independently selected from any amino acid.

[0110] According to a particular embodiment of the ORBIT-A module, the ORBIT-A consensus sequence corresponding to **SEQ ID NO 1** is characterized in that one more of the X_1 to X_{43} is selected in that:

X_1 is L;
X_2 is E;
X_3 is I;
X_5 is A;
X_6 is A;
X_7 is F;
X_8 is L;
X_{10} is Q;
X_{12} is N;
X_{13} is T;
X_{14} is A;
X_{15} is L;
X_{17} is T;
X_{19} is V;
X_{20} is A;
X_{22} is L;
X_{24} is Q;
X_{26} is V;
X_{27} is Q;
X_{29} is L;
X_{31} is N;
X_{33} is V;
X_{34} is S;
X_{35} is Q;
X_{36} is Y;
X_{38} is T;
X_{40} is Y;
X_{41} is G;
X_{42} is P;
X_{43}. is L.

[0111] According to particular embodiments, the ORBIT-A consensus sequence corresponding to **SEQ ID NO 1** is characterized in that 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the X_1 to X_{43} is selected as defined above.

[0112] According to a preferred embodiment of the ORBIT-A module, the ORBIT-A consensus sequence comprises **SEQ ID NO 2**.

[0113] The ORBIT-B consensus sequence corresponds to **SEQ ID NO 3**; wherein

$X_1X_2X_3\mathbf{E}X_5X_6X_7X_8\mathbf{ERE}X_{12}X_{13}X_{14}X_{15}\mathbf{E}X_{17}X_{18}X_{19}X_{20}\mathbf{E}X_{22}\mathbf{R}X_{24}\mathbf{R}X_{26}X_{27}\mathbf{R}X_{29}\mathbf{R}X_{31}\mathbf{R}X_{33}X_{34}X_{35}X_{36}\mathbf{R}X_{38}\mathbf{R}X_{40}X_{41}X_{42}X_{43}$

wherein X_1 to X_{43} are independently selected from any amino acid.

[0114] According to a particular embodiment of the ORBIT-B module, the ORBIT-B consensus sequence corresponding to **SEQ ID NO 3** is characterized in that one more of the X_1 to X_{43} is selected in that:

X_1 is L;
X_2 is E;
X_3 is I;
X_5 is A;
X_6 is A;
X_7 is F;
X_8 is L;
X_{12} is N;
X_{13} is T;
X_{14} is A;
X_{15} is L;
X_{17} is T;
X_{18} is R;
X_{19} is V;
X_{20} is A;
X_{22} is L;
X_{24} is Q;
X_{26} is V;
X_{27} is Q;
X_{29} is L;
X_{31} is N;
X_{33} is V;
X_{34} is S;
X_{35} is Q;
X_{36} is Y;
X_{38} is T;
X_{40} is Y;
X_{41} is G;
X_{42} is P;
X_{43} is L.

[0115] According to particular embodiments, the ORBIT-A consensus sequence corresponding to **SEQ ID NO 3** is characterized in that 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the X_1 to X_{43} is selected as defined above.

[0116] According to a preferred embodiment of the ORBIT-B module, the ORBIT-B consensus sequence comprises **SEQ ID NO 4**.

[0117] In certain embodiments, at least one, at least two, at least three, at least four, at least five, or more than five of the positions **X_1 to X_{43}** are defined as set forth in **SEQ ID NO: 2** or **SEQ ID NO: 4**.

[0118] According to a preferred embodiment, the ORBIT-A consensus sequence corresponds to **SEQ ID NO: 2**, and the ORBIT-B consensus sequence corresponds to **SEQ ID NO: 4**.

[0119] According to particular embodiments the ORBIT-A module, comprising the ORBIT-A consensus region, comprises at least 45 amino acids in length; for example at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 amino acids in length. According to particular embodiments the ORBIT-B module, comprising the ORBIT-B consensus region, comprises at least 45 amino acids in length; for example at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 amino acids in length. According to particular embodiments both the ORBIT-A module and ORBIT-B module comprises at least 45 amino acids in length; for example at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 amino acids in length.

[0120] According to a preferred embodiment, the invention relates to an *in vitro* method for isolating and/or purifying an organelle, comprising a step of:

a) providing a cell, culture medium or extract thereof, the cell or extract comprising **(i)** a target organelle, **(ii)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 2,** and **(iii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 4** ;

b) recovering from the cell, medium or extract thereof, a protein complex formed by the first and second polypeptide **SEQ ID NO 2** and **SEQ ID NO 4,** thereby recovering said target organelle.

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle, of which at least one is the target organelle.

[0121] According to a preferred embodiment, the invention relates to a composition comprising **(i)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 2,** and **(ii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 4:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0122] According to said most preferred embodiment, the invention relates to an expression vector **(i)** coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 2,** and **(ii)** coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 4:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle

[0123] According to said most preferred embodiment, the invention relates to a cell comprising:

(i) an heterologous nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 2,** and

(ii) an heterologous nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 4:**

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0124] According to said most preferred embodiment, the invention relates to a kit comprising **(i)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** a nucleic acid coding for a second polypeptide

comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

[0125] According to said most preferred embodiments, the invention relates to a kit comprising **(i)** an expression vector coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** an expression vector coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

DESCRIPTION OF THE FIGURES

[0126] **Figure 1. Purification of stimulus induced autophagosomes after in vivo delocalization. 1A.** Represents an autophagosome delocalization system including an heterologous ORBITA/CoilR construct and an heterologous ORBIT B/CoilE construct. **1B.** Represents one example of an autophagosome delocalization system including an heterologous ORBITA/CoilR construct and an heterologous ORBIT-B free construct, as a negative control.

[0127] **Figure 2. Expression of both ORBIT constructs after plant transformation and induction of Autophagy leads to effective organelle delocalization.**

EXAMPLES

Example 1. Purification of Autophagosomes from *Nicotiana benthamiana* leaves using affinity capture and 3C protease elution.

[0128] Material & Methods

[0129] Approximately **3.5 g** of fresh *N. benthamiana* leaves, from which the midrib has been removed, are homogenized in a pre-chilled mortar in the presence of **15 mL** of lysis buffer (**50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.3 M sorbitol, 1 mM PMSF, 10 mM DTT, protease inhibitor, and 0.6% Polyclar; autoclaved and**

sterile-filtered), using gentle pressure. The resulting lysate is filtered through a funnel fitted with a single layer of pre-wetted Miracloth into a **50 mL** tube. Residual extract is recovered by rinsing the mortar with **5 mL** of lysis buffer, applying the rinse to the Miracloth, and gently pressing the Miracloth. The filtrate is then passed sequentially through a **70 µm** sterile nylon mesh and distributed into four **5 mL** microcentrifuge tubes. The preparation is subjected to a first centrifugation at **1,500 × g** for **5 minutes**, after which the supernatants are collected and transferred to fresh tubes. The collected supernatants are then subjected to a second centrifugation at **2,500 × g** for **5 minutes**, and the resulting supernatants are again transferred to fresh tubes. Said supernatants are subsequently centrifuged at **17,000 × g** for **15 minutes** to obtain organelle-enriched pellets.**[0106]** The supernatants are discarded, and the pellets are gently resuspended, using a sterile brush, in a total volume of **1 mL** of washing buffer (**0.3 M sorbitol, 1.5 mM $MgCl_2$, 0.2 mM EDTA at pH 8.0, 150 mM NaCl, and 50 mM HEPES; autoclaved and sterile-filtered**) supplemented with **PMSF** (phenylmethylsulfonyl fluoride) and **PiC**. In some embodiments, **DTT is excluded** from the washing buffer in order to avoid leaching of the HA antibody from the beads. The suspension is then filtered through a **40 µm** nylon mesh, and the resulting filtrate constitutes the **INPUT fraction**.

[0130] The **50 µL** of equilibrated magnetic beads are added to **1 mL** of the INPUT fraction in a **2 mL** tube. The resulting mixture is incubated for **1 hour** on a rotating wheel at **15 rpm**. Thereafter, the tube is placed on a magnetic stand for **2 minutes**, and the flow-through is removed. The beads are then washed with **1 mL** of washing buffer and rotated for **2 minutes**. Said washing step is repeated **six times**. The beads are subsequently placed on a magnetic stand, the supernatant is removed, and the beads are resuspended in **200 µL** of washing buffer containing **2 mM DTT** and lacking **PiC** and **PMSF**, in order to avoid interference with protease activity. A cleavage protease, for example **3C protease** and/or **TEV protease**, is then added, and the resulting mixture is incubated overnight under slow rotation, for example at **5–10 rpm**. The protease is selected according to the cleavage site incorporated into the construct, for example the cleavage site present in the ORBIT-A construct corresponding to **SEQ ID NO: 5** and the ORBIT-B construct corresponding to **SEQ ID NO: 12**.

[0131] The tube containing the cleaved preparation is subsequently placed on a magnetic stand for **2 minutes**, and the eluate is recovered from the supernatant. The eluate is then subjected to centrifugation at **100,000 × g** for **30 to 60 minutes**. After centrifugation, the supernatant is discarded, and the resulting pellet corresponds to purified autophagosomes.

Example 2. ORBIT-A/B cloning and implementation in plant and mammalian cells.

ORBIT-A/B cloning and implementation in plant and mammalian cells

A. Plant cell implementation

[0132] Material & Methods

[0133] The plasmids used for cloning are derived from a Golden Gate modular cloning toolkit suitable for plant systems.

Plasmid construction by Golden Gate assembly

Amplification of DNA fragments

[0134] DNA fragments encoding **ORBIT-A-3C** (obtained by gene synthesis from GenScript, USA)**, mVenus,** and **ATG8** (Arabidopsis ATG8e isoform) were amplified by PCR using primers configured to introduce appropriate overhangs for Golden Gate assembly into the level 0 backbone **pICH41308**. Similarly, DNA fragments encoding **ORBIT-B, 3HA, mCherry,** and **TMmito** (transmembrane domain of mouse mitochondrial Monoamine oxidase A) were amplified by PCR using primers introducing compatible overhangs for cloning into **pICH41308**.

Assembly of level 1 constructs

(i) ORBIT-A-3C-Venus-ATG8 expression cassette

[0135] The **ORBIT-A-3C-Venus-ATG8** expression cassette was assembled by Golden Gate cloning using:

- the **ubiquitin promoter** from *Arabidopsis thaliana* contained in **pJOG684**;
- the **ACT2 terminator** from *A. thaliana*, comprising the 3′ untranslated region and polyadenylation signal, contained in **pICH44300**; and
- the level 1 acceptor vector **pICH47751** (position 3).

(ii) ORBIT-B-3HA-mCherry-TMmito expression cassette

[0136] The **CoilE-3HA-mCherry-TMmito** expression cassette was assembled by Golden Gate cloning using:

- a **minimal promoter** comprising a **LexA operator fused to a minimal 35S promoter**, contained in **pTEI036**;
- the **ACT2 terminator** from *A. thaliana*, comprising the 3′ untranslated region and polyadenylation signal, contained in **pICH44300**; and
- the level 1 acceptor vector **pICH47742**.

(iii) Inducible XVE cassette

[0137] The inducible expression cassette **pG10-90::XVE** was assembled by Golden Gate cloning using:

- the **XVE coding sequence** contained in **pTEI039**;
- the **G10-90 promoter** contained in **pTEI038**;
- the **RbcS3C terminator** from *Solanum lycopersicum* contained in **pICH71411**; and
- the level 1 acceptor vector **pICH47761** (position 4).

(iv) Hygromycin resistance cassette

[0138] A **hygromycin resistance cassette**, comprising the hygromycin resistance gene under the control of the **NOS promoter** and **NOS terminator**, was cloned into the level 1 acceptor vector **pICH47732** (position 1).

Assembly of level 2 multigene construct

[0139] The four level 1 expression cassettes were assembled into the destination vector **pAGM8031** by Golden Gate cloning, using **pICH50900** as an end-linker (position 4).

Golden Gate reaction conditions

[0140] Each Golden Gate assembly reaction was carried out in a total volume of **25 µL** comprising:

- **100 ng** of each plasmid DNA;
- **2.5 µL** of **10× T4 DNA ligase buffer**;
- **1.5 µL** of **T4 DNA ligase**;
- **1 µL** of restriction enzyme, namely **BsaI-HF** or **BbsI-HF**, as appropriate; and
- nuclease-free water to a final volume of **25 µL**.

[0141] The reactions were incubated in a thermocycler according to the following program:

- **37°C for 5 minutes**;
- **16°C for 5 minutes**;
  with the two-step cycle repeated **60 times**, followed by
- **80°C for 15 minutes** to inactivate the enzymes.

B. Mammalian cell implementation

[0142] In certain embodiments, the plasmids used for cloning are derived from a Golden Gate modular cloning toolkit suitable for mammalian systems.

Plasmid construction by Golden Gate assembly

Amplification of DNA fragments

[0143] DNA fragments encoding **ORBIT-A-3C, Venus,** and **LC3B** were amplified by PCR using primers configured to introduce appropriate overhangs for Golden Gate assembly into a level 0 mammalian entry vector compatible with the selected Golden Gate cloning framework. Similarly, DNA fragments encoding **ORBIT-B, 3HA, mCherry,** and **TMmito** were amplified by PCR using primers introducing compatible overhangs for cloning into a corresponding level 0 mammalian entry vector.

Assembly of level 1 constructs

(i) ORBIT-A-3C-Venus-LC3B expression cassette

[0144] The **ORBIT-B-3C-Venus-LC3B** expression cassette was assembled by Golden Gate cloning using:

- a **mammalian constitutive promoter**, for example a **CMV**, **EF1α**, or **CAG** promoter;
- a **mammalian transcription termination/polyadenylation sequence**, for example a **bGH polyadenylation signal** or an **SV40 polyadenylation signal**; and
- a mammalian level 1 acceptor vector compatible with the selected Golden Gate cloning system.

(ii) ORBIT-B-3HA-mCherry-TMmito expression cassette

[0145] The **ORBIT-B-3HA-mCherry-TMmito** expression cassette was assembled by Golden Gate cloning using:

- an **inducible mammalian promoter**, for example a **TRE** or **TRE3G promoter** operably linked to a **minimal CMV promoter**;
- a **mammalian transcription termination/polyadenylation sequence**, for example a **bGH** or **SV40** polyadenylation signal; and
- a mammalian level 1 acceptor vector compatible with the selected Golden Gate cloning system.

(iii) Inducible transactivator cassette

[0146] An inducible expression cassette was assembled by Golden Gate cloning using:

- a coding sequence encoding a **reverse tetracycline-controlled transactivator (rtTA)** or **Tet3G transactivator**;
- a **constitutive mammalian promoter**, for example an **EF1α**, **PGK**, or **CAG** promoter;
- a **mammalian transcription termination/polyadenylation sequence**, for example a **bGH** or **SV40** polyadenylation signal; and
- a mammalian level 1 acceptor vector compatible with the selected Golden Gate cloning system.

(iv) Hygromycin resistance cassette

[0147] A **hygromycin resistance cassette**, comprising a hygromycin resistance gene under the control of a **mammalian promoter**, for example a **PGK**, **SV40**, or **EF1α** promoter, and further comprising a mammalian transcription termination/polyadenylation sequence, was assembled into a mammalian level 1 acceptor vector compatible with the selected Golden Gate cloning system.

Assembly of level 2 multigene construct

[0148] The four level 1 expression cassettes were assembled into a mammalian level 2 destination vector by Golden Gate cloning. In certain embodiments, said destination

vector is configured for **transient transfection**, **stable genomic integration**, **lentiviral delivery**, or **transposon-based delivery**.

Golden Gate reaction conditions

[0149] Each Golden Gate assembly reaction was carried out in a total volume of **25 µL** comprising:

- **100 ng** of each plasmid DNA;
- **2.5 µL** of **10× T4 DNA ligase buffer**;
- **1.5 µL** of **T4 DNA ligase**;
- **1 µL** of restriction enzyme, namely **BsaI-HF**, **BbsI-HF**, or an equivalent type IIS restriction enzyme appropriate for the selected cloning system; and
- nuclease-free water to a final volume of **25 µL**.

[0150] The reactions were incubated in a thermocycler according to the following program:

- **37°C for 5 minutes**;
- **16°C for 5 minutes**; with the two-step cycle repeated **60 times**, followed by
- **80°C for 15 minutes** to inactivate the enzymes.

SEQUENCE LISTING

SEQ ID	Name	Sequence
1	ORBIT-A consensus	$X_1X_2X_3$**R**$X_5X_6X_7X_8$**R**X_{10}**R**$X_{12}X_{13}X_{14}X_{15}$**R**X_{17}**E**$X_{19}X_{20}$**E**X_{22}**E**X_{24}**E**$X_{26}X_{27}$ **R**X_{29}**E**X_{31}**E**$X_{33}X_{34}X_{35}X_{36}$**E**X_{38}**R**$X_{40}X_{41}X_{42}X_{43}$ wherein X_1 to X_{43} are independently selected from any amino acid; with X_1 being preferably L; with X_2 being preferably E; with X_3 being preferably I; with X_5 being preferably A; with X_6 being preferably A; with X_7 being preferably F; with X_8 being preferably L; with X_{10} being preferably Q; with X_{12} being preferably N; with X_{13} being preferably T; with X_{14} being preferably A; with X_{15} being preferably L; with X_{17} being preferably T; with X_{19} being preferably V; with X_{20} being preferably A; with X_{22} being preferably L; with X_{24} being preferably Q; with X_{26} being preferably V; with X_{27} being preferably Q; with X_{29} being preferably L; with X_{31} being preferably N; with X_{33} being preferably V; with X_{34} being preferably S; with X_{35} being preferably Q; with X_{36} being preferably Y; with X_{38} being preferably T; with X_{40} being preferably Y; with X_{41} being preferably G; with X_{42} being preferably P; with X_{43} being preferably L.
2	ORBIT-A	LEIRAAFLRQRNTALRTEVAELEQEVQRLENEVSQYETRYGPL
3	ORBIT-B consensus	$X_1X_2X_3$**E**$X_5X_6X_7X_8$**ERE**$X_{12}X_{13}X_{14}X_{15}$**E**$X_{17}X_{18}X_{19}X_{20}$**E**X_{22}**R**X_{24}**R**$X_{26}X_{27}$ **R**X_{29}**R**X_{31}**R**$X_{33}X_{34}X_{35}X_{36}$**R**X_{38}**R**$X_{40}X_{41}X_{42}X_{43}$ wherein X_1 to X_{43} are independently selected from any amino acid; with X_1 being preferably L; with X_2 being preferably E; with X_3 being preferably I; with X_5 being preferably A; with X_6 being preferably A; with X_7 being preferably F; with X_8 being preferably L;

		with X_{12} being preferably N; with X_{13} being preferably T; with X_{14} being preferably A; with X_{15} being preferably L; with X_{17} being preferably T; with X_{18} being preferably R; with X_{19} being preferably V; with X_{20} being preferably A; with X_{22} being preferably L; with X_{24} being preferably Q; with X_{26} being preferably V; with X_{27} being preferably Q; with X_{29} being preferably L; with X_{31} being preferably N; with X_{33} being preferably V; with X_{34} being preferably S; with X_{35} being preferably Q; with X_{36} being preferably Y; with X_{38} being preferably T; with X_{40} being preferably Y; with X_{41} being preferably G; with X_{42} being preferably P; with X_{43} being preferably L.
4	ORBIT-B	LEIEAAFLERENTALETRVAELRQRVQRLRNRVSQYRTRYGPL
5	ORBIT-A-3C-Venus-TEV-ATG8 construct (ORBIT-A)	MLEIRAAFLRQRNTALRTEVAELEQEVQRLENEVSQYETRYGPL LEVLFQGPESMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGE GDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCFARYPDHM KQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNR IELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKI RHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSALSKDP NEKRDHMVLLEFVTAAGITLGMDELKEAENLYFQGMNKGSIFK MDNDFEKRKAEAGRIREKYPDRIPVIVEKAEKSEVPNIDKKKYLV PSDLTVGQFVYVIRKRIKLSAEKAIFIFVDNVLPPTGELMSSVYED KKDEDGFLYITYSGENTFGASSI
6	3C	LEVLFQGPES
7	Venus	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT LKLICTTGKLPVPWPTLVTTLGYGLQCFARYPDHMKQHDFFKSA MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKE DGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGGV QLADHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMV LLEFVTAAGITLGMDEL
8	TEV	KEAENLYFQG
9	ATG8	MNKGSIFKMDNDFEKRKAEAGRIREKYPDRIPVIVEKAEKSEVPN IDKKKYLVPSDLTVGQFVYVIRKRIKLSAEKAIFIFVDNVLPPTGE LMSSVYEDKKDEDGFLYITYSGENTFGASSI
10	DNA sequence coding for ORBIT-A-3C-	ATGCTTGAGATTAGAGCTGCTTTCTTGAGACAAAGAAACACTG CTCTTAGAACAGAAGTTGCTGAATTGGAGCAAGAAGTTCAGA GACTTGAGAATGAGGTTTCTCAGTACGAGACTAGATATGGTCC TTTGCTTGAAGTTCTTTTTCAAGGACCTGAGTCTATGGTGAGC

	Venus-TEV-ATG8 construct (ORBIT-A)	AAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTC GAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCC GGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG AAGCTGATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCC ACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCC GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGC CATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAG GACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAG GGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGAC TTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTAC AACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAG AAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATC GAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAAC ACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACT ACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGA AGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCG GGATCACTCTCGGCATGGACGAGCTGTACAAGGAGGCTGAAA ATCTTTATTTTCAAGGAATGAATAAAGGAAGCATCTTTAAGAT GGACAACGATTTCGAAAAGAGAAAAGCTGAAGCTGGAAGGAT CAGGGAGAAATACCCTGATCGAATTCCTGTGATTGTGGAAAA GGCTGAGAAAAGTGAAGTCCCAAATATAGACAAGAAGAAGTA CCTTGTGCCATCAGACCTAACAGTTGGTCAATTTGTGTATGTG ATTCGGAAGAGAATCAAACTAAGTGCAGAGAAAGCTATCTTC ATTTTCGTAGACAATGTTCTTCCTCCAACAGGAGAGCTAATGT CAAGCGTTTACGAGGATAAGAAAGACGAAGATGGCTTCCTTT ACATCACTTACAGTGGCGAGAACACATTCGGTGCTTCTTCAAT CTAA
11	ORBIT-A-3C-Venus-TEV	MLEIRAAFLRQRNTALRTEVAELEQEVQRLENEVSQYETRYGPL LEVLFQGPESMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGE GDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCFARYPDHM KQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNR IELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKI RHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSALSKDP NEKRDHMVLLEFVTAAGITLGMDELKEAENLYFQG
12	DNA sequence coding for ORBIT-A-3C-Venus-TEV	ATGCTTGAGATTAGAGCTGCTTTCTTGAGACAAAGAAACACTG CTCTTAGAACAGAAGTTGCTGAATTGGAGCAAGAAGTTCAGA GACTTGAGAATGAGGTTTCTCAGTACGAGACTAGATATGGTCC TTTGCTTGAAGTTCTTTTTCAAGGACCTGAGTCTATGGTGAGC AAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTC GAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCC GGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG AAGCTGATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCC ACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCC GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGC CATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAG GACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAG GGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGAC TTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTAC AACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAG AAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATC GAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAAC ACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACT ACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGA AGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCG

		GGATCACTCTCGGCATGGACGAGCTGTACAAGGAGGCTGAAA ATCTTTATTTTCAAGGATAA
13	ORBIT-B-3HA-mCherry-TM construct (ORBIT-B)	MLEIEAAFLERENTALETRVAELRQRVQRLRNRVSQYRTRYGPL GYPYDVPDYAYPYDVPDYAYPYDVPDYAAGAGAGAGGMVSKG EEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTA KLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPE GFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSD GPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYD AEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAE GRHSTGGMDELYKGAGGSAVPAVEITHTFWERNLPSVSGLLKIIG FSTSVTALGFVLYKYKLLPRS
14	3HA	YPYDVPDYAYPYDVPDYAYPYDVPDYA
15	mCherry	MVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYE GTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYL KLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGT NFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDG GHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQY ERAEGRHSTGGMDELYK
16	TM	GSAVPAVEITHTFWERNLPSVSGLLKIIGFSTSVTALGFVLYKYKL LPRS
17	DNA coding for ORBIT-B-3HA-mCherry-TM construct (ORBIT-B)	ATGCTTGAAATTGAGGCTGCTTTTCTTGAAAGAGAGAATACTG CTCTTGAAACAAGAGTTGCTGAGTTGAGACAAAGAGTTCAGA GACTTAGGAACAGAGTTTCTCAATACAGAACAAGATACGGTC CTTTGGGATACCCATACGACGTCCCagactacgcctacccatatgatgtcccgg attacgcttacccataCGATGTTCCAGATTACGCTGCTGGAGCTGGTGCT GGAGCTGGAGGAATGGTGAGCAAGGGCGAGGAGGATAACAT GGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGA GGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGG CGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAA GGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTG TCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACC CCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGG CTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGT GGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTT CATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGAC GGCCCCGTAATGCAGAAGAAAACCATGGGCTGGGAGGCCTCC TCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAG ATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGAC GCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAG CTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCT CCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCG CCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACA AGGGAGCTGGAGGTTCTGcTGTTCCAGCTGTTGAAATCACTCA TACATTCTGGGAGAGAAATCTTCCTTCTGTTTCTGGTCTTTTGA AGATTATCGGATTTTCTACTTCTGTTACAGCTCTTGGATTCGTT TTGTATAAGTACAAACTTTTGCCAAGATCTTGA
18	3HA-mCherry-TM construct	MYPYDVPDYAYPYDVPDYAYPYDVPDYAAGAGAGAGGMVSK GEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQT AKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSF PEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPS

	(negative control)	DGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHY DAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERA EGRHSTGGMDELYKGAGGSAVPAVEITHTFWERNLPSVSGLLKII GFSTSVTALGFVLYKYKLLPRS
19	DNA sequence coding for 3HA-mCherry-TM construct	ATGTACCCATACGACGTCCCAGACTACGCCTACCCATATGATG TCCCGGATTACGCTTACCCATACGATGTTCCAGATTACGCTGC TGGAGCTGGTGCTGGAGCTGGAGGAATGGTGAGCAAGGGCGA GGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAA GGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGAT CGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGA CCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCG CCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGC CTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTG TCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCG AGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGC AGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCA ACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAAACCATGG GCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCG CCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACG GCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCA AGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCA AGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGG AACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCA TGGACGAGCTGTACAAGGGAGCTGGAGGTTCTGCTGTTCCAG CTGTTGAAATCACTCATACATTCTGGGAGAGAAATCTTCCTTC TGTTTCTGGTCTTTTGAAGATTATCGGATTTTCTACTTCTGTTA CAGCTCTTGGATTCGTTTTGTATAAGTACAAACTTTTGCCAAG ATCTTGA
20	mTagBFP2-ATG8	MVSKGEELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQ TMRIKVVEGGPLPFAFDILATSFLYGSKTFINHTQGIPDFFKQSFPE GFTWERVTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFTSNG PVMQKKTLGWEAFTETLYPADGGLEGRNDMALKLVGGSHLIAN AKTTYRSKKPAKNLKMPGVYYVDYRLERIKEANNETYVEQHEV AVARYCDLPSKLGHKLNGAGMNKGSIFKMDNDFEKRKAEAGRI REKYPDRIPVIVEKAEKSEVPNIDKKKYLVPSDLTVGQFVYVIRK RIKLSAEKAIFIFVDNVLPPTGELMSSVYEDKKDEDGFLYITYSGE NTFGASSI
21	mTagBFP2	MVSKGEELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQ TMRIKVVEGGPLPFAFDILATSFLYGSKTFINHTQGIPDFFKQSFPE GFTWERVTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFTSNG PVMQKKTLGWEAFTETLYPADGGLEGRNDMALKLVGGSHLIAN AKTTYRSKKPAKNLKMPGVYYVDYRLERIKEANNETYVEQHEV AVARYCDLPSKLGHKLN
22	DNA sequence coding for mTagBFP2-ATG8	ATGGTGTCGAAAGGAGAGGAATTAATCAAGGAAAATATGCAT ATGAAACTCTACATGGAAGGCACTGTTGACAATCACCATTTCA AATGCACTTCTGAGGGTGAAGGAAAACCATATGAAGGGACAC AAACAATGAGAATCAAAGTTGTTGAAGGTGGTCCGCTTCCATT TGCATTTGATATTTTGGCGACTTCATTTCTATATGGTTCTAAGA CATTCATAAATCATACTCAAGGAATTCCTGATTTTTTCAAGCA AAGTTTTCCAGAAGGATTTACTTGGGAGAGGGTGACGACATA TGAGGACGGCGGGGTACTGACAGCTACTCAGGACACATCACT ACAAGATGGTTGTCTCATTTACAATGTAAAGATTCGGGGTGTA

		AACTTCACCAGCAATGGACCAGTCATGCAGAAGAAAACCCTG GGATGGGAAGCTTTTACAGAAACTTTGTATCCTGCTGATGGGG GATTAGAGGGAAGAAATGATATGGCTTTGAAGTTAGTTGGTG GATCTCATTTGATTGCCAATGCAAAAACAACTTACAGATCCAA GAAGCCTGCCAAAAACTTGAAAATGCCTGGTGTTTATTATGTC GATTATAGGCTTGAGCGTATAAAGGAGGCAAACAATGAAACC TATGTGGAGCAGCATGAAGTTGCTGTGGCACGATACTGTGATC TTCCCAGTAAACTTGGCCACAAGCTTAACGGAGCTGGTATGAA TAAAGGAAGCATCTTTAAGATGGACAACGATTTCGAAAAGAG AAAAGCTGAAGCTGGAAGGATCAGGGAGAAATACCCTGATCG AATTCCTGTGATTGTGGAAAAGGCTGAGAAAAGTGAAGTCCC AAATATAGACAAGAAGAAGTACCTTGTGCCATCAGACCTAAC AGTTGGTCAATTTGTGTATGTGATTCGGAAGAGAATCAAACTA AGTGCAGAGAAAGCTATCTTCATTTTCGTAGACAATGTTCTTC CTCCAACAGGAGAGCTAATGTCAAGCGTTTACGAGGATAAGA AAGACGAAGATGGCTTCCTTTACATCACTTACAGTGGCGAGA ACACATTCGGTGCTTCTTCAATCTAA
23	CFP-ATG8	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT LKFICTTGKLPVPWPTLVTTLTWGVQCFSRYPDHMKQHDFFKSA MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKE DGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGSVQ LADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLL EFVTAAGITLGMDELYKGNKGSIFKMDNDFEKRKAEAGRIREKY PDRIPVIVEKAEKSEVPNIDKKKYLVPSDLTVGQFVYVIRKRIKLS AEKAIFIFVDNVLPPTGELMSSVYEDKKDEDGFLYITYSGENTFG ASSI
24	CFP	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT LKFICTTGKLPVPWPTLVTTLTWGVQCFSRYPDHMKQHDFFKSA MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKE DGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGSVQ LADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLL EFVTAAGITLGMDELYK
25	DNA sequence coding for CFP-ATG8	ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCC ATCCTGGTCGAGCTGGACGGCGACGTGAACGGCCACAAGTTC AGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG CTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGC CCTGGCCCACCCTCGTGACCACCCTGACCTGGGGCGTGCAGTG CTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTC AAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATC TTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTG AAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAG GGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAG CTGGAGTACAACTACATCAGCCACAACGTCTATATCACCGCCG ACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGATCCGCC ACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACC AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCG ACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACC CCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGA CCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGgg tAATAAAGGAAGCATCTTTAAGATGGACAACGATTTCGAAAAG AGAAAAGCTGAAGCTGGAAGGATCAGGGAGAAATACCCTGAT CGAATTCCTGTGATTGTGGAAAAGGCTGAGAAAAGTGAAGTC CCAAATATAGACAAGAAGAAGTACCTTGTGCCATCAGACCTA

		ACAGTTGGTCAATTTGTGTATGTGATTCGGAAGAGAATCAAAC TAAGTGCAGAGAAAGCTATCTTCATTTTCGTAGACAATGTTCT TCCTCCAACAGGAGAGCTAATGTCAAGCGTTTACGAGGATAA GAAAGACGAAGATGGCTTCCTTTACATCACTTACAGTGGCGA GAACACATTCGGTGCTTCTTCAATCTAA

CLAIMS

1. An *in vitro* method for isolating and/or purifying an organelle, comprising a step of:

a) providing a cell, culture medium or extract thereof, the cell, medium, or extract comprising **(i)** a target organelle, **(ii)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(iii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3** ;

b) recovering from the cell, medium or extract thereof, a protein complex formed by the first and second polypeptide **SEQ ID NO 1** and **SEQ ID NO 3,** thereby recovering said target organelle.

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle, of which at least one is the target organelle.

2. The *in vitro* method for recovering an organelle, according to claim **1**, wherein the target organelle is selected from the group consisting of: vesicle, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, vacuoles, chloroplast.

3. The *in vitro* method for recovering an organelle, according to claim **1** or **2**, wherein the target organelle is a vesicle selected from the group consisting of: lysosome, endosome, peroxisome, liposome, extracellular vesicle, exosome, phagosome.

4. The *in vitro* method for recovering an organelle, according to any of the preceding claims, wherein at least one of the first or second polypeptide comprises a surface marker polypeptide sequence expressed at the surface of an endosome or an autophagosome.

5. The *in vitro* method for recovering an organelle, according to claim **4**, wherein said surface marker of an endosome or a autophagosome is selected from the group consisting of: ATG8, LC3, GABARAP, LAMP1, and LAMP2.

6. The *in vitro* method for recovering an organelle, according to any of the preceding claims, wherein at least one of the first or second polypeptide comprises a surface marker of a mitochondria.

7. The *in vitro* method for recovering an organelle, according to any of the preceding claims, wherein:

- one of the polypeptides comprises a surface marker polypeptide sequence expressed at the surface of a mitochondria; and
- one of the polypeptides comprises a surface marker polypeptide sequence expressed at the surface of an endosome or a phagosome;

said surface marker polypeptide sequences being the same or different, preferably different.

8. The *in vitro* method for recovering an organelle, according to claim **6** or **7**, wherein said surface marker of a mitochondria is selected from the group consisting of: VDAC1, TOMM70, TOMM20, TOM22, OMP25, FIS1.

9. The *in vitro* method for recovering an organelle, according to any of the preceding claims, wherein the cell or extract is a plant cell, or an extract thereof.

10. The *in vitro* method for recovering an organelle, according to any of the preceding claims, wherein the cell or extract comprises **(i)** a target organelle, **(ii)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID**

NO 2, and **(iii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 4.**

11. The *in vitro* method for recovering an organelle, according to any of the preceding claims, wherein the first and second polypeptide each comprise at least one distinct organic fluorophore, a pH-sensitive fluorophore, an enzyme activated fluorophore, a redox-sensitive fluorophore, a fluorogenic probe, a nonfluorescent protein and/or electron dense particle.

12. A composition comprising **(i)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

13. An expression vector **(i)** coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

14. A cell comprising:

(i) an heterologous nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and

(ii) an heterologous nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:**

wherein the first and second polypeptide comprise each a surface marker polypeptide sequence expressed at the surface of an organelle.

15. A kit comprising **(i)** a nucleic acid coding for a first polypeptide comprising a polypeptide sequence **SEQ ID NO 1,** and **(ii)** a nucleic acid coding for a second polypeptide comprising a polypeptide sequence **SEQ ID NO 3:** wherein the first and second polypeptide comprise each a surface marker expressed at the surface of an organelle.

ABSTRACT

SYSTEMS AND METHODS FOR SELECTIVE ISOLATION AND CHARACTERIZATION OF ORGANELLES USING PROGRAMMABLE TARGETING MODULES

The present invention relates to the field of cellular and molecular biology and, more specifically, to systems and methods for the selective isolation, enrichment, and purification of organelles, in particular intracellular organelles and vesicular compartments, in particular from eukaryotic cells.

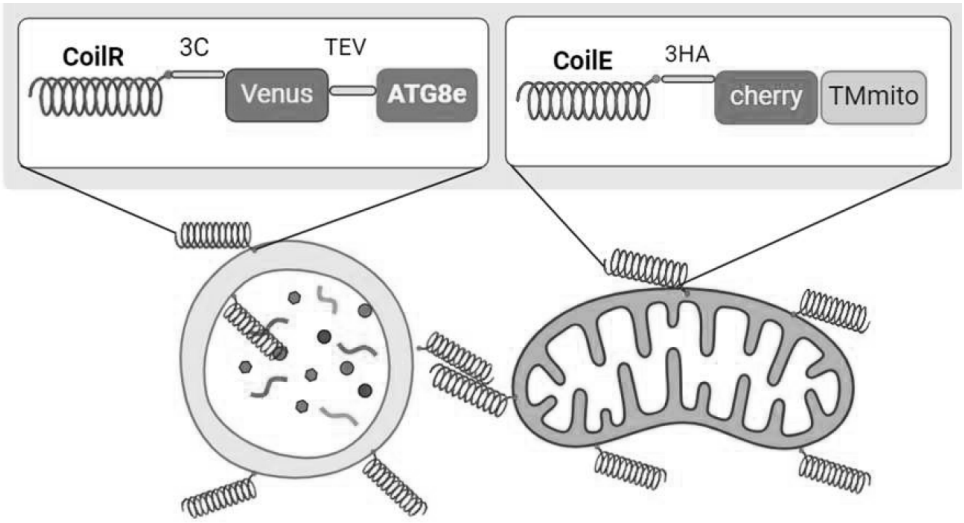


FIGURE 1A

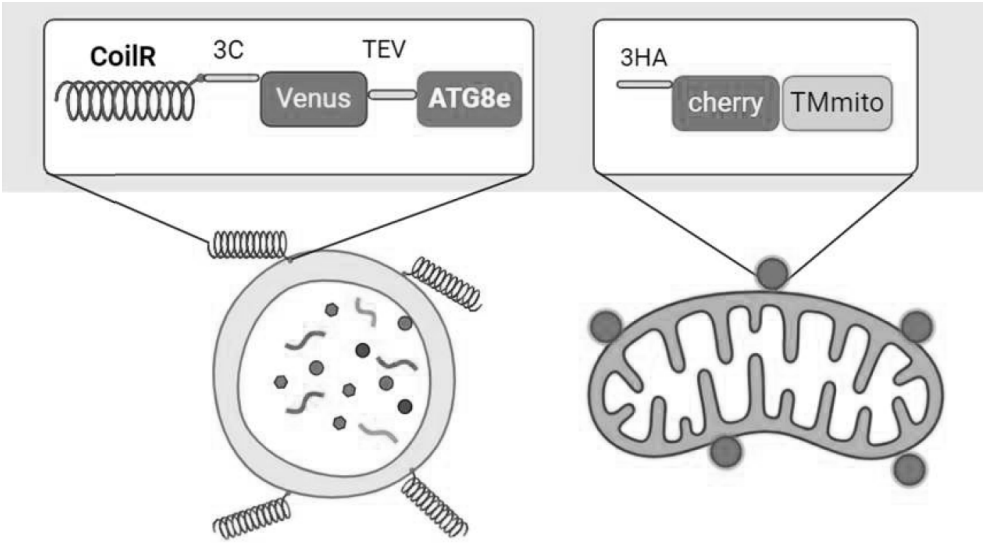


FIGURE 1B

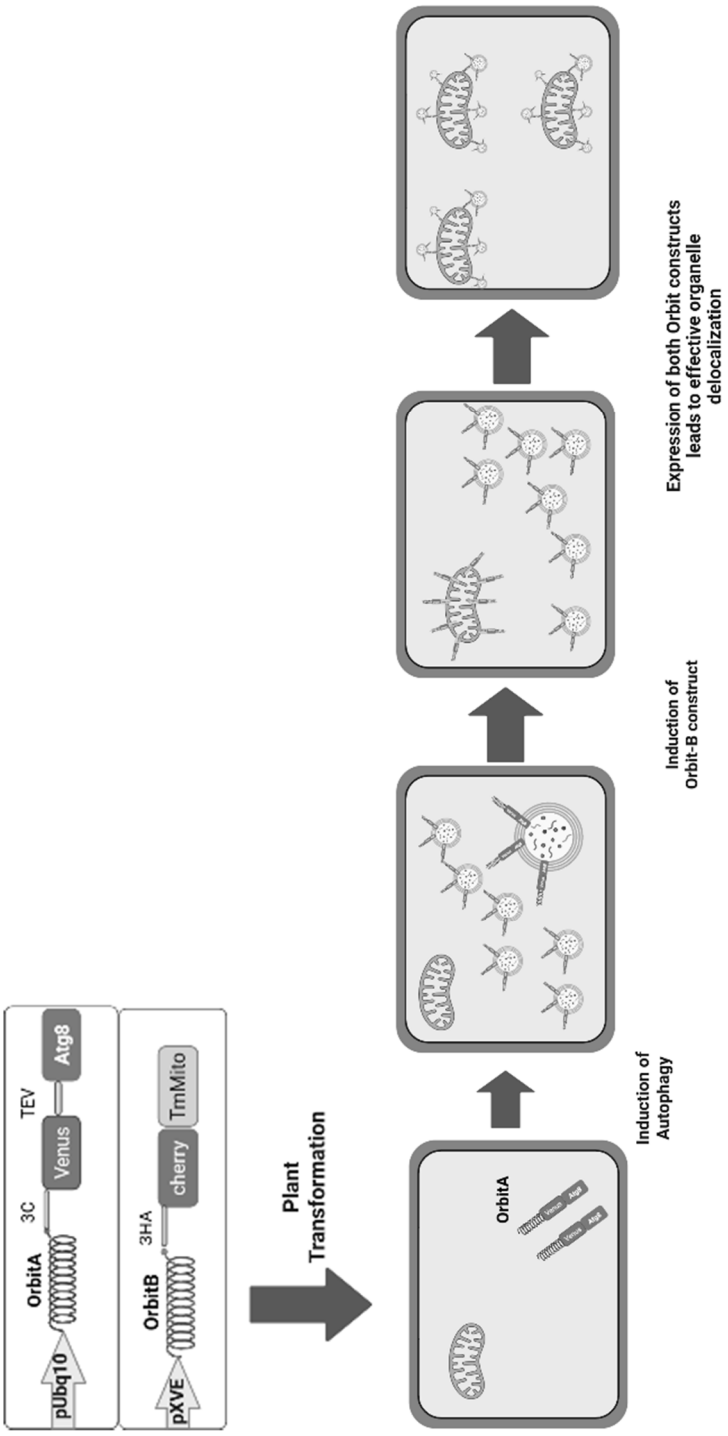

pUbq10
OrbitA
3C
Venus
TEV
Atg8
pXVE
OrbitB
3HA
cherry
TmMito
Plant
Transformation
OrbitA
Induction of
Autophagy
Induction of
Orbit-B construct
Expression of both Orbit constructs
leads to effective organelle
delocalization

FIGURE 2