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(54) **GENETICALLY ALTERED CILIATES AND USES THEREOF**

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(57) **ABSTRACT**

Ciliate organisms are provided that comprise reduced proteolytic processing in granules. For example, ciliates are provided that lack detectable expression of one or more sortilin (SOR) gene product. Methods for producing such genetically altered ciliates and methods for protein production in a these organisms are also provided.

Fold-induction during regranulation	Fold-induction in exo-mutant (neg. control)	Statistical significance	Gene identity
11.1	0.7	0.0008	AP-3 adaptin large subunit
30.0	1.2	0.001	AP-3 medium subunit
6.2	0.4	0.0003	SEC14
11.2	1.1	0.0007	Vps9
7.3	0.6	0.0006	beta-arrestin-related
16.1	0.7	0.0006	GRIP domain protein
7.2	0.4	0.004	V-type ATPase
7.8	1.0	0.0007	Vps10/sortilin (#1)
7.5	1.2	0.0005	Vps10/sortilin (#2)
16.7	1.0	0.002	tSNARE
12.5	1.1	0.001	synaptobrevin
4.1	1.0	0.002	Dynamin-related protein (DRP7)
4.5	0.4	0.0006	cathepsin
5.8	0.7	0.0004	carboxypeptidase

FIG. 1

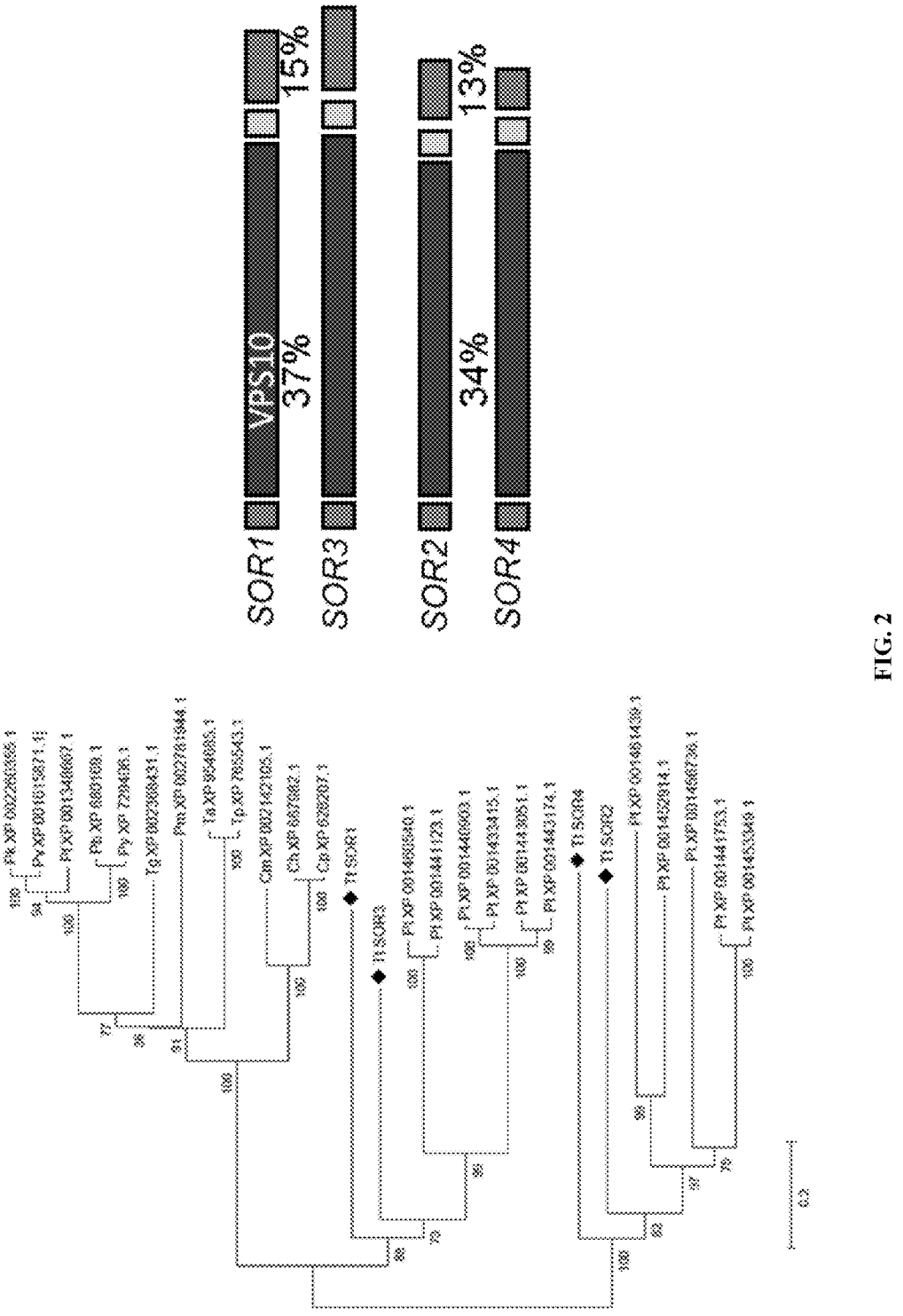
VPS10
37%
15%
34%
13%
SOR1
SOR3
SOR2
SOR4
Tt SOR1
Tt SOR3
Tt SOR4
Tt SOR2
0.2

FIG. 2

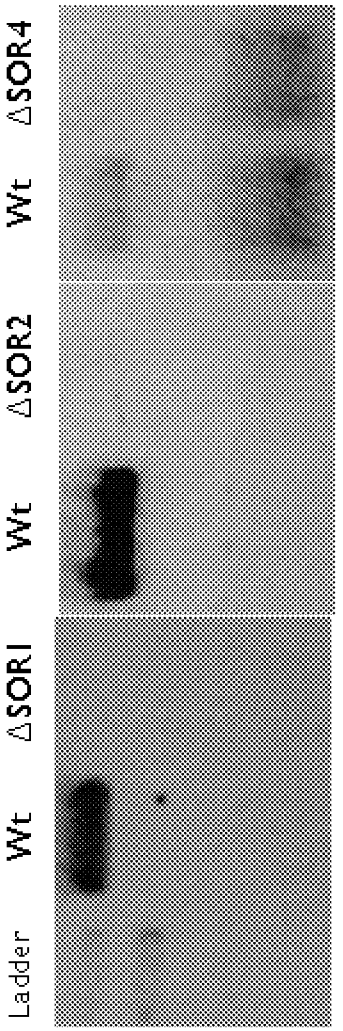

FIG. 3

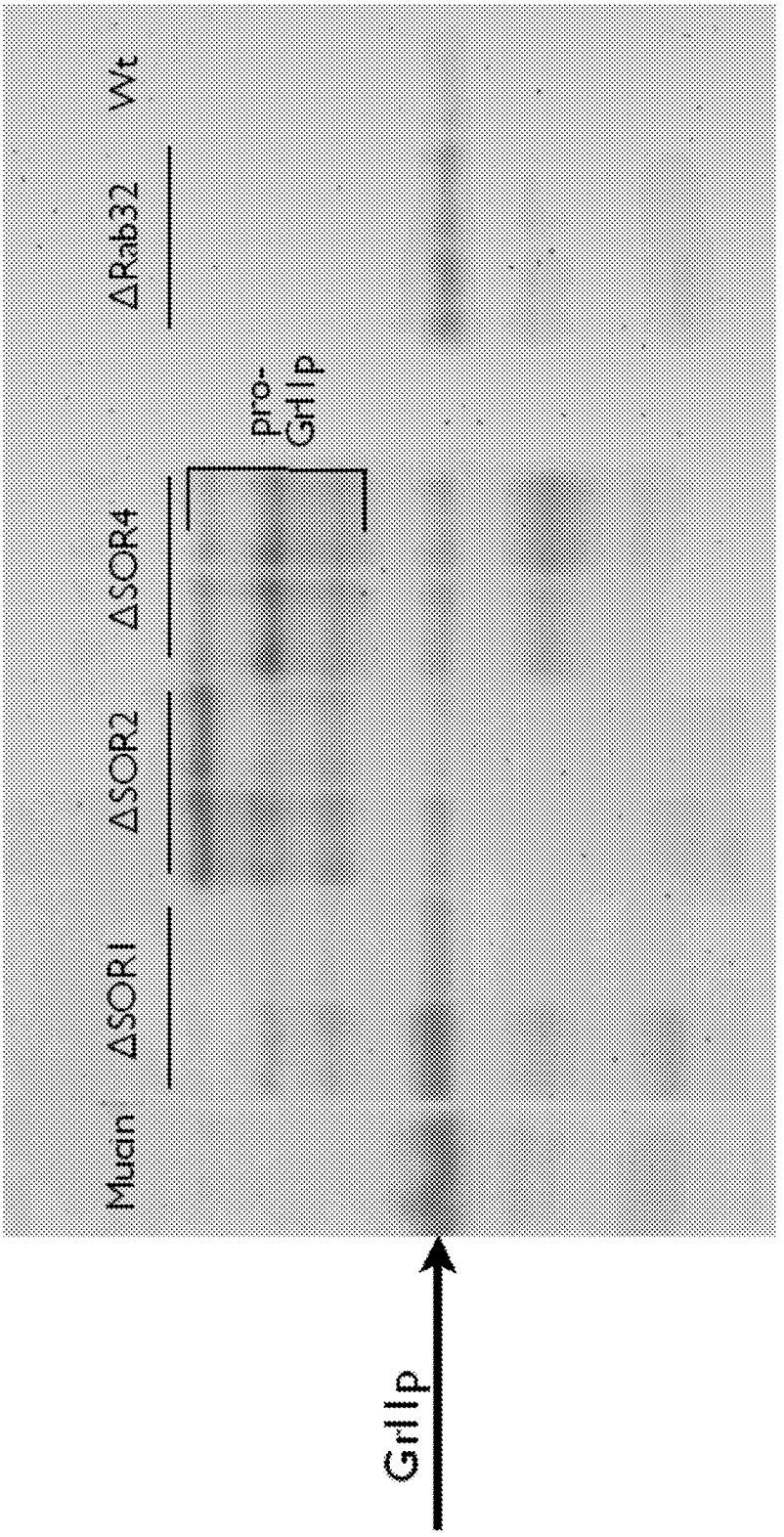

FIG. 4

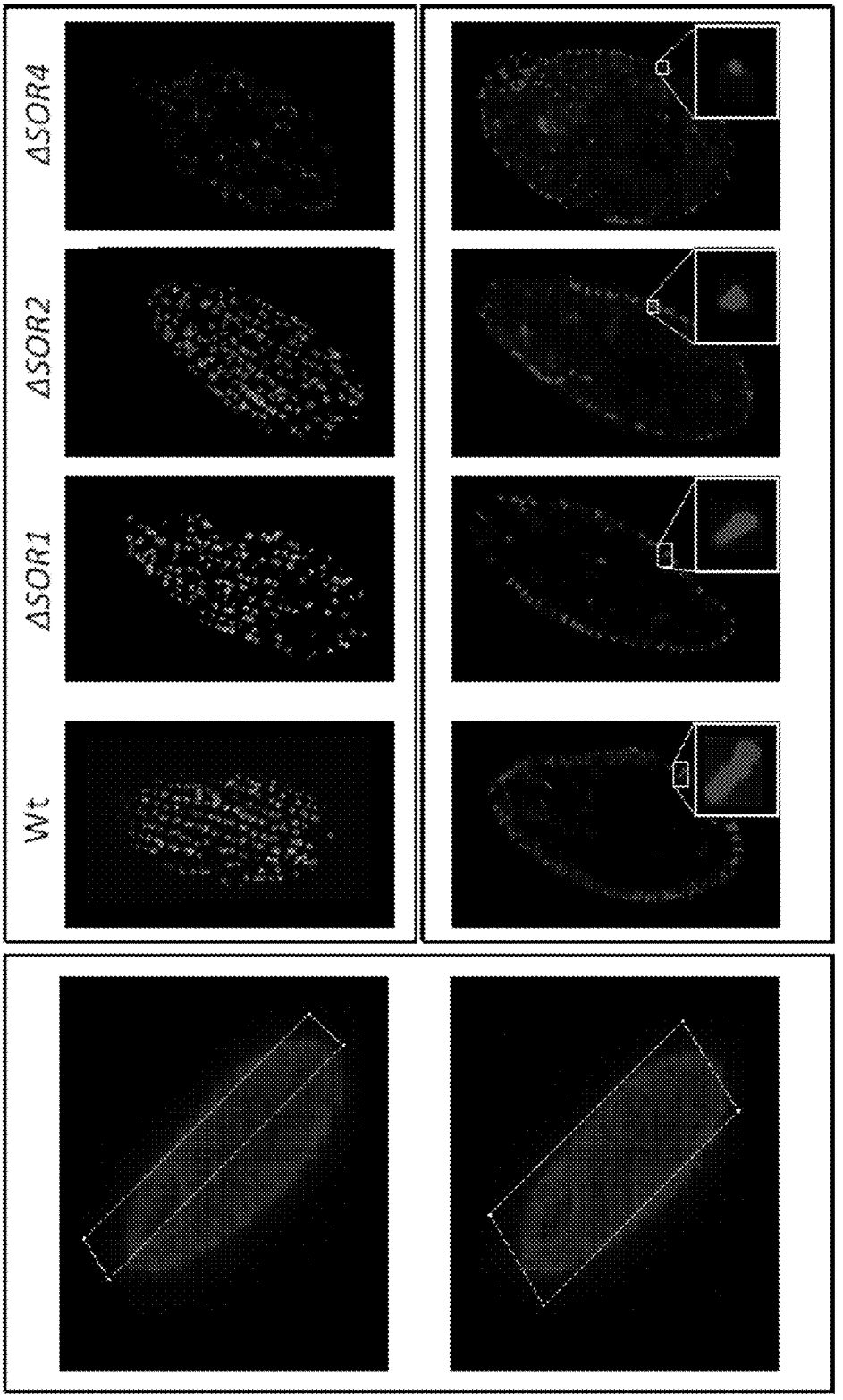

FIG. 5

GENETICALLY ALTERED CILIATES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Ser. No. 61/601,921, filed on Feb. 22, 2012, which is hereby incorporated by reference in its entirety.

[0002] The invention was made with government support under Grant No. GM077607 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] 1. Field of the Invention

[0004] The present invention relates generally to the field of genetics and molecular biology. More particularly, it concerns genetically altered ciliate organisms and the use of such organisms in recombinant protein production.

[0005] 2. Description of Related Art

[0006] Recombinant proteins are useful for a wide range of applications including as industrial enzymes and as therapeutics. For example, production of genetically engineered vaccine antigens, therapeutics (including antibodies and antibody fragments), industrial enzymes, biopolymers, and bioremediation agents now constitute a multibillion dollar-per-year industry. There is also a large market for recombinant proteins in the basic research arena (Pavlou and Reichert (2004); Langer (2005)).

[0007] Currently available platforms for the production of recombinant proteins are limited to a relatively small number of cell-based systems that include bacteria, fungi, and insect and mammalian tissue culture cells. Although bacteria can offer high yield and low cost alternatives for production of mammalian proteins, cell culture systems based on higher organisms such as insect cells or mammalian cell systems generally provide proteins having greater fidelity to the natural proteins in terms of protein folding and/or post-translational processing (e.g., glycosylation). Whole transgenic plants and animals have also been harnessed for the production of recombinant proteins, but the long development time from gene to final product can be a major drawback with these multicellular organisms, as can their high cost, low yield and many inherent difficulties in purification.

[0008] There remains a need in the art for improved methods for rapid, high-fidelity and cost-effective production and purification of recombinant polypeptides.

SUMMARY OF THE INVENTION

[0009] In a first embodiment a genetically altered ciliate is provided wherein the ciliate lacks detectable expression (or has reduced expression) of one or more SOR gene product. For example, the SOR gene product may be a product corresponding to SOR1 (SEQ ID NO: 1, 2), SOR2 (SEQ ID NO: 3, 4), SOR3 (SEQ ID NO: 5, 6) and/or SOR4 (SEQ ID NO: 7, 8; indicating the protein and nucleic acid coding sequence respectively). In some aspects, the ciliate may lack detectable expression of a SOR polypeptide or a SOR RNA corresponding to SOR1, SOR2, SOR3 and/or SOR4. In a further aspect, the ciliate lacks detectable expression of 2, 3 or 4 of the SOR1, SOR2, SOR3, or SOR4 genes. For example, a ciliate of the embodiments may lack detectable expression (or have reduced expression) of SOR1 and SOR2; SOR1 and SOR3; SOR1 and SOR4; SOR2 and SOR3; SOR2 and SOR4; SOR3 and SOR4; SOR1, SOR2 and SOR3; SOR1, SOR2 and SOR4; SOR1, SOR3 and SOR4; SOR2, SOR3 and SOR4; or SOR1, SOR2 SOR3 and SOR4.

[0010] In certain aspects, a ciliate of the embodiments comprises a genomic alteration, such as an insertion or a deletion in both copies of the germline genome that disrupts expression of one or more SOR gene product. For instance, the ciliate can comprise an insertion or deletion located in the open reading frame of a gene corresponding to SOR1, SOR2, SOR3 and/or SOR4. In some aspects, a genomic insertion comprises a selectable marker, such a drug resistance marker (e.g., a gene for tetracycline or neomycin resistance). Accordingly, in some aspects, a ciliate of the embodiments comprises an insertion or a deletion in all macronuclear copies of a gene corresponding to SOR1, SOR2, SOR3 and/or SOR4.

[0011] In further aspects a ciliate of the embodiments expresses a polynucleotide complementary to all or part of an RNA gene product corresponding to SOR1, SOR2, SOR3 and/or SOR4. For example, the ciliate can express an antisense RNA or a double stranded RNA (dsRNA) molecule, such as a small interfering RNA (siRNA), short hairpin RNA (shRNA) or micro RNA (miRNA), complementary to all or part of an RNA gene product corresponding to SOR1, SOR2, SOR3 and/or SOR4.

[0012] In still further aspects, a ciliate of the embodiments comprises a transgenic expression cassette, such as an expression cassette encoding a polypeptide. For example, the polypeptide can be a polypeptide for recombinant production in the ciliate. Polypeptides for use in accordance with the embodiments include, but are not limited to, enzymes, immunoglobulin (e.g., immunoglobulin light chains, immunoglobulin heavy chains or single chain antibodies), cytokines, chemokines, and antigens (e.g., bacterial or viral antigens). In some aspects the polypeptide coding sequence can comprise a sequence for cellular trafficking, such as a mucocyst-targeting sequence. For example, the polypeptide can encode a mucocyst-targeting sequence derived from a *Tetrahymena* Grl protein, such as Grl1, Gr12, Gr13, Gr14, Gr15, Gr16, Gr17, Gr18, Gr19 or Grl 10. In still further aspects, the polypeptide encodes a cleavable linker (e.g., between the polypeptide for expression and a mucocyst-targeting sequence).

[0013] In some specific aspects, a ciliate of the embodiments is a *Tetrahymena*, such as a *T. thermophila* or *T. pyriformis*.

[0014] In yet a further embodiment there is provided a recombinant *Tetrahymena* germline genome (e.g., a recombinant *T. thermophila* or *T. pyriformis* genome) comprising a genomic insertion or deletion in both copies of one or more SOR gene selected from the group consisting of SOR1, SOR2, SOR3, and SOR4. For example, the genomic insertion or deletion can be located in the open reading frame of the gene. In some aspects, a genomic insertion comprises the insertion of a selectable marker, such a drug resistance marker. In still further aspects, a *Tetrahymena* germline genome comprises a genomic insertion or deletion in both copies of 2, 3 or 4 SOR genes corresponding to SOR1, SOR2, SOR3 or SOR4. Thus, the genome can comprise an insertion of deletion in the genes for SOR1 and SOR2; SOR1 and SOR3; SOR1 and SOR4; SOR2 and SOR3; SOR2 and SOR4; SOR3 and SOR4; SOR1, SOR2 and SOR3; SOR1, SOR2 and

SOR4; SOR1, SOR3 and SOR4; SOR2, SOR3 and SOR4; or SOR1, SOR2 SOR3 and SOR4.

[0015] In a further embodiment there is provided a recombinant *Tetrahymena* germline genome comprising, an expression cassette comprising a sequence encoding a polynucleotide molecule complementary to all or part of an RNA gene product corresponding to SOR1, SOR2, SOR3, or SOR4. For example, genome can comprise sequences encoding an antisense RNA or a dsRNA, such as a siRNA, shRNA or miRNA, complementary to all or part of an RNA gene product corresponding to SOR1, SOR2, SOR3 and/or SOR4.

[0016] In still a further aspect of the embodiments a recombinant *Tetrahymena* germline genome can comprise a transgenic expression cassette, such as a cassette encoding a polypeptide, optionally including a mucocyst-targeting sequence.

[0017] In still yet a further embodiment there is provided a method of producing a genetically altered ciliate comprising: (a) transforming a ciliate with a polynucleotide comprising a sequence complementary to a SOR gene corresponding to SOR1, SOR2, SOR3 and/or SOR4; and (b) isolating a genetically altered ciliate wherein the ciliate lacks detectable expression of the gene product of said SOR gene. For example, step (b) can comprise isolating a genetically altered ciliate comprising an insertion or deletion in a SOR gene or isolating a genetically altered ciliate expressing a polynucleotide molecule complementary to all or part of an RNA gene product of a SOR gene. In further aspects, step (a) comprises transforming the ciliate with a polynucleotide comprising a sequence complementary to a SOR gene and comprising a selectable marker (e.g., a drug resistance marker). Thus, in some aspects, a genetically altered ciliate is isolated based on expression of a selectable marker (such as by drug selection). Detailed methods for genetic alteration of ciliates are well known in the art and are detailed in PCT Patent Publn. No. WO2010108182, the entirety of which is incorporated herein by reference.

[0018] In a further embodiment there is provided a method of producing a genetically altered ciliate of the embodiments comprising obtaining the genetically altered ciliate and vegetatively propagating the ciliate. In further aspects, a genetically altered ciliate can be a produced by sexually propagating a genetically altered ciliate and isolating progeny that comprise the genetic alterations.

[0019] In still yet a further embodiment a method of producing a polypeptide is provided comprising: (a) expressing a polynucleotide encoding the polypeptide in a ciliate of the embodiments; and (b) incubating the ciliate in a media under conditions permissible for expression of the polypeptide. In some aspects, the majority (or at least a portion) of the polypeptide is secreted from the ciliate and the method can comprise (c) purifying the expressed polypeptide from the media. In certain aspects, the majority (or at least a portion) of the polypeptide is not secreted by the ciliate and the method can comprise (c) purifying the ciliate from the media and, optionally, (d) purifying the protein from the ciliate. In still further aspects, a method of the embodiments further comprises transforming a ciliate with a polynucleotide encoding a polypeptide. Further methods for polypeptide expression in ciliates are detailed in PCT Patent Publn. No. WO2010108182, the entirety of which is incorporated herein by reference.

[0020] In some aspects a expressing a polynucleotide for expression in a ciliate is further defined as an expression cassette encoding a polypeptide. For example, the polypeptide can be a polypeptide of mammalian origin, such as a human polypeptide. In some aspects, the polypeptide comprises sequence encoding an enzyme, an immunoglobulin, a cytokine, a chemokine, or an antigen.

[0021] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one.

[0022] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

[0023] Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0024] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0026] FIG. **1**: Two sortilin genes in *Tetrahymena* are dramatically up-regulated during induced granule formation (regranulation) in *Tetrahymena*. First column show fold induction of the genes, significance values are shown in third column.

[0027] FIG. **2**: Analysis of the *Tetrahymena* genome indicates that it codes four sortilin/Vps10 genes. Diagram shows the homology between the four sortilin genes (Tt SOR1-4) and structurally related genes.

[0028] FIG. **3**: *Tetrahymena* RNA expression was examined in putative SOR knockout lines (SORT, SOR2 and SOR4). In each case wild type (WT) *Tetrahymena* exhibited SOR RNA expression, whereas no expression was observed in the knockout lines.

[0029] FIG. **4**: *Tetrahymena* sortilin knockout lines secrete unprocessed precursors of the granule protein Grl1p. Immunoblot media samples show that in the case of each of the SOR knockout lines unprocessed (high molecular mass) Grl1p precursors are released into the media.

[0030] FIG. **5**: The sortilin knockout lines each make aberrant secretory granules, which are visualized here by immunefluorescence. Granule-specific immunefluorescence results for the indicated knockout cells (or for wild type "wt") are shown in tangential section (upper panels) or equatorial section (lower panels).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0031] Recombinant protein production has become very important for a variety of applications. For example, many modern therapeutics, such as enzymes and monoclonal antibodies, are proteins that are produced recombinantly. However, adequate and cost-effective protein production systems are lacking Bacterial expression systems, while low cost, often result in proteins that do not incorporate crucial post-translation modifications or are improperly folded. On the other hand, mammalian cell expression systems are very expensive to maintain and produce notoriously low yields of protein products that must be extensively purified. Accordingly, ciliate-based protein production systems could provide an attractive alternative existing systems. However, there remains a need for a ciliate system adapted to provide high quality recombinant protein yield.

[0032] Studies detailed herein identify four ciliate gens from *Tetrahymena* that are important regulators of cell trafficking and secretion pathways. In particular, the SOR genes mediate transport of proteases to granules allowing for proteolytic processing of the granule contents. The studies here demonstrate that SOR gene expression can effectively knocked-out (see, FIG. **3**), and that such knockout is not lethal to the organism. Indeed, knockout of SORT, SOR2 and SOR4 all resulted in decreased proteolytic processing of products located in granules (FIG. **4**) and a change in granule structure indicative of reduced proteolytic processing (FIG. **5**).

[0033] Accordingly, modified ciliate organisms, such as *Tetrahymena*, are provided that have reduced expression of one or more sortilin gene product. Importantly, these organism exhibit reduced proteolytic processing in granules and are thereby ideal for recombinant protein production. Such organisms can be used to produce a wide range of protein products without aberrant cleavage of the products during expression. Moreover, recombinant proteins can be easily and cost-effectively purified by either isolating the ciliate cells comprising large quantities of highly concentrated (and uncleaved) protein product or by targeting the proteins for secretion and isolating the product from cell media.

I. Ciliates for Use According to the Embodiments

[0034] The embodiments may be practiced with a variety of different ciliates which include secretory granules called mucocysts. Heterologous polypeptides can be targeted to these secretory granules by encoding fusion proteins of the desired heterologous polypeptide and an appropriate targeting sequence. After exposing the ciliate to a secretory stimulus that causes the mucocysts to discharge their contents to the extracellular environment, the heterologous polypeptide can be recovered from the resulting matrix and medium.

[0035] The free-living ciliate protists are a large and diverse phylum (Ciliata) whose members display a structural and functional complexity comparable to that of higher metazoa (Fankel (2000); Turkewitz et al. (2002)), and include over 7,000 species with 11 major subdivisions. Tetrahymenids and Paramecium belong to the Oligohymenophoreans. Ciliates that include mucocysts useful in the invention include *Tetrahymena* species such as *Tetrahymena thermophila* and *Tetrahymena pyriformis*. Paramecium has dense core granules but does not secrete a proteinaceous gel. Both *Tetrahymena thermophila* and *Tetrahymena pyriformis* produce mucocysts, and both secrete a proteinaceous gel.

[0036] *Tetrahymena* spp. are amenable to genetic manipulation, can be grown on a large scale and have a doubling time of 1.5-3 hrs. Unlike *T. thermophila*, which has an optimal growth temperature of 35° C., the optimal growth temperature for *T. pyriformis* is lower (maximal growth temperature of 34° C.). Cells reach high-density in a short time on a variety of inexpensive media and can be expanded for growth in bioreactors up to several thousand liters in size (Hellenbroich et al. (1999); de Coninck et al. (2000)). Methods for transformation, along with robust, inducible promoters for driving high-level gene expression have recently been described for this system (Bruns and Cassidy-Hanley (2000); Gaertig and Kapler (2000); Shang et al. (2002); Boldrin et al. (2006)).

[0037] *Tetrahymena* spp. devote a large part of their metabolism to membrane protein production due to the hundreds of cilia that extend from its surface (Williams et al. (1980)). Additionally, *Tetrahymena* spp. lack a cell wall and display high-mannose N-glycan protein modifications that lack branched, immunogenic structures (Taniguchi et al. (1985); Becker and Rusing (2003); Weide et al. (2006)). Glycosylation patterns of secreted proteins in *Tetrahymena* spp. are uniform and consist of high-mannose N-glycan structures comprising Man3GlycNac2 core N-glycans similar to those which are produced in the endoplasmic reticulum of mammalian cells.

[0038] This glycosylation pattern is unlike the glycosylation pattern produced in other microbial systems. For example, such glycosylation is non-existent in bacteria, and is highly branched and immunogenic in fungi.

II. Genetic Alteration of Ciliates

[0039] Methods for genetic alteration of ciliates are well known in the art and may be used in accordance with the instant embodiments. For example, ciliates can be transformed with vectors that express nucleic acid to disrupt expression of a SOR gene (such as siRNAs). In some aspects, the ciliates is transformed with a vector to disrupt an endogenous SOR gene (e.g., by generating an insertion of deletion in a genomic copy of the gene). In still further aspects, a ciliate can be transformed with a vector for the expression of heterologous polypeptides, such as peptides that will be harvested from the cells.

[0040] Certain aspects of the embodiments concern ciliates that lack detectable expression (or have reduced expression) of one or more SOR gene product corresponding to SOR1, SOR2, SOR3 or SOR4. In some aspects the SOR gene product is an RNA at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence of SOR1 (SEQ ID NO: 2), SOR2 (SEQ ID NO: 4), SOR3 (SEQ ID NO: 6) or SOR4 (SEQ ID NO: 8). In a further aspect, the SOR gene product is an polypeptide at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence of SOR1 (SEQ ID NO: 1), SOR2 (SEQ ID NO: 3), SOR3 (SEQ ID NO: 5) or SOR4 (SEQ ID NO: 7). In yet further aspects the SOR gene product is an polypeptide comprising at least 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890 or 900, contiguous amino acids identical to

the amino acid sequence of SOR1 (SEQ ID NO: 1), SOR2 (SEQ ID NO: 3), SOR3 (SEQ ID NO: 5) or SOR4 (SEQ ID NO: 7). In some specific aspects, the gene product corresponding to SOR1, SOR2, SOR3 or SOR4 is one of the gene products listed in the NCBI accession numbers of FIG. **2**, each of which is incorporated herein by reference. Thus, in some aspects, a ciliate of the embodiments comprises an insertion or a deletion in such a gene corresponding to *Tetrahymena* SOR1, SOR2, SOR3 and/or SOR4. In yet further aspects, a ciliate can comprise an expression cassette encoding a polynucleotide (e.g., a dsRNA, siRNA, shRNA or miRNA) complementary to all or part of an RNA corresponding to a *Tetrahymena* SORT, SOR2, SOR3 and/or SOR4 RNA.

[0041] Transformation

[0042] Genes can be introduced into ciliates using established protocols or any method known to one skilled in the art. Transformation of ciliates can be achieved by microinjection (Tondravi and Yao (1986)), electroporation (Gaertig and Gorovsky (1992)), or biolistically (Cassidy-Hanley et al. (1997)).

[0043] Thus, in some embodiments, ciliate cells can be transformed with a chimeric gene by particle bombardment (also known as biolistic transformation) (Cassidy-Hanley et al. (1997)). Particle bombardment transformation can be achieved by several ways. For example, inert or biologically active particles can be propelled at cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the chimeric gene. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Other variations of particle bombardment, now known or hereafter developed, can also be used.

[0044] Microcarrier bombardment can also be used to transform ciliate cells by means of DNA-loaded gold particles (U.S. Pat. No. 6,087,124; European Pat. EP 847 444; WO 1998/001572). In this approach, microcarrier bombardment with DNA-coated gold is used as a means of introducing foreign genes into ciliates. In one embodiment, microcarrier bombardment can be used to transform ciliates and introduce genes into the (germline) micronucleus

[0045] Methods for selection of transformed cells harboring foreign genes are known in the art. For example, the vector can further comprise a selectable cassette marker to permit selection for transformed cells {e.g., a neo 2 cassette) (Gaertig et al. (1994)).

[0046] Selection of transformants can be achieved by growing the cultured ciliates in a medium which allows only the transformants to survive. Suitable selection agents include antibiotics which will kill most all non-transformants but allow transformants (which also possess an antibiotic resistance gene) to survive. A number of antibiotic-resistance markers are known in the art. Any known antibiotic-resistance marker can be used to transform and select transformed host cells in accordance with the present invention. For example, selection of the transformants can be performed by means of a resistance marker such as a point mutation in the 17s rDNA, which confers resistance to paromomycin, can allow for selection of rDNA transformants (Spangler and Blackburn (1985); Bruns et al. (1985)). Other methods include the use of a mutant cell line that allows targeting of genes to the beta tubulin-1 locus of *T. thermophila* by homologous recombination, and allows efficient selection of transformed cell lines by growth in the microtubule-stabilizing agent (taxol) (U.S. Pat. No. 6,846,481). Another method for selection of transformed cells harboring foreign genes is to insert full length coding regions into the pD5HA vector (Cowan et al. (2005)). In this method, transcription is driven by the inducible MTT1 promoter. Once cells have been transformed with the pD5HA vector selection of positive transformants is determined by paromomycin resistance (i.e., cell growth in media containing the drug). Presence of the transgene is then verified by PCR and then induced with cadmium chloride to over-express the recombinant gene product.

[0047] Many other selectable marker systems are known in the art. Selectable marker genes that confer resistance or tolerance to a normally toxic selection agent allow only successfully transfected cells to survive in the presence of the selection agent, and are referred to as positive selectable markers. Examples of positive selectable marker genes and their corresponding selection agents are: aminoglycoside phosphotransferase (APH) and G418; dihydro folate reductase (DHFR) and methotrexate (Mtx); hygromycin-B-phosphotransferase (HPH) and hygromycin-B; xanthine-guanine phosphoribosyltransferase (XGPRT) and mycophenolic acid; and adenosine deaminase (ADA) and 9-β-D-xylofuranosyl adenine (Xyl-A).

[0048] In another example of a positive selectable marker system, thymidine kinase (TK) and aminopterin (included, e.g., in hypoxanthine-aminopterin-thymidine (HAT) medium) can be used in cells that are initially thymidine kinase deficient (tk~). The aminopterin will normally kill tk~ cells and, therefore, only successful TK transfectants will survive. Selectable marker genes that confer sensitivity or susceptibility to a normally nontoxic selection agent cause only successfully transfected cells to die in the presence of the selection agent, and are referred to as negative selectable markers. An example of a negative selectable marker system is thymidine kinase (TK) and gancyclovir. Phenotypic selectable marker genes permit selection based upon morphological or biochemical traits rather than cell death or survival. In some cases, the phenotypic marker is detectable only in the presence of an additional selection agent. An example of a phenotypic selectable marker system is β-galactosidase (lacZ) and X-gal.

III. Vectors and Polypeptide Expression

[0049] Heterologous nucleic acids can be introduced into the ciliate host on an expression vector that is capable of integrating into the host's genome. For example, expression vectors capable of homologous recombination with a highly expressed gene that is endogenous to the protozoan host, such as a P-tubulin gene are known in the art. Alternatively, a heterologous nucleic acid transformed into a ciliate can be maintained extrachromosomally on an autonomous plasmid.

[0050] Expression vectors useful for transforming ciliates in accordance with the methods described herein include but are not limited to replacement vectors, rDNA vectors, and rDNA-based vectors. Replacement vectors accomplish DNA-mediated transformation by replacing or altering endogenous genes using homologous recombination. Integration of the heterologous nucleic acid into the host's genome at the targeted site is accomplished via homologous recombination involving a double crossover event with the vector containing the heterologous nucleic acid. An example of an expression vector useful for genomic incorporation of a heterologous nucleic acid by replacement is one that includes

a heterologous coding sequence flanked by portions of the endogenous BTU1 gene of *Tetrahymena* thermophile.

[0051] A replacement vector can include a 5' region, followed by a heterologous coding region, followed by a 3' region, wherein at least a portion of each of the 5' and 3' regions is complementary to 5' and 3' regions on an endogenous gene of the host, to allow for genomic integration of the heterologous coding region via homologous recombination. The 5' and 3' regions of the vector can also comprise regulatory elements, such as a promoter and a terminator. The necessary regulatory elements can also be supplied by the endogenous gene into which the heterologous coding region integrates. Suitable regulatory regions include, but are not limited to promoters, termination sequences, signal peptides and proprotein domains involved in the expression and secretion of proteins. For example, such regulatory elements can provide efficient heterologous expression of proteins in *Tetrahymena* spp. under control of promoters and/or terminators which are derived from genes in *Tetrahymena* ssp. Such vectors can comprise naturally occurring promoters and/or terminators from proteins secreted at a high level in *Tetrahymena* ssp. The expression of recombinant polypeptides in *Tetrahymena* spp. can be driven by strong promoters, pre/pro sequences and terminators. In one embodiment, the promoters and/or terminators can be selected from proteins secreted at a high level independent of the cell-cycle in *Tetrahymena* spp. (US Patent Application 2006/0127973; WO2003/078566). Inducible promoters from *Tetrahymena* spp. genes have also been described that allow robust expression of foreign genes. For example, heat-inducible promoters of the heat shock protein family of the ciliate *Tetrahymena* spp. are also suitable for use with the methods described herein. Suitable heat shock promoters from *Tetrahymena* spp. are known in the art (see WO2007/006812).

[0052] Methods for creating mitotically stable *Tetrahymena* spp. transformants, for example, by integration of a heterologous gene by homologous DNA recombination, are known in the art. Methods for generating *Tetrahymena* spp. having targeted gene knockouts by homologous DNA recombination are also known in the art (Bruns and Cassidy-Hanley (2000); Hai et al. (2000); Gaertig et al. (1999); Cassidy-Hanley et al. (1997)). The somatic macronucleus or the generative micronucleus can be transformed in alternation. For example, sterile transformants, which may provide improved safety parameters, can be obtained with macronucleus transformation.

[0053] Expression vectors can also be maintained extrachromosomally in the ciliates. An expression vector maintained as an extrachromosomal element can be a rDNA-based vector containing an on from *Tetrahymena* spp. rDNA, which is known to support extrachromosomal replication. Such a vector can further comprise a 5' regulatory region from an endogenous *Tetrahymena* spp. gene containing a promoter region operably linked to the heterologous coding region and, optionally, a 3' regulatory region from the same or a different *Tetrahymena* spp. gene. For example, regulatory regions from ciliate genes in such vectors can include, but are not limited to, regulatory regions from genes such as HHFI, rp129, BTU1, BTU2, SerH3, and actin.

[0054] There are a number of suitable vectors suitable for transformation of ciliates known in the art. For example, *Tetrahymena* spp. can be transformed with an rDNA vector (Tondravi and Yao (1986); Yu and Blackburn (1989)). The shuttle vector pXS76 allows insertion of transgenes downstream of a cadmium-inducible promoter from the MTT1 metallothionein gene of *T. thermophila* via homologous recombination and selection in paromomycin. Alternatively, inserts can be introduced into high copy number ribosomal DNA vectors (such as pD5H8) under control of the cadmium-inducible MTT1 promoter. The pD5H8 vector takes advantage of a biological feature of *Tetrahymena* spp. in which the ribosomal cistrons become amplified to extraordinarily high copy numbers following conjugation. An rDNA-based vector can be a circular vector that contains a 5' non-translated sequence comprising two or more on sequences from *Tetrahymena* spp. rDNA. A nucleic acid fragment containing a heterologous coding region, for example a selectable marker or transgene, can also be added to the vector. The vector can further comprise a 5' untranslated region of a *Tetrahymena* spp. gene and a 3' untranslated region of a *Tetrahymena* spp. gene, inserted upstream and downstream of the selectable marker and/or the transgene. Methods for transformation, along with robust, inducible promoters for driving high-level gene expression have recently been described for this system (Bruns and Cassidy-Hanley (2000); Gaertig and Kapler (2000); Shang et al. (2002); Boldrin et al. (2006)).

[0055] Sequence variations within the origins of replication of rDNA from wild-type B- and C3-strains of *T. thermophila* convey a replicative advantage to the C3-form in B/C3 heterozygotes. Although both B- and C3-forms of rDNA are initially present in the macronucleus in approximately equal amounts, within 30 fissions only the C3 variant remains (Pan et al. (1982); Orias et al. (1988)). pIC19-based shuttle vectors containing the C3 origin of replication have been used as high-copy number vectors for the delivery of foreign DNA to *Tetrahymena* spp. (Yu and Blackburn (1989)) (FIG. **5**).

[0056] Although such vectors can become unstable and be lost within about 50 to about 80 generations, micronuclear versions of the C3 rDNA is accurately processed (to form a palindrome) following introduction into *T. thermophila* B cell lines. The micronuclear version is maintained as a stable linear chromosome over many generations (Bruns et al. (1985)). Functional transgenes can be inserted into the 3'-nontranscribed spacer (3'-NTS) of such vectors with no effect on rDNA processing. Within 6-10 generations, recombinant molecules can comprise 50-100% of the total rDNA complement, with as many as 18,000 copies of the transgene per cell (Blomberg et al. (1997)). The use of this approach enables an increase in the number of cloned genes in transformed cell lines by orders of magnitude and leads to increased expression at the protein level. For example, the use of rDNA-based vectors in combination with the MTT1 promoter can be used to drive expression of the endogenous granule lattice protein Grl Ip to approximately 20% of total cell protein (Lin et al. (2002)). Similarly, pD5H8 rDNA-based vectors (Blomberg et al. (1997)) can be used to boost expression of proteins by at least 3-10 fold compared with trans formants in which respective transgenes are integrated at somatic gene loci. Other vectors suitable for use with the methods described here include vectors comprising a ribosomal DNA sequence. Such vectors can replicate at high copy numbers and can be used to deliver a heterologous DNA sequence to *Tetrahymena* spp. for purposes of RNA expression.

[0057] Heterologous Polypeptides

[0058] Suitable heterologous polypeptides for use with these methods include, but are not limited to, antibodies, antibody fragments, cytokines, growth factors, protein

kinases, proteases, protein hormones or any fragment thereof. Similarly, the methods described herein are suitable for the production of specialty proteins. The use of such specialty proteins can include, but is not limited to, prototype vaccines for animal model studies, structural studies, or as therapeutic proteins. For example, quantities of antigens can be produced according to the methods described herein.

[0059] Isolation of Desired Polypeptides from the Mucocyst Matrix

[0060] In one aspect, the invention provides methods for protein purification from the extracellular matrix formed by the discharge of mucocysts. Because heterologous polypeptides targeted to the mucocyst compartment will be associated within the matrix, the invention provides matrix-based purification strategies. Advantageously, the matrix can be used for rapid purification of recombinant polypeptides associated with it.

[0061] Proteins within the gel matrix can be separated from cellular constituents by low-speed centrifugation (See Turkewitz et al. (2000)). Any other method known in the art suitable for separating intact cells, from the discharged material, including, but not limited to filtration harvesting using an appropriately selected mesh, can also be used in conjunction with the methods described herein. After isolation of the matrix, the desired heterologous polypeptide can be liberated from the secreted matrix gel. Methods for liberation of the protein can include chemical methods {e.g., high salt concentrations) and/or enzymatic methods {e.g., site-specific proteases).

[0062] Proteins can also be isolated in intact secretory granules. For example, the use of an exocytosis-defective mutant, MN 173, of *T. thermophile* where granules accumulate in the cytoplasm has been described for such purposes (Melia et al. (1998)).

EXAMPLES

[0063] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Sortilin Gene Control Granule Trafficking of Proteases in *Tetrahymena*

[0064] Initial studies were undertaken to identify genes that are up-regulated upon regranulation in *Tetrahymena* cells. Results shown in FIG. **1** demonstrate that two sortilin genes are among the genes that are dramatically up-regulated during this process. In view of these studies, the *Tetrahymena* genome was analyzed in comparison with genes from other organisms and four *Tetrahymena* sortilin genes were identified (FIG. **2**). The identified genes were SOR1, SOR2, SOR3, and SOR4, corresponding to NCBI accession nos. XM_001033316.2, XM_001020814.3, XM_001025035.2 and XM_001033494.2, each incorporated herein by reference.

[0065] To further determine the function of the SOR genes vectors were constructed to knockout each of the genes *Tetrahymena* (by homologous recombination targeting to the SOR ORFs). The vectors used in the studies are provided as SEQ ID NOs: 9-12, for targeting SOR1, SOR2, SOR3 and SOR4 respectively. Following transformation, knockout cells were successfully isolated for SOR1, SOR2 and SOR4. Studies shown in FIG. **3** confirm that in each case the knockout lines lack detectable expression of the indicated sortilin RNA. Moreover, knockout of the sortilin genes hampered proteolytic processing in the knockout cells. As shown in FIG. **5**, unprocessed forms of Grl1p (pro-Grl1p) were observed in the media of the knockout lines, but not in that of wild type cells or a knockout of the Rab32 gene. Thus the sortilin knockouts result in an inability to effect the normal proteolytic processing of granule proteins.

[0066] Immunefluorescence studies were also performed to visualize granules in knockout and wild type cells. Results, shown in FIG. **5**, demonstrate that wild type granules are elongated, a shape that is generated by the proteolytic processing of the content proteins. The granules in the sortilin knockout lines are spherical, consistent with the failure to proteolytically process the contents.

[0067] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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[0068] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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Glu Asn Thr Asp Gln Leu Ser Phe Thr Ile Leu Lys Ser Asp Gly Ala
305                 310                 315                 320

Met Val Phe Leu Ala Ile His His Glu Thr Gln Asn Met Trp Gln Ser
                325                 330                 335

Asn Ile Tyr Val Ser Asp Trp Arg Gly Tyr Asp Leu Thr Leu Ala Leu
            340                 345                 350

Leu Tyr Asn Val Arg Ala Pro Asn Gly Asp Cys Asp Phe Glu Lys Ile
        355                 360                 365

Glu Ser Asn Glu Gly Val Tyr Ile Ala Asn Thr Tyr Asp Val Glu Lys
    370                 375                 380
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Val Glu Lys Leu Arg Asn Glu Val Lys Lys Met Asp Ile Ser Thr Ala
385                 390                 395                 400

Lys Asn Lys Leu Gln Thr Lys Asp Lys Lys Asn Leu His Lys Glu Leu
                405                 410                 415

Thr Asn Tyr Arg Lys Ser Val Ile Ser Phe Asp Ser Gly Ser Ser Trp
            420                 425                 430

His Pro Ile Arg Ala Pro Ser Gln Arg Trp Asn Gly Lys Thr Val Val
        435                 440                 445

Cys Ser Gly Glu Cys Ser Leu His Leu Ala Gly Arg Thr Tyr Tyr Lys
    450                 455                 460

Lys Ser Gln Met Tyr Ser Ser Ser Asn Ala Pro Gly Leu Ile Val Ala
465                 470                 475                 480

Leu Gly Ser Ile Gly Thr His Leu Glu Asn Asn Phe Asn Leu Leu Asn
                485                 490                 495

Thr Tyr Leu Ser Asn Asp Gly Gly His Gln Trp Arg Glu Ile Leu Lys
            500                 505                 510

Gly Pro His Ile Phe Glu Ile Gly Asp His Gly Gly Ile Ile Val Ala
        515                 520                 525

Ala Ser Val Ala Asn Lys Thr Asn Ile Ile Lys Tyr Ser Trp Asp Glu
    530                 535                 540

Gly Lys Thr Trp Ser Glu Tyr Lys Leu Ser Ala Leu Pro Phe Glu Ile
545                 550                 555                 560

Asp Gln Ile Ile Thr Glu Pro Ser Asn Met Glu Gln Arg Phe Val Val
                565                 570                 575

Tyr Gly Lys Gly Arg Asn Gly Thr Glu Thr Ser Met Ile Val Ser Val
            580                 585                 590

Asp Leu Gln Asp Leu His Ile Arg Gly Cys Val Gly Ala Glu His Pro
        595                 600                 605

Asn Arg Pro Asn Ser Asp Tyr Glu Ile Trp Ile Pro Thr Asn Phe Lys
    610                 615                 620

Gly Glu Gln Cys Ile Phe Gly Arg Lys Val Lys Tyr Val Arg Arg Lys
625                 630                 635                 640

Pro Asp Ala Lys Cys Phe Asn Ser Ile Thr Thr Asp Gln Lys Thr Val
                645                 650                 655

Ile Glu Glu Cys Pro Cys Thr Gln Glu Asp Trp Glu Cys Asp Phe Gly
            660                 665                 670

Phe Tyr Arg Lys Glu Asn Glu Leu Glu Cys Ile Pro Met Asn Glu His
        675                 680                 685

Tyr Ser Pro Asp Asn Leu Ala Lys Pro Pro Ala Asp Cys Ser Trp Ser
    690                 695                 700

Tyr Leu Val Ser Lys Gly Tyr Arg Lys Ile Pro Gly Val Phe Cys Gln
705                 710                 715                 720

Gly Gly Val Asp Leu Ser Pro Glu Tyr Lys Glu Cys Pro Pro Lys Ile
                725                 730                 735

Ser Val Pro Arg Thr Glu Glu Glu Thr Asp Gln Tyr Lys Ser Phe Lys
            740                 745                 750

Glu Ala Gln Lys Glu Ile Ile Ser Gln Tyr Gln Gln Gln Gln Gln Gln
        755                 760                 765

Ser Asn Ser Gln Asn Gly Lys Thr Asp Ser Ser Ser Ser Ile Asn Trp
    770                 775                 780

Gly Val Ile Phe Thr Gln Ile Phe Tyr Ala Gly Leu Ile Leu Thr Ala
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785 790 795 800

Leu Ala Leu Ala Phe Ile Phe Arg Glu Asn Ile Lys Gln Val Val Lys
805 810 815

Ser Ile Gly Glu Ile Gly His Asn Lys Glu Arg Lys Gln Tyr Gln Gln
820 825 830

Leu Gln Ser Ser Gln Asn Lys Gln Ser Ser Tyr Thr Gln Gln Lys Asn
835 840 845

Thr Gln Asn Val Arg Ile Gln Glu Thr Glu Glu Arg Asn Tyr Asp Leu
850 855 860

Glu Glu Gln Asp Met His Tyr Pro Glu Asp Glu Lys Pro Val Leu Gln
865 870 875 880

Arg Asp Gln Glu Asp Tyr Tyr Tyr Gln Glu Asp Tyr Asp
885 890

<210> SEQ ID NO 4
<211> LENGTH: 2682
<212> TYPE: DNA
<213> ORGANISM: Tetrahymena thermophila

<400> SEQUENCE: 4

atgaaaataa aaaggaatta gcaaattgca attatatttg ctattttcat cttgactgct 60

atttaggcag cagatgatgt tgcagatgat aaggtttagt aagctataaa aagttattaa 120

aagtaagtag atggaggtat tttagaattc gagtggtgtg gtacaaatga aatttataac 180

gatgaaactg accgtgttgt tgttgattaa gaagttgaag aatcattcga tactcgtata 240

tttgttctta cagatgaagg ttaagttttt aaaagtacaa actatggtaa aagttgggtc 300

catgtcacta aatcctttta tggttcaaat aattagccat ttttctctac tgaagtttcc 360

atttctcctg ttgatggtaa aacagtctat atttggggac acaaggatac cagctatgtt 420

tctgaggaat gtggtaagac ttggaaaaag ttaaaccatc ctgctggttt gtttgatttt 480

agatttcacc gtaaaaataa aaattgggta ttagctttca ctaatataga atgtaagaga 540

tttgatgaag attgtgaatc taatatgaga aatctttacg tttcttaaga tgcgggtgtt 600

actttcacat tcttagctac taaagtttta gaagcttcat ggaatagaat gaataacttt 660

tacaacgttg acagtcctgg tattttaatg gccgttcaat aagaatcata aagtaatgta 720

gtttacactg aagacttcgg taaaactatg cacacagttt aagaaggtgg tgataatttc 780

ttttaagcag agtacttcct ctttttaaca gttaagccta aaaacagtaa aagaacctat 840

gatatgaaaa tcgcaactat gtttgacgat tttaattact atgttgaacc caaaagctta 900

aagcttccct ttgaaaacac tgattaactt tcgtttacaa ttctaaagag cgatggtgcc 960

atggttttcc ttgccataca ccacgaaact caaaatatgt ggtaaagcaa tatctatgtt 1020

tctgattgga gaggttatga tttgacttta gctttacttt acaatgttag agctccaaac 1080

ggagattgcg actttgaaaa gatagaaagc aatgaaggtg tttatatagc aaatacatat 1140

gatgttgaaa aagttgaaaa attaagaaac gaagttaaaa aaatggatat cagcactgca 1200

aagaataaat tataaacaaa agataaaaag aatttgcaca aagaactaac taattatagg 1260

aaatcagtca tttcatttga cagcggttct agttggcatc caattagagc tccttcatag 1320

agatggaatg gaaagactgt tgtttgcagt ggagaatgca gtttgcattt agctggtaga 1380

acatattata aaaaatctta gatgtattct tcctctaacg ctcctggttt aattgttgca 1440

ttaggaagca ttggaactca tcttgaaaac aacttcaatc ttcttaacac atatctttca 1500

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aacgatggtg gtcactaatg gcgtgaaatt cttaagggtc ctcatatttt tgaaattggt   1560

gatcatggtg gtatcatcgt agctgcttct gttgccaata aaacaaatat catcaaatac   1620

agttgggatg aaggaaaaac atggagcgaa tataaattga gtgctttacc atttgaaata   1680

gattaaataa ttactgagcc tagcaatatg gaacagagat ttgttgttta tggaaaagga   1740

agaaatggaa cagaaacttc tatgattgtt tctgtagatt tataagattt gcacattaga   1800

ggttgtgtag gagctgaaca tcctaataga cctaatagtg attatgaaat ctggattcct   1860

actaatttta aaggtgaaca atgtattttc ggtcgtaaag ttaaatatgt tagaagaaag   1920

cctgatgcaa aatgctttaa ttctatcaca acagattaaa aaacagttat tgaagaatgc   1980

ccatgcacat aagaagattg ggaatgtgac ttcggtttct acagaaaaga aaacgaatta   2040

gaatgtattc caatgaatga gcattattct cctgataatc ttgctaaacc tcctgcagat   2100

tgtagttggt cttacttagt ctcaaaggga tatagaaaaa taccaggagt attttgttaa   2160

ggaggtgttg atttaagtcc agaatataaa gaatgtcctc caaaaatatc agtgcctaga   2220

actgaagaag aaacagatta atataaaagc ttcaaagaag cataaaaaga gattattagc   2280

taatattaat agtaatagta gtaatcaaat agttaaaatg gaaaaactga ttcatcatct   2340

tcaataaact ggggtgttat ttttacataa attttctatg ctggattaat tttaacagct   2400

ttagctttag ctttcatatt tagagagaat atcaaataag tagtaaaaag cattggtgaa   2460

ataggacata ataaagaacg caaataatat taataactct aatcatctta gaataaataa   2520

tcatcataca cttaatagaa aaatactcaa aatgtccgca tttaagaaac tgaagaaaga   2580

aattatgatt tagaagaata agacatgcat tatccagaag atgaaaagcc tgtcttgtaa   2640

agagatcaag aagattacta ttattaagaa gattacgatt ga                      2682
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<210> SEQ ID NO 5
<211> LENGTH: 936
<212> TYPE: PRT
<213> ORGANISM: Tetrahymena thermophila

<400> SEQUENCE: 5

```
Met Lys Lys Glu Ile Arg Ile Ala Leu Ile Ala Leu Phe Cys Cys Ile
1               5                   10                  15

Leu Thr Val Asn Cys Arg Asn Glu Tyr Ser Ser Ser Val Ile Gly Asn
            20                  25                  30

Pro Ser Ser Leu Asp Ser Pro Leu Gln Asp Ile Gln Trp Cys Gly Glu
        35                  40                  45

Asn Ser Ser Asn Asp Asn Leu Val Val Leu Leu Thr Gln Lys Gly Ser
    50                  55                  60

Val Tyr Arg Ser Glu Asp Arg Gly Ala Ser Trp Ile Lys Met Val Asp
65                  70                  75                  80

Ser Phe Ala Arg Val Gly Val Asn Val Lys Met Asp Leu Ser Ser Asn
                85                  90                  95

Val Gly Ile Val Thr Gln Met Ile Ala Ser Pro Ile Asp Ser Asn Glu
            100                 105                 110

Ile Val Phe Met Gly Ser Asp Gly Ile Asn Trp Ile Thr Thr Asp Cys
        115                 120                 125

Gly Val Thr Ile Gln Ala Leu Gly Ile Asn Leu Asn Leu Arg Glu Phe
    130                 135                 140

Met Tyr His Pro Thr Glu Lys Asn Trp Met Leu Ala Ser Ser Phe Asn
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145                 150                 155                 160

Asn Cys Glu Lys Gln Asn Asn Gln Lys Asp Lys Arg Lys Lys Asp Thr
                165                 170                 175

Glu Cys Phe Lys Thr Lys Asp Leu Phe Phe Ser Glu Asn Lys Gly Lys
            180                 185                 190

Ser Trp Arg Val Leu Leu Lys Tyr Val Val Gln Phe Gly Trp Ala His
        195                 200                 205

Lys Val Asn Ser Lys Leu Thr Asn Val Pro Thr Ser Arg Ile Ile Tyr
    210                 215                 220

Ser Lys Glu Val Gly Ser Asn Ser Phe Phe Phe Asn Glu Ala Ser Gln
225                 230                 235                 240

Gln Thr Asn Ile Ile Ile Lys Asp Ser Gly His Gln Val Met Lys Gly
                245                 250                 255

Trp Ser Met Lys Thr His Leu Phe Tyr Thr Asp Asp Phe Met Lys Asn
            260                 265                 270

Gln Asn Met Ile Val Asn Gln Gly Asn Lys Phe Leu Ile Thr Glu Asn
        275                 280                 285

Tyr Leu Phe Ala Ala Gln Val His Ser Ser Asp Asn Gln Leu Val Lys
    290                 295                 300

Leu Met Val Ser Gln Ser Asn Gln Lys Glu Tyr Ser Phe Thr Tyr Ala
305                 310                 315                 320

Glu Ile Pro Glu Asp Ile His Gln His Ser Phe Thr Ile Leu Asp Thr
                325                 330                 335

Lys Glu Gly Gln Val Phe Leu Asn Ile Asn His Leu Gly Ser Asn Ser
            340                 345                 350

Pro Met Gly Asn Ile Tyr Gln Ser Asp Ser Thr Gly Thr Arg Phe Ser
        355                 360                 365

Leu Ser Leu Glu Asp Asn Val Arg Gly Arg Asp Gly Gln Cys Asp Phe
    370                 375                 380

Glu Ser Val Asn Gly Val Glu Gly Ile Phe Ile Ser Asn Ile Phe Ala
385                 390                 395                 400

Pro Ser Lys Lys Leu Lys Gly Ile Lys Gln Met Leu Lys Ser Lys Asn
                405                 410                 415

Pro Asp Thr Ser Asp Glu Asp Ile Pro Thr Glu Asn Thr Arg Lys Lys
            420                 425                 430

Gly Gln Ala Gln Asn Ser Glu Asp Val Leu Lys Glu Ser Leu Lys Ser
        435                 440                 445

Leu Arg Asp Asn Met Val Thr Arg Ile Thr Phe Asp Lys Gly Gly Met
    450                 455                 460

Trp Ser Leu Leu Arg Ala Pro Ala Lys Asp Ser Asn Gly Lys Gln Ile
465                 470                 475                 480

Asn Cys Asp Ile Asn Lys Lys Cys Ser Leu His Leu His Ser Val Ser
                485                 490                 495

Ser Gln Leu Ser Phe Gly Pro Ala Tyr Ser Ser Glu Asn Ser Leu Gly
            500                 505                 510

Leu Ile Ile Ala Thr Gly Asn Thr Gly Gln Phe Leu Ser His Lys Ala
        515                 520                 525

Gly Ser Val Asn Thr Tyr Leu Ser Arg Asp Gly Gly Leu Val Trp Glu
    530                 535                 540

Glu Ile Arg Lys Gly Ser His Ile Tyr Glu Val Ala Asp His Gly Ser
545                 550                 555                 560
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Ile Ile Val Met Ala Thr Asp Gln Glu Pro Thr Lys Asn Ile Ile Phe
                565                 570                 575

Ser Trp Asp Glu Gly Arg Thr Trp Asn Thr Lys Gln Ile Ser Asp Thr
            580                 585                 590

Pro Val Met Ile Ser Asn Ile Ile Thr Glu Pro Gly Asn Thr Ser Asp
        595                 600                 605

Lys Phe Leu Val Tyr Gly Ser Ile Glu Gly Glu Ser Asp Ile Ser Gly
    610                 615                 620

Ile Ile Val Leu Leu Asp Phe Ala Ser Leu His Pro Arg Asp Cys Gln
625                 630                 635                 640

Gly Tyr Glu Asn Pro Asp Thr Ser Asp Ser Asp Tyr Glu Tyr Trp Thr
                645                 650                 655

Pro His Asn Pro Ser Glu Phe Cys Leu Leu Gly Arg Glu Ile Lys Tyr
            660                 665                 670

Val Arg Arg Lys Arg Asp Ala Ala Cys Phe Asn Pro Glu Thr Phe Glu
        675                 680                 685

Arg Ser Tyr Val Val Arg Lys Cys Glu Cys Thr Glu Leu Asp Trp Glu
    690                 695                 700

Cys Asp Val Gly Phe Ala Arg Ala Lys Asp Asp Ser Lys Glu Arg Thr
705                 710                 715                 720

Gly Pro Cys Val Pro Leu Lys Asp Phe Lys Val Asp Tyr Asn Pro Pro
                725                 730                 735

Gln Thr Cys Ser Gly Ser Tyr Gln Val Thr Gln Gly Tyr Arg Arg Val
            740                 745                 750

Ala Gly Asn Gln Cys Ile Gly Gly Ile Asp His Ala Pro Ile Gln Tyr
        755                 760                 765

Pro Cys Pro Met Phe Gly Phe Leu Ser Tyr Asn Asn Leu Phe Thr Asn
    770                 775                 780

Val Leu Ile Leu Gly Ala Met Ala Gly Val Phe Tyr Leu Ile Ile Gln
785                 790                 795                 800

Asn Lys Glu Val Val Ile Thr Phe Val Ala Thr Ser Asn Leu Asp Ala
                805                 810                 815

Tyr Ile Asn Leu Gly Lys Thr Tyr Leu Lys Lys Gly Tyr Thr Phe Val
            820                 825                 830

Thr Ser Ile Val Leu Pro Gln Ala Ser Asn Gln Gln Gln Gly Tyr Phe
        835                 840                 845

Gln Ala Asn Gln Asp Glu Glu Asn Arg Lys Ser His Ser Leu Lys Asp
    850                 855                 860

Gln His His Gln Phe His Asp Asn Leu Ile Glu Ser His Asp His Asp
865                 870                 875                 880

Asp Glu Glu Glu Gln Ser Asp Ala Val Gln Gln Gln Leu Thr Ser Ser
                885                 890                 895

Gln Val Pro Gln Asn Asn Ser Asn Lys Asn Asn Asn Asn Ser Asn Thr
            900                 905                 910

Pro Asn Gln Ala Gln His Lys Asp Leu Leu Asp Glu His Asp Gly Glu
        915                 920                 925

Glu Asp Pro Phe Asp Pro Arg Asn
    930                 935


<210> SEQ ID NO 6
<211> LENGTH: 3010
<212> TYPE: DNA
<213> ORGANISM: Tetrahymena thermophila
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<400> SEQUENCE: 6

atgaaaaaag aaataagaat agctcttata gctttatttt gctgcatttt gacagtaaat 60

tgtagaaatg aatactcaag cagtgtcatt ggaaacccct caagtttgga ttcacctctt 120

taggacattt aatggtgtgg tgaaaattca tcaaatgata atttggttgt cctcttaact 180

taaaagggta gcgtttacag atcagaagat agaggagcat cttggataaa gatggttgac 240

tcttttgcga gagttggtgt aaatgtaaag atggatctga gctcaaacgt aggtattgtt 300

acttaaatga ttgcaagtcc tattgattct aatgaaatag tctttatggg ctctgatggt 360

attaactgga tcactactga ttgtggtgtt accatttaag cccttggaat caacttaaat 420

ttgagagaat ttatgtatca cccaactgaa aagaattgga tgcttgcttc ttcctttaac 480

aactgtgaaa agcaaaacaa ccaaaaagat aagagaaaaa aggacactga atgctttaag 540

actaaagatt tgtttttctc tgaaaataag ggtaaaagct ggagagtttt acttaaatat 600

gttgtacaat tcggatgggc tcacaaagtt aattctaagc taacaaatgt cccaacttca 660

agaattatat actctaagga agtcggaagt aattcgtttt tctttaatga agcatctcaa 720

taaactaata taataataaa agatagtggt caccaagtga tgaagggttg gagcatgaaa 780

actcatttat tctatactga tgatttcatg aaaaactaga atatgattgt taactaagga 840

aataagtttt tgattactga aaactacttg ttcgctgcat aagttcacag tagtgataat 900

taactagtca agttaatggt ttcttaatct aattaaaaag aatactcttt cacttatgct 960

gaaattcctg aagatataca ctagcactca ttcactattt tagatactaa ggaaggttag 1020

gtattcttaa atattaatca cttgggcagt aactctccta tgggtaatat ttactaatct 1080

gactcaactg gtactcgttt ctctctttct cttgaagata atgtaagagg aagagatggt 1140

taatgcgatt ttgaatcagt taatggtgtt gaaggtattt ttatctcaaa tatattcgct 1200

cctagcaaaa agttaaaggg tatcaagcaa atgttgaaat ccaaaaatcc tgatacaagc 1260

gatgaagata ttccaactga aaacacaaga aagaaaggtc aagcataaaa ttctgaagat 1320

gtcttaaaag aatccttaaa aagtcttaga gataacatgg taactcgtat cactttcgac 1380

aagggtggta tgtggagttt gcttagggct cctgctaaag attctaatgg aaaataaatt 1440

aattgtgata ttaataaaaa gtgttctctt caccttcact cagtttcttc ataactaagt 1500

tttggacctg cttactcaag tgaaaattca ttaggtttaa ttattgctac tggtaacaca 1560

ggataattct taagtcataa agcaggtagc gtcaacactt atctttctcg tgatggtggt 1620

cttgtttggg aagaaatccg taaggggtct cacatatatg aagttgctga tcatggctct 1680

atcatagtta tggctactga ttaagaacct actaagaaca ttattttctc ttgggatgaa 1740

ggccgcacat ggaacaccaa gtaaattagc gatactcctg tcatgatttc aaatattatc 1800

actgaacctg gcaatacttc tgacaagttc ttagtttatg gatctattga aggtgaatct 1860

gatatttcag gaataattgt ccttcttgac tttgcttctc ttcatcctcg cgattgctaa 1920

ggttatgaaa accctgacac ttctgattct gattatgaat actggactcc tcataatccc 1980

agtgaattct gtttattagg acgtgaaatt aaatatgtca gaagaaaaag agatgctgct 2040

tgctttaatc ccgaaacttt tgaaagatct tatgttgtta gaaaatgtga atgtactgaa 2100

cttgattggg aatgtgatgt cggatttgct cgtgctaaag acgatagcaa agaaagaact 2160

ggcccttgcg ttcctttaaa agacttcaaa gtggattaca atcctccata aacttgcagt 2220

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ggctcttacc aagttacata aggttacaga agagtagctg gtaattaatg tataggcggt   2280

attgatcatg ctccaattta atacccttgt cctatgtttg gcttcttgag ctataacaac   2340

cttttcacca atgttcttat tttaggagct atggctggtg ttttctactt aattatataa   2400

aataaagaag tagtaataac atttgtagct acatcaaatc ttgatgccta cattaactta   2460

ggtaaaactt acctaaagaa gggttatact tttgttacat caattgtcct tccacaagct   2520

tcaaattaat aataaggata tttccaagct aaccaagatg aggaaaatag aaaatctcat   2580

tccttaaagg atcaacatca ttaattccat gataatttaa ttgaaagcca tgatcatgat   2640

gatgaggaag agtaaagtga tgcagtataa taataattaa cttcttctta agtcccttaa   2700

aataatagta acaaaaacaa taataatagt aatacaccaa actaagctca gcacaaagat   2760

cttcttgatg aacatgatgg tgaagaagat ccttttgatc ctagaaattg aaaaataatt   2820

gactgaataa tattgctaat ttatttttt acttaaataa taaataaata aaaataaata   2880

aattaatttt tgtctttcat taatattatt tagaaagttt ttctaagtaa tttaatatag   2940

tgtgtcaagt atctttttct cttaacttat gtattttatc aaatcctttt ttactttatt   3000

attcctagtt                                                          3010
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<210> SEQ ID NO 7
<211> LENGTH: 872
<212> TYPE: PRT
<213> ORGANISM: Tetrahymena thermophila

<400> SEQUENCE: 7

```
Met Lys Lys Gln Asp Leu Thr Val Tyr Val Ala Ala Phe Leu Leu Leu
1               5                   10                  15

Phe Ser Cys Val Ile His Phe Ala Asn Ala Gln Asp Lys Val Ser Glu
            20                  25                  30

Ile Phe Lys Asp Lys Tyr Asp Val Lys Tyr Arg Val Thr Glu Leu Asp
        35                  40                  45

Ser Pro Val Gln Glu Ile Leu Trp Cys Gly Ser Ser Gln Ala Thr Ser
    50                  55                  60

Glu Asp Gly Asp Ile Ile Thr Tyr Asp Gln Thr Ala Lys Val Arg Lys
65                  70                  75                  80

Leu Tyr Val Leu Thr Asp Lys Gly Lys Leu Tyr Tyr Ser Glu Asp Tyr
                85                  90                  95

Gly Ile Thr Leu Lys Leu Ile Asn Asp Asp Ile Arg Gln Ser Thr Asn
            100                 105                 110

Ser Lys Gln Thr Gln Val Glu Val Asp Asp Ile Met Ile Ser Pro Val
        115                 120                 125

Lys Asn Arg Lys Val Phe Ile Phe Thr Lys Ser Gly Glu Ser Tyr Tyr
    130                 135                 140

Thr Glu Asn Cys Gly Ala Thr Tyr Thr Ser Phe Lys His Glu Ile Leu
145                 150                 155                 160

Leu Tyr Asp Ile Gln Pro Asn Pro Ser Asp His Lys Ser Leu Ile Gly
                165                 170                 175

Leu Val Pro Val Gln Cys Gln Lys Gly Asp Pro Glu Cys Gln Gly Gly
            180                 185                 190

Asp Ser Asp Leu Tyr Leu Thr Val Asp Ser Gly Met Thr Trp Arg Lys
        195                 200                 205

Ile Val Ser Asn Val Asn Gln Ala Gln Trp Asp Lys Thr Lys Gln Thr
    210                 215                 220
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Leu Met Asn Thr Gln Asn Arg Ile Ile Leu Ser His Gln Glu Gln Glu
225                 230                 235                 240

Lys Asn Glu Lys Gly Glu Asn Val Phe Leu Asn Lys Val Ser Tyr Thr
                245                 250                 255

Asp Asn Tyr Gly Lys Asp Leu Lys Val Val Glu Lys Asn Gly Val Arg
            260                 265                 270

Phe Tyr Gln Thr Glu Glu Tyr Ile Phe Val Leu Ile Gln Gly Lys Glu
        275                 280                 285

Phe Gly Lys Tyr Lys Leu Asn Ile Gly Pro Ser Phe Val Thr Gln Ser
    290                 295                 300

Ser Ser Arg Lys Glu Ile Asp Leu Pro Leu Gln Arg Val Lys Asp Glu
305                 310                 315                 320

Ser Phe Thr Val Leu Asp Ile Asp Ala Gly Gln Ile Leu Ile Ala Ile
                325                 330                 335

Asn His Glu Gly Asp Ser Ala Gly Tyr Thr Asn Val Tyr Ile Ser Asn
            340                 345                 350

Ser Gln Gly Glu Gln Phe Thr Leu Ser Leu Gln Tyr Thr Val Gly Asp
        355                 360                 365

Asp Asp Ser Asn Ile Asp Phe Glu Pro Ile Asn Ser Asn Glu Gly Val
    370                 375                 380

Tyr Ile Ala Asn Thr Tyr Thr Ala Ala Ser Ile Ser Lys Tyr Gln Lys
385                 390                 395                 400

Leu Leu Gln Arg Lys Glu Gly Gln Lys Ser Ser Gly Ser Ser Leu Thr
                405                 410                 415

Leu Asp Ser Phe Lys Ile Glu Asn Met Lys Lys Thr Lys Ile Thr Phe
            420                 425                 430

Asn Lys Gly Gly Asp Trp His Ala Ile Lys Ala Pro Glu Phe Asn Tyr
        435                 440                 445

Ala Gly Asn Pro Ile Arg Cys Ser Gly Asp Cys Ser Leu Asn Phe Lys
    450                 455                 460

Gly Arg Thr Glu Ser Gln Gly Thr Pro Val Tyr Ser Thr Asp Asn Ala
465                 470                 475                 480

Pro Gly Ile Ile Leu Ala Thr Gly Asn Val Gly Ser Tyr Leu Thr Asn
                485                 490                 495

Asn Gln Asp Glu Leu Arg Thr Tyr Leu Ser Ile Asp Gly Gly His Thr
            500                 505                 510

Trp Lys Glu Ile Gln Val Gly Ser His Glu Tyr Glu Ile Gly Asp Gln
        515                 520                 525

Gly Gly Ile Ile Ala Met Ala Arg Asp Asp Lys Leu Thr Asn Glu Val
    530                 535                 540

Ile Tyr Ser Val Asp Glu Gly Glu Thr Trp Arg Lys Leu Asn Phe Lys
545                 550                 555                 560

Asp Glu Asn Lys Phe Lys Val Asp Ser Phe Val Thr Glu Glu Gly Asn
                565                 570                 575

Asp Glu Arg Thr Phe Leu Phe Tyr Gly Thr Lys Thr Gly Ala Asp Gly
            580                 585                 590

Asn Thr Lys Gly Val Ile Gly Ala Ile Asn Phe Ser Asn Leu Phe Lys
        595                 600                 605

Lys Glu Cys Thr Gly Phe Glu Asn Pro Gly Glu Asp Gly Ser Asp Tyr
    610                 615                 620

Glu Arg Trp Val Pro Leu Asn Phe Glu Gly Lys Lys Cys Leu Phe Gly
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625 630 635 640

Ser Lys Ile Ser Tyr Ile Arg Lys Lys Thr Asp Ser Ser Cys Phe Asn
645 650 655

Asn Arg Lys Val Gly Asp Leu Arg Met Val Gln Gly Ser Cys Glu Cys
660 665 670

Thr Glu Glu Asp Phe Glu Cys Asp Tyr Gly Phe Thr Lys Asp Leu Ile
675 680 685

Asp Glu Thr Lys Cys Val Pro Ile Asn Ala Lys Phe Ala Lys Lys Arg
690 695 700

Asp Gln Pro Pro Leu Asn Cys Lys Asp Phe Tyr Phe Val Ser Ser Gly
705 710 715 720

Lys Arg Lys Ile Ala Asn Asn Gln Cys Gln Gly Gly Ile Glu Glu Leu
725 730 735

Tyr Thr Lys Lys Lys Val Arg Cys Pro Gly Asn Glu Glu Ala Gln Gln
740 745 750

Thr Gln Gln Gln Thr Gln Asn Thr Gln Ala Asn Thr Ala Gln Asn Asn
755 760 765

Gln Gln Asp Leu Phe Ser Arg Lys Pro Glu Asp Ile Lys Lys Glu Ile
770 775 780

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ccaccgctgg tagcggtggt ttttttgttt gcaagcagca gattacgcgc agaaaaaaag   5400

gatctcaaga agatcctttg atcttttcta cggggtctga cgctcagtgg aacgaaaact   5460

cacgttaagg gattttggtc atgagattat caaaaaggat cttcacctag atccttttaa   5520

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tgactggtga gtactcaacc aagtcattct gagaatagtg tatgcggcga ccgagttgct   6180

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ggaaatgttg aatactcata ctcttccttt ttcaatatta ttgaagcatt tatcagggtt   6480

attgtctcat gagcggatac atatttgaat gtatttagaa aaataaacaa atagggggttc   6540

cgcgcacatt tccccgaaaa gtgc                                          6564
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1. A genetically altered ciliate wherein the ciliate lacks detectable expression of one or more SOR gene product corresponding to SOR1 (XM_001033316.2), SOR2 (XM_001020814.3), SOR3 (XM_001025035.2), or SOR4 (XM_001033494.2).

2. The ciliate of claim **1**, wherein the ciliate lacks detectable expression of a polypeptide corresponding to a SOR1. SOR2, SOR3, or SOR4 polypeptide.

3. The ciliate of claim **1**, wherein the ciliate comprises a genomic alteration.

4. The ciliate of claim **3**, wherein the ciliate comprises a deletion in both copies of the germline genome that disrupts expression of a SOR gene product.

5. The ciliate of claim **3**, wherein the ciliate comprises an insertion in both copies of the germline genome that disrupts expression of a SOR gene product.

6. The ciliate of claim **5**, wherein the insertion is located in the open reading frame of a gene corresponding to SOR1, SOR2, SOR3, or SOR4.

7.-**8**. (canceled)

9. The ciliate of claim **1**, wherein the ciliate lacks detectable expression of the gene product corresponding to 2, 3, or 4 of the SOR 1, SOR2, SOR3, or SOR4 genes.

10. The ciliate of claim **1**, wherein the ciliate expresses a polynucleotide complementary to all or part of an RNA gene product corresponding to SOR1, SOR2, SOR3 or SOR4.

11. The ciliate of claim **10**, wherein the polynucleotide molecule complementary to all or part of an RNA gene product is an antisense RNA or a double stranded RNA (dsRNA).

12.-**14**. (canceled)

15. The ciliate of claim **1**, wherein the ciliate is *Tetrahymena*.

16. (canceled)

17. A recombinant *Tetrahymena* germline genome comprising a genomic insertion or deletion in both copies of one or more SOR genes selected from the group consisting of SOR1, SOR2, SOR3, and SOR4.

18. The recombinant genome of claim **17**, wherein the genomic insertion or deletion is located in the open reading frame of the gene.

19. (canceled)

20. The recombinant genome of claim **17**, wherein the genome comprises a genomic insertion or deletion in both copies of 2, 3, or 4 SOR genes corresponding to SOR1, SOR2, SOR3 or SOR4.

21.-**27**. (canceled)

28. A method of producing a genetically altered ciliate comprising:

(a) transforming a ciliate with a polynucleotide comprising a sequence complementary to a SOR gene corresponding to SOR1, SOR2, SOR3 or SOR4; and

(b) isolating a genetically engineered ciliate wherein the ciliate lacks detectable expression of the gene product of said SOR gene.

29. The method of claim **28**, wherein step (b) comprises isolating a genetically engineered ciliate comprising an insertion or deletion in a SOR gene.

30.-**32**. (canceled)

33. A method of producing a polypeptide comprising:

(a) expressing a polynucleotide encoding the polypeptide in a ciliate of claim **1**; and

(b) incubating the ciliate in a media under conditions permissible for expression of the polypeptide.

34.-**39**. (canceled)

40. The method of claim **33**, further comprising:

(c) purifying the expressed polypeptide from the media.

41. The method of claim **33**, wherein the majority of the polypeptide is not secreted by the ciliate.

42. The method of claim **41**, further comprising:

(c) purifying the ciliate from the media.

43. The method of claim **42**, further comprising:

(d) purifying the protein from the ciliate.

44. (canceled)

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