

(51) International Patent Classification:
C12Q 1/682 (2018.01)

(21) International Application Number:
PCT/EP2024/066358

(22) International Filing Date:
13 June 2024 (13.06.2024)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2308860.2 14 June 2023 (14.06.2023) GB

(71) Applicant: **WAGENINGEN UNIVERSITEIT** [NL/NL];
Droevendaalsesteeg 4, 6708 PB Wageningen (NL).

(72) Inventors: **STEENS, Aiko Jurre**; Droevendaalsesteeg
4, 6708 PB Wageningen (NL). **OOST, VAN DER,
John**; Droevendaalsesteeg 4, 6708 PB Wageningen (NL).
STAALS, Raymond Hubert Josèphe; Droevendaalses-
teeg 4, 6708 PB Wageningen (NL).

(74) Agent: **HGF LIMITED**; 1 City Walk, Leeds West York-
shire LS11 9DX (GB).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG,
KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY,
MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA,
NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO,
RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH,
TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS,
ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, CV,
GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST,
SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ,
RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ,
DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT,
LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE,
SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: METHOD AND SYSTEM OF NUCLEIC ACID SEQUENCE DETECTION

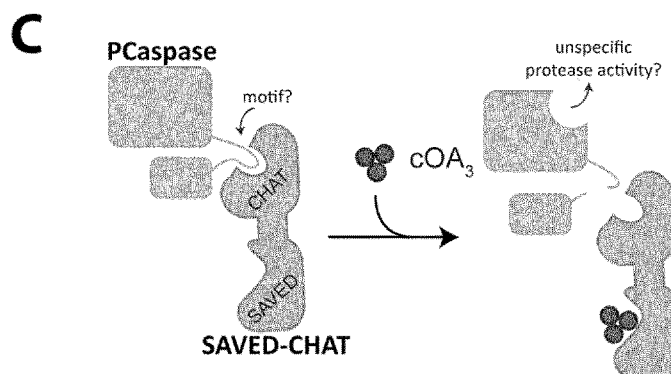


FIGURE 1

(57) Abstract: A type III-B system from *Haliangium ochraceum* contains two caspase-like proteases, SAVED-CHAT and PCaspase (prokaryotic caspase). Cyclic tri-adenosine monophosphate (AMP)-induced oligomerization of SAVED-CHAT activates proteolytic activity of the CHAT domains, which specifically cleave and activate PCaspase. Subsequently, activated PCaspase cleaves a multitude of proteins, which results in a strong interference phenotype in vivo in *Escherichia coli*. This provides for a CRISPR-Cas-based method of detecting a target RNA, whereby detection signalling is triggered via a cascade of caspase-associated proteolytic activities. Polynucleotides, plasmids, vectors and nucleoprotein complexes comprising nucleic acids encoding the components of the system are provided.

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

METHOD AND SYSTEM OF NUCLEIC ACID SEQUENCE DETECTION

FIELD OF THE INVENTION

[0001] The invention described herein is directed to a CRISPR/Cas-related nucleic acid
5 detection system and its broad use in diagnostic applications.

BACKGROUND

[0002] Since their discovery more than a decade ago, the intensity of research has revealed a great number and variety of CRISPR-Cas systems within the kingdoms of bacteria and archaea (Fenner *et al.*, 2007. J Biomol Screen 20: 1027-1039; van der Oost
10 *et al.*, 2009. Trends Biochem Sciences 34: 401—407). A categorization of CRISPR/Cas systems has been made. Class I systems utilize multisubunit Cas complexes, whereas the class II systems use only a single Cas protein to mediate its activity. Different types are generally characterized based on the presence of signature genes (Wright *et al.*, 2016. Cell 164: 29-44).

15 [0003] CRISPR-Cas nucleic acid detection diagnostics offer a solution for setting up a decentralized screening platform. CRISPR-Cas based diagnostics have been claimed to be faster than PCR, and cheaper to perform on site (see Sheridan C. (2020) Nature Biotechnology 38: 382 - 384).

[0004] Of the CRISPR systems, class 2 systems have primarily been applied for
20 diagnostics, as these systems are simpler to reconstitute. They include enzymes with collateral activity, which serves as the backbone of many CRISPR-based diagnostic assays. Chen J S *et al.* (2018) Science 360:436 – 439 describe a CRISPR-Cas diagnostic tool named DNA endonuclease-targeted CRISPR trans reporter (DETECTR). This method depends on the collateral activity of a Cas12a protein, activated after recognition of target
25 RNA by the Cas12a. The particular Cas12a protein from *Lachnospiraceae bacterium* strain ND2006 (LbCas12a) has nonspecific collateral activity and degrades all adjacent DNA molecules after recognizing a desired target RNA. The DNA molecules are set up as fluorescent reporter probes and stable and strong fluorescent signal can be generated. Additionally, DETECTR has been combined with an isothermal preamplification step to
30 enrich target sequences (RPA).

[0005] CRISPR defence can be described as a process consisting of three stages: adaptation, expression, and interference (Rath *et al.*, 2015. Biochimie 117: 119— 128; Makarova *et al.*, 2011. Nat Rev Microbiol 9: 467—477). During the adaptation stage, genetic fragments are acquired from foreign invading entities and stored in the CRISPR
35 memory (Jackson *et al.*, 2017. Science 356: eaal5056). This memory comprises the

foreign DNA sequences, called spacers, which are separated by repetitive DNA sequences (repeats). Expression of the CRISPR locus (stage II) leads to transcription of a long RNA molecule which is subsequently processed into multiple CRISPR RNAs (crRNAs) (Brouns *et al.*, 2008. Science 321: 960-964). Additionally, the cell expresses

5 Cas proteins, the effectors of the CRISPR/Cas system, which form ribonucleoprotein (RNP) complexes by incorporating the crRNAs.

[0006] Kellner M J *et al.* (2020) Nature Protocols 15 1311 describes SHERLOCK which is a diagnostic tool based on a CRISPR-Cas type VI system. SHERLOCK is in principle similar to DETECTR, but uses a Cas13 nuclease from *Leptotrichia wadei*. This Cas13

10 specifically recognizes and cleaves RNA and not DNA. RNA fragments, Cas13 protein crRNA and fluorescent RNA probes are mixed together, and where target RNA is present in the sample, Cas13 recognizes them via crRNA and collateral activity cleaves the fluorescent RNA probes, releasing the fluorophore from the quencher and allowing a signal to be read. Intensity of signal can show the quantity of RNA target in the sample. *In vitro*

15 transcription of the sample can adapt the test to recognise DNA targets and isothermal amplification by RPA can be used to enrich target molecules and increase sensitivity.

[0007] Type III CRISPR-Cas systems are adaptive immune systems in bacteria and archaea. These systems use CRISPR-derived RNA (crRNA) guides to target complementary nucleic acids of invading viruses and plasmids. Interestingly, type III

20 systems have many unique features, including a rapidly expanding network of signal transduction pathways to trigger dormancy and cell death (see van Beljouw, S. P. B., *et al.*, (2022) Nat. Rev. Microbiol. 21: 21 – 23; Steens, J. A., *et al.* (2022) Biochem. Soc. Trans. 50: 1353–1364 (2022); and Steens, J. A., *et al.*, (2022) Mol. Cell 82: 4405 – 4406.

[0008] A typical type III operon encodes multiple Cas proteins that form a type III effector

25 complex together with the mature crRNA. These complexes will bind complementary target RNA sequences, which initiate at an exposed seed region at the 3' end of the crRNA guide. Seed binding initiates complete base pairing between the target RNA and the crRNA, resulting in the activation of Cas10, the characteristic multidomain subunit of the type III complex (see Steens, J. A. *et al.* (2021) Nat. Commun. 12: 1–12. The HD domain

30 of activated Cas10 degrades ssDNA substrates in a non-sequence specific manner, whereas its Palm domain acts as a cyclase to convert ATP into signalling molecules called cyclic oligoadenylates (cOA) of 3-6 AMP moieties (see Elmore, J. R. *et al.*, (2016) Genes Dev. 30: 447–459; Estrella, M. A., *et al.*, (2016) Genes Dev. doi:10.1101/gad.273722.115; Kazlauskienė, M., *et al.*, (2016) Mol. Cell 62: 295–306; Niewoehner, O. *et al.* (2017) Nature

35 548: 543–548; Kazlauskienė, M., *et al.*, (2017) Science 80(357): 605–609). These cOA signalling molecules activate a particular set of effector proteins carrying an appropriate

cOA binding domain: CARF and SAVED proteins. These sensory domains are generally fused to a wide range of catalytic domains, e.g. RNases, DNases and nickases, NADases and toxins (see Makarova, K. S., *et al.*, (2014) *Front. Genet.* 5: 1–9 and Makarova, K. S. *et al.* (2020) *Nucleic Acids Res.* 48: 8828–8847. Over the last years, a handful of these

5 CARF and SAVED proteins have been characterized, and despite their different activities, they all are geared towards killing the host, stopping the spread of the invading nucleic acid (e.g., phage progeny, plasmid propagation, etc.) in a process known as abortive infection. Recent work on type III systems indicated that proteases also play a role in type

10 type III-E complex, and a cOA-activated Lon-like protease (CalpL) in a type III-B system (see van Beljouw, S. P. B. *et al.* (2021) *Science* 373: 1349–1353 and Rouillon, C., *et al.*, (2022) *Nature* 1–23 doi:10.1038/s41586-022-05571-7). More particularly, Rouillon C R *et al.* (2018) *eLife* e36734 DOI: <https://doi.org/10.7554/eLife.36734> reports on the study of control of the type III CRISPR system from the thermophile *Sulfolobus solfataricus*. This

15 type III CRISPR responds immediately to viral RNA in the cell to cleave it with a concomitant production of cOA which is tightly linked to the abundance of viral RNA. The cOA amplifies the cell's response by activating a CARF-domain nuclease Csx1 which results in more general RNA degradation. Rouillon *et al.* suggest that a better understanding of type III CRISPR systems could be used to improve the yield of products,

20 like yoghurt, that depend on healthy bacteria. Also suggested is that further research in this area could also lead to new antibiotics that over-activate type III CRISPR to destroy bacterial cells.

[0009] Niewoehner O *et al.*, (2017) *Nature* 548: 543 – 548 describes CRISPR-associated protein Csm6 in prokaryotes and its function as a standalone RNase that degrades invader

25 RNA transcripts. The authors show how Csm6 proteins are activated through a second messenger generated by a type III interference complex. Upon target RNA binding by the interference complex, the Cas10 subunit converts ATP into a cyclicoligoadenylate which then activates the RNase activity of the Csm6.

[0010] Hochstrasser M L *et al.* (2021) *Nature Microbiology* 6 1481 – 1482 describes a

30 fusion of what is usually a multisubunit CRISPR complex in prokaryotes, into a single protein, which cleaves RNA and interacts with ancillary PCaspase protease (TPR-CHAT/Csx29) and which may trigger cell death or dormancy.

[0011] Hu C *et al.* (2022) *Science* 377: 1278 – 1285 describes a CRISPR-Cas type III-E RNA-targeting effector complex gRAMP/Cas7-11 which associates with a caspase-like

35 protein (TPR-CHAT/Csx29) to form a CRISPR-guided caspase (Craspase). The authors use cryo-electron microscopy to work out a mechanism of action whereby target-guide

pairing extends into the 5' region of the guide RNA and displaces a gating loop in the Type III-E gRAMP (Cas7 – 11), which triggers an extensive conformational relay that allosterically adjusts the protease activity. Csx30 is described as being an endogenous protein substrate that is site-specifically cleaved by the RNA-activated Craspase. This protease activity is switched off with a target RNA cleavage by gRAMP and is not activated by RNA targets containing a matching protospacer flanking sequences. Thus the Craspase is proposed as a target RNA-activated protease with self-regulating activity.

[0012] Recently, certain Type III CRISPR/Cas systems have been used in developing diagnostic systems. WO2020/256553 A1 (Wageningen Universiteit) discloses a Type III CRISPR/Cas-related ribonucleic acid detection system and its use in diagnostic applications. The detection system comprises (a) an effector complex comprising a Type III CRISPR-associated effector protein (Cas) and at least one CRISPR RNA (crRNA) that binds to a target nucleic acid molecule, and (b) means for directly or indirectly determining a level of cyclic oligoadenylate (cOA). The detection system is a Type III Cas, a Type IIIB Cas or a Type IIIB Cmr. The Type III Cas is preferably from a thermophilic organism such as *Thermus thermophilus*. The level of cOA is detected by using an inorganic pyrophosphatase which releases pyrophosphate (PPi) which is then measured by a colorimetric-, fluorometric-, fluorescent- or bioluminescent-based assay. The pyrophosphatase can be chosen to be from a thermophilic organism such as *Thermus thermophilus* and this conveniently allows for an isothermal reaction when used with a preferred Type III CRISPR/Cas system. Another way of detecting the level of cOA is to use a cOA-dependent, non-specific effector endoribonuclease such as Csx1, and a detectable substrate for said endoribonuclease. Also disclosed are devices and kits for performing methods of ribonucleic acid detection.

[0013] Steens J A *et al.*, (2022) Biochemical Society Transactions 50: 1353 – 1364 is a review article that considers the range of CRISPR-associated Rossmann fold (CARF) and second messenger oligonucleotide or dinucleotide synthetase-associated and fused to various effector domains (SAVED) effectors which may be found associated with type III CRISPR systems. A non-nuclease CARF protein associated with type III immunity is speculated about by the authors as a result of some bioinformatic analysis. Suggested is the possibility of a CARF-Lon fusion protein, wherein the Lon domain is active in a family of housekeeping proteases. Further, the authors speculate that a CARF-Lon protein might be activated by cOA towards an aggressive host killing activity.

[0014] Another non-nuclease protein associated type III immunity described by Steens J A *et al.*, (2022) is a Lon-SAVED effector which contains a C-terminal SAVED sensing domain, consisting of two CARF-like domains, fused to a N-terminal Lon protease domain.

A cOA4 messenger molecule binds to the protein and induces an allosteric change that activates it to cleave a specific target protein, CRISPR-T. The 32 kDa CRISPR-T protein is cleaved by activated CRISPR-Lon into two fragments (~23 and ~10 kDa). The ~23 kDa fragment bears structural similarity to MazF, which is known to be a toxin known that
 5 cleaves specific rRNA, mRNA and tRNA molecules, leading to abortive infection

[0015] Makarova K. S. et al (2020) Nucleic Acids Research 16: 8828 – 8847 is survey and review about the Crispr-associated Rossmann Fold (CARF) domain superfamily of proteins, which includes SMODS-associated and fused to various effector domains (SAVED). These proteins are key components of cyclic oligonucleotide-based antiphage
 10 signalling systems that sense cyclic oligonucleotides and transmit the signal to an effector inducing cell dormancy or death. There are 10 major families of CARF domains and multiple smaller groups that differ in structural features.

BRIEF SUMMARY OF THE DISCLOSURE

[0016] In accordance with the present invention there is provided a clustered regularly
 15 interspaced short palindromic repeats (CRISPR) based nucleic acid sequence detection system comprising:

(a) a type III CRISPR-associated effector protein complex which when comprised with a targeting RNA substantially complementary to a desired target polynucleotide sequence recognises that target sequence in a nucleic acid molecule and cleaves the
 20 nucleic acid molecule and produces a cyclic oligoadenylate (coA_x) messenger;

(b) a cyclic oligoadenylate (coA_x)-dependent protease; and

(c) a protein or polypeptide susceptible to cleavage by the protease of (b) and from which a detectable signal is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (b).

[0017] In preferred aspects, a detection system of the invention further comprises the
 25 targeting RNA which is substantially complementary to the desired target polynucleotide.

[0018] In detection systems of the invention, the coA_x-dependent protease may be a ssecond messenger oligonucleotide or dinucleotide synthetase-associated and fused to various effector domains-caspase HetF associated with Tetratricopeptide repeat (TPR)
 30 (SAVED-CHAT) protein; or a CHAT-SAVED protein.

[0019] The SAVED-CHAT protein may have an amino acid sequence of SEQ ID NO: 14, or a sequence of at least 70% identity therewith, or a functional fragment thereof.

[0020] The SAVED-CHAT protein may, independently or additionally to the aforementioned reference sequence or variants or fragments thereof, may comprise one or

more of the following amino acid (peptide) motifs in any combination, wherein X is any amino acid:

GX(V/A)X(I/L)PXXX(H/Y)LGXXL [SEQ ID NO: 16]

(P/N)XAXXXDXXXL(R/E)XXXPX(I/V)(V/L)HXX(S/G)HG [SEQ ID NO: 17]

5 DXX(A/S)XXF(S/A)XX(F/L)YXXLXXXXX(I/V)XXAF [SEQ ID NO: 18]

[0021] The SAVED-CHAT protein may comprise an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6, or a sequence of at least 70% identity with any of SEQ ID Nos 1 – 6, or any functional fragment thereof.

10 **[0022]** The CHAT-SAVED protein may have an amino acid sequence of SEQ ID NO: 15, or a sequence of at least 70% identity therewith, or a functional fragment thereof.

[0023] The CHAT-SAVED protein may, independently or additionally to the aforementioned reference sequence or variants or fragments thereof, may comprise one or more of the following amino acid (peptide) motifs in any combination:

15 LRLVVLXACX [SEQ ID NO: 19]

IPAVIASQMPLS [SEQ ID NO: 20]

XPLPLAXXXGX [SEQ ID NO: 21]

IHLFLAXPXSXAX- [SEQ ID NO: 22]

wherein X is any amino acid, wherein underlined amino acids are not substituted, and
20 wherein specified amino acids may independently be substituted conservatively with another amino acid, as herein defined.

[0024] As used herein, in relation to any protein or polypeptide component, a "functional fragment" or "active fragment" refers to a protein or polypeptide fragment that substantially retains the native protein function or activity.

25 **[0025]** Due to the range of possible type III CRISPR-associated effector complexes useful in the invention, a respective range of possible coA_x messengers is available for tailoring to a cyclic oligoadenylate (coA_x)-dependent protease. Accordingly, x in coA_x may be selected from 2, 3, 4, 5 or 6; preferably wherein the coA_x is coA₃.

[0026] The protein or polypeptide susceptible to cleavage by the protease of (b)
30 preferably comprises an amino acid sequence susceptible to cleavage at a site therein by the coA_x-dependent protease, so as to generate at least two peptide portions. At least one of these peptide portions may comprise a detectable marker. In this way, the detection of the marker and thereby the peptide portion signals the activity of the coA_x-dependent

protease, which in turn signals the recognition and cleavage activity of the RNA-guided type III CRISPR-associated effector protein complex on a target nucleic acid in a sample.

[0027] Of the possible detectable markers which can be used, preferred markers are fluorescent markers which may be fluorescent molecules or moieties. Prior to cleavage the protein or polypeptide may also have a quencher molecule or moiety attached so that in the absence of cleavage there is no fluorescence, or the wavelength of fluorescence is different from the wavelength of fluorescence without the quencher. Ideally the quencher may be attached to a different cleavage portion of the peptide than the fluorescent marker. The fluorescent marker may be attached to the N- or C-terminus of the protein or polypeptide, and the quencher may be attached to the respective C- or N-terminus of the protein or polypeptide. More details of fluorescent labels and quenchers is provided hereinafter.

[0028] In preferred aspects, the protein or polypeptide (c) may be a PCaspase protein comprising an amino acid sequence of SEQ ID NO: 7 or a sequence of at least 70% identity therewith, or polypeptide fragment thereof, and wherein protease activity of PCaspase protein is activated by the action of the coA_x-dependent protease.

[0029] Where a PCaspase protein is employed, the detection system preferably further comprises (d) a protein or polypeptide substrate for the PCaspase protein or polypeptide to act on. When this substrate (d) is cleaved it provides the or a detectable signal.

Therefore, within the scope of the invention is the possibility that the proteins or polypeptides of (c) and/or (d) may serve to provide for the detection of the protease of (b). In other words the detection can be direct and/or indirect, or both direct and indirect.

[0030] The protein or polypeptide substrate (d) is preferably cleaved into at least two peptide portions, wherein at least one peptide portion comprises a detectable label. The detectable label may be a fluorescent moiety; optionally wherein the protein substrate also comprises a quencher moiety which on cleavage is not comprised in the cleavage portion comprising the fluorescent moiety. Ideally the quencher may be attached to a different cleavage portion of the peptide than the fluorescent marker. The fluorescent marker may be attached to the N- or C-terminus of the protein or polypeptide, and the quencher may be attached to the respective C- or N-terminus of the protein or polypeptide. More details of fluorescent labels and quenchers is provided hereinafter.

[0031] The protein or polypeptide substrate (d) may be of any amino acid sequence and suitable structure to be able to function as a reporter protein. Usually there will be one cleavage site, but there may be a multiplicity of cleavage sites, in which case a suitable indirect measurement of effector complex activity can be monitored as loss of full length substrate (d) and/or appearance of one or more fragments thereof, which may or may not

necessarily be labelled, although labelling with detectable labels may allow easier and more rapid measurement by spectral or radiolabel measuring instrumentation. In general terms, the susceptibility of the substrate (d) to protease activity may be provided by an portion of the amino acid sequence that is not folded or structured in a particular way, and
5 which comprises a lysine (K) and/or arginine (R) amino acid. An example of a useful substrate (d) is casein.

[0032] With the exception of type III-E effectors, the type III CRISPR-associated effector comprises Cas10 with an amino acid sequence of SEQ ID NO: 9 or a sequence of at least 70% identity therewith. The Cas10 is the large subunit of the Cmr complex of proteins and
10 comprised within the effector complex along with other Cas proteins (Cmr 1, Cmr3, Cmr 4, Cmr 5 and Cmr 6).

[0033] In a preferred detection system the type III CRISPR complex is the type III-B system from *Haliangium ochraceum* DSM 14365, although other suitable type III CRISPR complexes may be used from other bacteria. The components of this particular type III-B
15 CRISPR complex are Cmr1, Cmr2 (Cas10), Cmr3, Cmr4, Cmr5 and Cmr6 with amino acid sequences as set forth in SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, respectively. Within the scope of any aspect of the invention described herein, is a CRISPR complex comprised of any one or more of these proteins, preferably all six of these proteins. Variants of each protein are possible of at
20 least 70% identity with any of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, respectively. Any of the six individual proteins may be a variant or not, and may be a variant of any degree of sequence identity from 70% to 99% with the relevant reference sequence. Therefore a wide variety of possible type III-B CRISPR complex variants are possible based on the system from *Haliangium*
25 *ochraceum* DSM 14365.

[0034] The detection systems herein are defined both with and without a sample of nucleic acid. The samples of nucleic acid may be of any kind, whether RNA and/or DNA containing. In some preferred modes of the detection systems of the invention the target nucleic acid molecule in a sample is an RNA.

30 **[0035]** The invention also provides a method of detecting a target polynucleotide sequence in a sample of nucleic acid, comprising combining in a reaction mixture:

- (a) a type III CRISPR-associated protein (Cas);
- (b) a targeting RNA substantially complementary to a desired target polynucleotide sequence, wherein (b) in combination with (a) forms an effector complex;
- 35 (c) a cyclic oligoadenylate (coA_x)-dependent protease; and

(d) a protein or polypeptide susceptible to cleavage by the protease of (c) and from which a detectable signal is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (c).

[0036] The coA_x-dependent protease is preferably a second messenger oligonucleotide or
 5 dinucleotide synthetase-associated and fused to various effector domains-caspase HetF
associated with Tetratricopeptide repeat (TPR) (SAVED-CHAT) protein comprising an
 amino acid sequence of any of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO:
 4, SEQ ID NO: 5, or SEQ ID NO: 6, or a sequence of at least 70% identity with any of SEQ
 ID Nos 1 – 6, or any active fragment thereof.

10 **[0037]** The coA_x may be selected from a coA_x wherein x is 2, 3, 4, 5 or 6; preferably
 wherein the coA_x is coA₃.

[0038] In preferred methods, the protein or polypeptide of (d) comprises an amino acid
 sequence susceptible to cleavage at a site therein by the coA_x-dependent protease so as
 to generate at least two peptide portions. The cleavage of the protein or polypeptide (d)
 15 provides a signal based on the reduction of the amount of detectable full length protein or
 polypeptide and/or the appearance of one or more fragments of the protein or polypeptide.
 Detection may be of the protein or polypeptide or fragments thereof directly based on size
 and/or via a label which can be measured qualitatively and/or quantitatively.

[0039] In preferred methods, at least one portion of a cleaved peptide comprises a
 20 detectable marker. Particularly useful markers include fluorescent molecules or moieties
 which are, ideally, prior to cleavage, covalently linked to the protein or polypeptide (d), and
 remain covalently linked to the peptide(s) after protease cleavage. Förster resonance
 energy transfer (FRET) is a well-known technique involving two fluorescent chromophores
 for studying protein structure and interactions. The technique is well employed in all
 25 aspects of the present invention for detecting protease cleavage activity of the protein or
 polypeptide (d). Many fluorescent molecule pair combinations, including dye-quencher
 combinations, will be well known to a person of skill in the art and adapted for use in the
 present invention for detecting cleavage activity, whether by appearance and/or
 disappearance of fluorescence of suitably tagged proteins or polypeptides (d).

30 **[0040]** A preferred protein or polypeptide (d) for use in methods or other aspects of the
 invention is a PCaspase protein comprising an amino acid sequence of SEQ ID NO: 7 or a
 sequence of at least 70% identity therewith, or polypeptide fragment thereof, and wherein
 protease activity of PCaspase protein is activated by the action of the coA_x-dependent
 protease. SEQ ID NO: 7 is the peptidase C14 caspase catalytic subunit p20 from
 35 *Haliangium ochraceum* (strain DSM 14365 / JCM 11303 / SMP-2). Functional variants of
 this PCaspase protein, optionally from other species of microorganism, are included within

the scope of the invention, as may be defined in terms of percentage identity with SEQ ID NO: 7. Functional variants include fragments of the full length sequence or full length variants thereof. Variations in sequence may comprise conservative and/or non-conservative amino acid substitutions.

5 **[0041]** In further methods of the invention, a protein or polypeptide substrate (e) is provided for the PCaspase protein or polypeptide. When this protein or polypeptide substrate (e) is cleaved then a detectable signal for this cleavage activity of the PCaspase protein is generated. In preferred aspects, in similar ways described for the protein or polypeptide substrate (c) above, the protein or polypeptide substrate (e) is cleaved into at
10 least two peptide portions, and wherein at least one peptide portion comprises a detectable label. As already described elsewhere herein, the detectable label may be a fluorescent molecule or moiety ideally covalently attached to the protein or polypeptide (e).

[0042] The amino acid sequence of the protease cleavage site in the protein or polypeptide (e) may be replicated and within a suitable motif may be engineered into other
15 possible protein or polypeptide substrates. However, generally speaking, the protein substrate (e) comprises an amino acid sequence portion, optionally an unstructured portion, comprising a lysine (K) or arginine (R) amino acid. A particularly useful protein substrate is a casein.

[0043] In preferred methods and any of the of the other aspects of the invention, the type
20 III CRISPR-associated protein (Cas) is Cas10 (SEQ ID NO: 9 or sequence of at least 70% identity therewith). The Cas10 is the large subunit of the Cmr complex of proteins and comprised within the effector complex along with other Cas proteins. In particularly referred methods the type III CRISPR complex is the type III-B system from *Haliangium ochraceum* DSM 14365, although other suitable type III CRISPR Cas proteins and
25 complexes with targeting RNA may be used from other bacteria. With this in mind, a suitable CRISPR complex may be comprised of any one or more of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, or sequences of at least 70% identity therewith. Any of the six individual proteins making up the complex may be a variant or not, and may be a variant of any degree of sequence
30 identity from 70% to 99% with the relevant reference sequence. Therefore a wide variety of possible type III-B CRISPR complex variants are possible based on the system from *Haliangium ochraceum* DSM 14365.

[0044] In the wider aspect of the method of the invention, the essential reagents minus the sample are assembled and optionally combined, following which there may be the step
35 of combining the reagents with a sample (g) comprising nucleic acid; preferably comprising RNA.

[0045] The method of the invention may be carried out whereby the reagents (reaction components) can be combined at different times and in different combinations. For example, the type III CRISPR-associated protein (Cas) may be combined with the targeting RNA first to form an effector complex, prior to combining together with the other components of the reaction mixture. The particular order of addition/combination of reaction components is readily ascertained and understood by a person of skill in the art for optimisation of the individual reactions in the method/system, taking into consideration the format, e.g. liquid phase, or solid phase, or combination of solid and liquid phase reactions and the methods/instrumentation used for detection of the marker or markers.

[0046] For example, the sequential order of combining of reaction components is: (g), then (a)/(b), then (c), then (d), then (e).

[0047] Other sequential orders of the combining of reaction components may be arranged as follows:

- (g), then (a), then (b), then (c), then (d), then (e); or

- (g), then (a), then (b), then (c), then (d), then (e).

[0048] The invention includes a polynucleotide comprising a nucleotide sequence encoding one or more of:

i. a type III CRISPR complex comprising proteins with amino acid sequences of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, or sequences of at least 70% identity therewith;

ii. a SAVED-CHAT protein with an amino acid sequence of SEQ ID NO: 14 or a sequence of at least 70% identity therewith, or a functional fragment thereof;

iii. a SAVED-CHAT protein with an amino acid sequence comprising one or more of the following peptide motifs: GX(V/A)X(I/L)PXXX(H/Y)LGXXL [SEQ ID NO: 16]; (P/N)XAXXXDXXXL(R/E)XXXPX(I/V)(V/L)HXX(S/G)HG [SEQ ID NO: 17]; or DXX(A/S)XXF(S/A)XX(F/L)YXXLXXXXX(I/V)XXAF [SEQ ID NO: 18], wherein X is any amino acid;

iv. a CHAT-SAVED protein with an amino acid sequence of SEQ ID NO: 15, or a sequence of at least 70% identity therewith, or a functional fragment thereof;

v. a CHAT-SAVED protein with an amino acid sequence comprising one or more of the following peptide motifs: LRLVVLXACX [SEQ ID NO: 19]; IPAVIASQMPLS [SEQ ID NO: 20]; XPLPLAXXXGX [SEQ ID NO: 21]; or IHLFLAXPXSXAX [SEQ ID NO: 22], wherein X is any amino acid, wherein underlined amino acids are not substituted, and wherein specified amino acids may independently conservatively substituted;

vi. a PCaspase with an amino acid sequence of SEQ ID NO: 7 or a sequence of at least 70% identity therewith;

vii. a PCk with an amino acid sequence of SEQ ID NO: 42, or a sequence of at least 70% identity therewith;

5 viii. a PC- σ with an amino acid sequence of SEQ ID NO: 43, or a sequence of at least 70% identity therewith.

[0049] A polynucleotide as aforementioned may comprise a combination of nucleotide sequences selected from:

(i) and (ii), optionally further comprising (vi) and/or (vii) and/or (viii);

10 (i) and (iii); optionally further comprising (vi) and/or (vii) and/or (viii);

(i) and (iv) optionally further comprising (vi) and/or (vii) and/or (viii);

(i) and (v) optionally further comprising (vi) and/or (vii) and/or (viii).

[0050] A polynucleotide as aforementioned may further comprise a nucleotide sequence encoding a guide RNA with a sequence specific for a desired target nucleic acid sequence.

15 **[0051]** The invention also includes a plasmid comprising any of the aforementioned polynucleotides which lack a gRNA encoding sequence; optionally wherein the plasmid is a native conjugative plasmid, or an expression plasmid.

[0052] The invention further includes a viral vector comprising any of the aforementioned polynucleotides or plasmids which lack a gRNA encoding sequence; optionally wherein the
20 viral vector is a bacteriophage.

[0053] The invention also provides a composition comprising any of the aforementioned polynucleotides, plasmids, viral vectors, or nanoparticle compositions which lack a gRNA encoding sequence.

[0054] The invention further provides a plasmid comprising an aforementioned
25 polynucleotide which additionally comprises a gRNA encoding sequence; optionally wherein the plasmid is a native conjugative plasmid, or an expression plasmid.

[0055] The invention also provides a viral vector comprising an aforementioned polynucleotide which additionally comprises a gRNA encoding sequence, or a plasmid comprising such a polynucleotide; optionally wherein the viral vector is a bacteriophage.

30 **[0056]** The invention further provides a composition comprising an aforementioned polynucleotide which additionally comprises a gRNA encoding sequence, or a plasmid comprising such a polynucleotide, and a nanoparticle.

[0057] The invention also provides a composition comprising an aforementioned polynucleotide which additionally comprises a gRNA encoding sequence, a plasmid, viral vector or nanoparticle composition comprising such a polynucleotide.

5 [0058] The invention also provides a kit comprising (a) a plasmid as aforementioned which does not comprise a gRNA encoding sequence, a viral vector as aforementioned which does not comprise a gRNA encoding sequence, a nanoparticle composition as aforementioned which does not comprise a gRNA encoding sequence; and (b) a separate polynucleotide, plasmid, vector or nanoparticle composition comprising a nucleotide
10 sequence encoding a guide RNA with a sequence specific for a desired target nucleic acid sequence.

[0059] The invention further includes a composition comprising:

- (a) a type III CRISPR-associated protein (Cas);
- (b) a targeting RNA substantially complementary to a desired target polynucleotide sequence, wherein the combination of (a) and (b) provides an effector
15 complex; and
- (c) a cyclic oligoadenylate (coA_x)-dependent protease.

[0060] Such compositions of the invention may, as already noted, be in solid and/or liquid phase, depending on the particular format of the detection assay used, and also depending on the particular marker used to detect the various proteolytic cleavage events.

20 [0061] In a preferred aspect, a composition of the invention may further comprise (d) a protein or polypeptide susceptible to cleavage by the protease of (c) and from which a detectable signal is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (c). The coA_x-dependent protease (c) may be a second messenger oligonucleotide or dinucleotide synthetase-associated and fused to various
25 effector domains-caspase HetF associated with TPR (SAVED-CHAT) protein comprising an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6, or a sequence of at least 70% identity with any of SEQ ID Nos 1 – 6, or any active fragment thereof. Functional variants of this TPR (SAVED-CHAT) protein, optionally from other species of microorganism, are included within the
30 scope of the invention, as may be defined in terms of percentage identity with SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. Functional variants include fragments of the full length sequence or full length variants thereof. Variations in sequence may comprise conservative and/or non-conservative amino acid substitutions.

- [0062]** The protein or polypeptide (d) may be a PCaspase protein comprising an amino acid sequence of SEQ ID NO: 7 or a sequence of at least 70% identity therewith, or polypeptide fragment thereof, and wherein protease activity of PCaspase protein is activated by the action of the coA_x-dependent protease. Functional variants of this PCaspase protein, optionally from other species of microorganism, are included within the scope of the invention, as may be defined in terms of percentage identity with SEQ ID NO: 7. Functional variants include fragments of the full length sequence or full length variants thereof. Variations in sequence may comprise conservative and/or non-conservative amino acid substitutions.
- [0063]** Composition as herein described may further comprise (e) a protein or polypeptide substrate for the PCaspase protein or polypeptide, which when cleaved provides the detectable signal. Ideally, the protein or polypeptide substrate (e) is cleaved into at least two peptide portions, and wherein at least one peptide portion comprises a detectable label.
- [0064]** Though not exclusively, a useful detectable label is a fluorescent molecule or moiety and, ideally, prior to cleavage, these are covalently linked to the protein or polypeptide (d), and may remain covalently linked to the peptide(s) after protease cleavage. Förster resonance energy transfer (FRET) is a well-known technique involving two fluorescent chromophores for studying protein structure and interactions. The technique is well employed in all aspects of the present invention for detecting protease cleavage activity of the protein or polypeptide (d). Many fluorescent molecule pair combinations, including dye-quencher combinations, will be well known to a person of skill in the art and adapted for use in the present invention for detecting cleavage activity, whether by appearance and/or disappearance of fluorescence of suitably tagged proteins or polypeptides (d). Optionally a quencher may be used in combination with the fluorescent molecule or moiety wherein on cleavage the quencher is not comprised in the cleavage portion comprising the fluorescent moiety.
- [0065]** Considering the susceptibility of the protein substrate (e) to protease activity, the substrate (e) preferably comprises a portion of amino acid sequence, optionally an unstructured portion, comprising a lysine (K) or arginine (R) amino acid. A useful protein substrate is casein.
- [0066]** In compositions of the invention herein defined, the type III CRISPR-associated protein (Cas) is preferably Cas10 (SEQ ID NO: 9 or sequence of at least 70% identity therewith); more preferably the type III CRISPR complex is the type III-B system from *Haliangium ochraceum* DSM 14365. The sequences of the six Cmr proteins and their variants are elsewhere described herein.

[0067] Compositions of the invention may further comprise (f) a sample comprising nucleic acid; preferably wherein the nucleic acid is RNA.

[0068] The invention also includes a device for detecting a polynucleotide molecule comprising a defined nucleic acid sequence in a sample, comprising a container, wherein
5 the container comprises (i) a type III CRISPR-associated effector protein (Cas), (ii) a targeting RNA substantially complementary to the defined RNA sequence, wherein the combination of (i) and (ii) provides an effector complex; (iii) a cyclic oligoadenylate (coA_x)-dependent protease; and (iv) a protein or polypeptide susceptible to cleavage by the protease of (v) and from which a detectable signal moiety is generated whether directly or
10 indirectly by cleavage of said protein or polypeptide by the protease of (iii). The container may itself form the device of the invention, or the container may be comprised in a device which further includes mechanical, electrical and/or software elements needed in combination to provide a fully operational detection device. The device is preferably presented and available without a sample such that it is in readiness to receive any given
15 sample. Therefore the invention also includes a detection device as described herein further comprising a sample being interrogated for a defined nucleic acid sequence therein. A person of skill in the art will readily appreciate that the targeting RNA substantially complementary to the defined RNA sequence is therefore selected to discriminate positively in respect of the defined nucleic acid sequence in the sample. This
20 provides in all aspects of the invention the degree selectivity and specificity needed for detecting the sequence of interest in a sample.

[0069] Possible within the scope of the invention is the provision of separate reactions based on respective different targeting RNAs as a way of providing a multi-parallel interrogation of sample DNA for a number of possible nucleic acid sequences
25 simultaneously. Therefore the invention includes any device comprising a detection system as herein described and such devices may preferably comprise multiple arrayed ribonucleic acid detection systems, each of the arrayed ribonucleic acid detection systems preferably being directed to specifically detect different respective target nucleic acid molecules.

[0070] As well as devices envisaged comprised around a single container or test well based on being a liquid phase assay, other devices can be designed around on a solid phase assay such as a lateral flow device, or a combination of solid phase and liquid phase assays. A device for detecting a polynucleotide molecule comprising a defined sequence in a sample, comprising a lateral flow substrate comprising (a) a sample
30 receiving portion, (b) a flow through portion, and (c) a capture portion, wherein the flow through portion comprises (i) a type III CRISPR-associated protein (Cas), (ii) a targeting
35

RNA substantially complementary to the defined RNA sequence, wherein the combination of (i) and (ii) provides an effector complex; (iii) a cyclic oligoadenylate (coA_x)-dependent protease; and (iv) a protein or polypeptide susceptible to cleavage by the protease of (iii) and from which a detectable signal is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (iii); and wherein the capture portion retains the detectable signal moiety.

[0071] As well as the aforementioned elements, devices in accordance with the invention may comprise any of the necessary other components necessary for a working device, whether standalone or for coupling to other equipment for generating a test result. For example, any necessary reservoirs, conduits, pumps, mixing chambers, filters, substrates, illumination means, e.g. lasers etc., light detectors, CCDs, electronic circuits, microprocessors or signal transmission devices.

[0072] The invention also provides a kit of parts for the purposes of detecting of a polynucleotide molecule comprising a defined nucleic acid sequence in a sample, the kit comprising at least one container, (i) a type III CRISPR-associated protein (Cas), (ii) a cyclic oligoadenylate (coA_x)-dependent protease; and (iii) a protein or polypeptide susceptible to cleavage by the protease of (ii) and from which a detectable signal moiety is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (ii). As well as the at least one container, the kit may include written material in physical or virtual form providing instructions for operation and use of the kit. The kit may be for use with other devices and assay equipment. Therefore the at least one container may be a reaction vessel within which the detection of labelled substances also takes place. The at least one container may contain one or all of the aforementioned components, but where less than all of the components are comprised in a single container then the kit provides a plurality of containers. Some containers may comprise a single component, other containers may comprise combinations of components. A single empty container may be provided as a reaction vessel into which a sample is introduced, whether before, after or simultaneously with one or more of the other reaction components.

[0073] A kit in accordance with the invention as defined above, may further comprise (iv) a targeting RNA substantially complementary to the defined RNA sequence which is being detected for in the sample. In such a way, kits can be presented to the user for a predetermined sample sequence and therefore ready to use, as compared to a kit which is ready to configure by the user with a selected targeting RNA in order to detect a chosen sequence in a sample.

[0074] As noted previously, a kit of the invention may have a separate container for each of components (i), (ii), (iii) and (iv).

[0075] The *Haliangium* species of bacteria from which a preferred type III Cas protein is derived are from the marine environment, and so temperatures for operation of the method and system of the invention mainly correspond to those ambient native temperatures. If a type III Cas protein is used from a thermophilic organism then the usable temperatures are consequently higher within the range of tolerance of the other components of the system, the preferred elements being the SAVED-CHAT protein and optionally the PCaspase protein. Consequently, the methods and systems of the invention may be operated at temperatures in the range from about 4 °C to about 38 °C. Included therefore are temperatures in the range from about 10 °C to about 38 °C, from about 15 °C to about 38 °C, from about 20 °C to about 38 °C and from about 25 °C to about 38 °C, for example. Narrower ranges of temperature such as from about 10 °C to about 36 °C, from about 15 °C to about 36 °C, from about 20 °C to about 36 °C and from about 25 °C to about 36 °C may provide a suitable reaction temperature to be used. Therefore included in the invention are methods and systems operating at individual temperatures of about 4 °C, about 5 °C, about 6 °C, about 7 °C, about 8 °C, about 9 °C, about 10 °C, about 11 °C, about 12 °C, about 13 °C, about 14 °C, about 15 °C, about 16 °C, about 17 °C, about 18 °C, about 19 °C, about 20 °C, about 21 °C, about 22 °C, about 23 °C, about 24 °C, about 25 °C, about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, about 35 °C, about 36 °C, about 37 °C, about 38 °C. The term "about" is used because it is clear to a person of skill in the art that temperatures may not be so precise during the reactions of the assay carried out in accordance with the invention that they cannot vary within ± 0.5 °C or ± 0.6 °C and yet achieve reproducible and accurate results.

[0076] The type III Cas protein and resulting effector complex described herein may be modified to lack cleavage activity but still generate coA_x and therefore drive the activation of the coA_x activated protease. A skilled person will be able to identify the necessary cleavage-dead mutations through a process of design, expression and testing of various mutated versions of the type III Cas effector complex and constituent proteins.

[0077] In any of the aspects of the invention, "base pairing affinity" and "complementarity" may be used interchangeably and refer to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick base pairing or other non-traditional types. A percent identity (i.e. complementarity) in relation to a reference sequence, in the various descriptions of the invention, represents the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% identity). "Perfectly complementary"

means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence, and this is a preferred condition for antisense oligonucleotide binding to the targeting RNA which corresponds to 100% identity for a length of targeting RNA molecule which is the same length as the antisense oligonucleotide. Also, the term "substantially complementary" as used herein refers to a degree of identity that is at least 90%, 95%, 97%, 98%, 99%, or 100% between the portion of the antisense oligonucleotide and the equivalent length of targeting RNA molecule. This may also correspond to nucleic acids that hybridize under stringent conditions.

[0078] As used herein, "stringent conditions" for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions surrounding the nucleic acids, temperature, the nature of the hybridization method, and the composition and length of the nucleic acid molecules used. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001); and Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology - Hybridization with Nucleic Acid Probes Part I, Chapter 2* (Elsevier, New York, 1993), each of which are incorporated herein by reference. The T_m is the temperature at which more than 50% of a given strand of a nucleic acid molecule is hybridized to its complementary strand. The following is an exemplary set of hybridization conditions and is not limiting:

[0079] Very High Stringency (allows sequences that share at least 90% identity to hybridize) Hybridization: 5x SSC at 65 °C for 16 hours; wash twice: 2x SSC at room temperature (RT) for 15 minutes each; wash twice: 0.5x SSC at 65 °C for 20 minutes each.

[0080] High Stringency (allows sequences that share at least 80%> identity to hybridize) Hybridization: 5x - 6x SSC at 65 °C - 70 °C for 16 - 20 hours; wash twice: 2x SSC at RT for 5 - 20 minutes each; wash twice: 1x SSC at 55 °C - 70 °C for 30 minutes each.

[0081] Low Stringency (allows sequences that share at least 50%> identity to hybridize); hybridization: 6x SSC at RT to 55 °C for 16 - 20 hours; wash at least twice: 2x - 3x SSC at RT to 55 °C for 20 - 30 minutes each.

[0082] In terms of percentage identity characterising the extent of variation of a protein or polypeptide described herein with a specified reference sequence, the degree of identity

may be any of: at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, most preferably at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

[0083] In any of the aspects of the invention, the targeting RNA molecule is designed to have complementarity, where hybridization between a target sequence and the RNA targeting molecule promotes the formation of a RNA-targeting complex. Targeting RNA molecules in accordance with the invention may include mature crRNA, guide RNA (gRNA) or single guide RNA (sgRNA) and these terms can be used interchangeably. In general, a targeting RNA has a sufficient complementarity with the target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of the CRISPR enzyme or Cascade complex to the target sequence. The degree of complementarity between a targeting RNA and its corresponding target sequence may be more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more, with optimal algorithmic alignment.

[0084] Throughout this specification in any context, optimal alignment may be determined using, for example, any of the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g., the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net).

[0085] The term “Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)”, as is used herein, refers to one or more specialized regions of DNA in the genome of prokaryotic microorganisms. These regions are characterized by the presence of nucleotide repeats that are interspersed by spacer sequences, typically ~25- to ~38 bp direct DNA repeats, separated by unique spacer sequences of a similar length (see Grissa *et al.*, (2007) BMC Bioinformatics May 23;8:172. doi: 10.1186/1471-2105-8-172) which are derived from previous encounters with invading elements. They serve as a memory to quickly attack these invaders upon a next infection. The genomic region includes one or more CRISPR-associated effector protein (Cas)-encoding genes that are located in the vicinity of the CRISPR loci.

- [0086] The term “CRISPR crRNA”, as is used herein, refers to a CRISPR-derived RNA molecule comprising a spacer sequence and 5 and 3 repeat-derived termini. Said CRISPR crRNA preferably has a length of at least 30 nucleotides, more preferred at least 34 nucleotides, more preferred at least 40 nucleotides, more preferred at least 46
5 nucleotides. Said CRISPR crRNA preferably is less than 1000 nucleotides, preferably less than 200 nucleotides, preferably less than 100 nucleotides. Said RNA molecule may include ribonucleic acid nucleotide analogues such as inosine, uridine, xanthine, hypoxanthine, 2,6-diaminopurine, and 6,8- diaminopurine-based ribonucleotides and desoxyribonucleotides.
- 10 [0087] The term “CRISPR-associated effector protein (Cas)”, as is used herein, refers to a protein that is associated with CRISPR crRNA. CRISPR/Cas systems are presently grouped into two classes. Class I systems utilize multisubunit Cas complexes, whereas Class II systems use only a single Cas protein to mediate its activity. Class I, type III CRISPR-Cas systems which is what the present invention utilizes have evolved to target
15 especially RNA sequences. Cas10 is unique to the Class I, type III systems which the present invention is based on.
- [0088] The term “effector complex”, as is used herein, refers to a CRISPR-Cas ribonucleoprotein complex that has nuclease activity and may cleave and inactivate an
20 invading nucleic acid sequence that comprises complementary sequences to the spacer sequence in the CRISPR crRNA.
- [0089] The term “cyclic oligoadenylate (cOA)”, as is used herein, refers to a ring structure comprising 2, 3, 4, 5 or 6 molecules of Adenosine Mono Phosphate (AMP). The formation of cOA is catalysed by the cyclase domain of Cas10, which is part of type III effector systems.
- 25 [0090] The term “Type III Cas”, as is used herein, refers to a RNA-targeting, multiple subunit CRISPR-associated complex that comprises at least a Cas10 protein.
- [0091] A type IIIA Cas, as is used herein, which falls under within the scope of the aforementioned “type III Cas”, is an RNA-targeting Type 3 CRISPR/Cas complex that has unspecific DNase activity upon binding to a target RNA molecule. Type IIIA Cas include,
30 for example, Type IIIA Csm complexes from *Staphylococcus thermophilus*, *Thermus thermophilus* and *Staphylococcus epidermis*.
- [0092] The term “Type IIIB Cas”, as is used herein, refers to a RNA-targeting Type 3 CRISPR-Cas complex that lacks unspecific DNase activity. Said Type IIIB Cas complex is composed of six to seven proteins. Type IIIB Cas include, for example, Type IIIB Cmr

complexes from *Pyrococcus furiosus*, *Thermus thermophilus* and *Sulfolobus solfataricus*, as well as the preferred type III-B Cas from *Haliangium ochraceum* DSM 14365.

[0093] The term “biosensor”, or “biological sensor”, as is used herein, refers to a sensing device comprising a CRISPR-based ribonucleic acid system according to the invention. A signal that is generated when the CRISPR-based ribonucleic acid system of the invention interacts with a RNA molecule that is complementary to the crRNA, for example a colorimetric, fluorometric, fluorescent or bioluminescent signal, may be coupled to a transducer, allowing quantification of the signal. The signal is preferably converted by means of a suitable transducer into a measurable electrical parameter such as a current or voltage.

[0094] A general structure of Type III CRISPR/Cas complexes is that they comprise multiple subunits of Cas7 and Cas11 (Staals et al., 2013. Mol. Cell 52: 135-145; Staals et al., 2014. Mol. Cell 56: 518-530), which are capped off at one side by Cas5 and Cas10. Of these, Cas7 provides RNase activity upon recognition of target RNA by the pre-loaded RNA guide. It has been shown that target recognition promotes the production of cyclic oligoadenylates (cOA) by the Cas10 Palm domain (Kazlauskienė et al., (2017) Science 357(6351): 605-609).

[0095] Preferred fluorescent labels in accordance with any aspect of the invention may be selected from any of Atto425 (ATTO-TEC GmbH, Siegen, Germany), Atto 647N (ATTO-TEC GmbH, Siegen, Germany), YakimaYellow (Epoch Biosciences Inc, Bothell, WA, USA), Cal610 (BioSearch Technologies, Petaluma, CA, USA), Cal635 (BioSearch Technologies, Petaluma, CA, USA), FAM (Thermo Fisher Scientific Inc., Waltham, MA USA), TET (Thermo Fisher Scientific Inc., Waltham, MA USA), HEX (Thermo Fisher Scientific Inc., Waltham, MA USA), cyanine dyes such as Cy5, Cy5.5, Cy3, Cy3.5, Cy7 (Thermo Fisher Scientific Inc., Waltham, MA USA), Alexa dyes (Thermo Fisher Scientific Inc., Waltham, MA USA), Tamra (Thermo Fisher Scientific Inc., Waltham, MA USA), ROX (Thermo Fisher Scientific Inc., Waltham, MA USA), JOE (Thermo Fisher Scientific Inc., Waltham, MA USA), fluorescein isothiocyanate (FITC, Thermo Fisher Scientific Inc., Waltham, MA USA), Yakima Yellow® (YY, Epoch Biosciences, Bothell, Washington) and tetramethylrhodamine (TRITC, Thermo Fisher Scientific Inc., Waltham, MA USA). Said substrate is preferably labelled at the 5' end with a detectable label, preferably a fluorescent label.

[0096] Quenchers useful in the invention including, for example, tetramethylrhodamine TAMRA. Preferred quenchers include Black Hole Quencher®- 1 (BHQ1) and BHQ2 (Biosearch Technologies, Petaluma, CA, USA). The BHQ1 dark quencher has strong absorption from 480 nm to 580 nm, which provides quenching of fluorophores that

fluoresce in this range, such as FAM, TET, CAL Fluor® Gold 540, JOE, HEX, CAL Fluor Orange 560, and Quasar® 570 dyes. The BHQ2 dark quencher has strong absorption from 599 nm to 670 nm, which provides quenching of fluorophores that fluoresce in this range, such as Quasar® 570, TAMRA, CAL Fluor® Red 590, CAL Fluor Red 610, ROX, CAL Fluor Red 635, Pulsar® 650, Quasar 670 and Quasar 705 dyes. BHQ1 and BHQ2 may quench fluorescence by both FRET and static quenching mechanisms.

[0097] Ribonucleic acid detection systems according to the invention involve an *in vitro* assembly of a CRISPR Type III ribonucleoprotein complex, preferably a Type IIIB complex. Said proteins preferably are expressed and purified from a suitable expression system. Commonly used expression systems for heterologous protein production include *E. coli*, *Bacillus* spp., baculovirus, yeast, fungi, most preferably filamentous fungi or yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris*, eukaryotic cells such as Chinese Hamster Ovary cells (CHO), human embryonic kidney (HEK) cells and PER.C6® cells (Thermo Fisher Scientific, MA, USA) and plants. The efficiency of expression of recombinant proteins in heterologous systems depends on many factors, both on the transcriptional level and the translational level.

[0098] Cas proteins preferably are produced using prokaryotic cells, preferably *E. coli*. Said Cas proteins are preferably produced by expression cloning of the proteins to a prokaryotic cell of interest, preferably *E. coli*. Said expression construct, preferably DNA, is preferably produced by recombinant technologies, including the use of polymerases, restriction enzymes, and ligases, as is known to a skilled person. Alternatively, said expression construct is provided by artificial gene synthesis, for example by synthesis of partially or completely overlapping oligonucleotides, or by a combination of organic chemistry and recombinant technologies, as is known to the skilled person.

[0099] As an alternative, or in addition, Cas proteins may be isolated from a thermophilic organism by expression of a tagged Cas protein in said thermophilic organism, and isolation of ribonucleoprotein complex comprising said Cas proteins on the basis of the tag. Said isolated ribonucleoprotein complexes can be isolated using the tagged Cas protein.

[00100] Said expression construct is preferably codon-optimised to enhance expression of the Cas proteins in a prokaryotic cell of interest, preferably *E. coli*. Further optimization preferably includes removal of cryptic splice sites, removal of cryptic polyA tails and/or removal of sequences that lead to unfavourable folding of the mRNA. In addition, the expression construct preferably encodes a protein export signal for secretion of the Cas proteins out of the cell into the periplasm of prokaryotes, allowing efficient purification of the Cas proteins.

[00101] Methods for purification of Cas proteins are known in the art and are generally based on chromatography such as affinity chromatography and ion exchange chromatography, to remove contaminants. In addition to contaminants, it may also be necessary to remove undesirable derivatives of the product itself such as degradation products and aggregates. Suitable purification process steps are provided in Berthold and Walter (1994) *Biologicals* 22: 135 - 150.

[00102] As an alternative, or in addition, recombinant Cas proteins may be tagged with one or more specific tags by genetic engineering to allow the protein attach to a column specific to the tag and therefore be isolated from impurities. The purified protein is then exchanged from the affinity column with a decoupling reagent. The method has been increasingly applied for purifying recombinant protein.

[00103] Conventional tags for proteins, such as histidine tag, are used with an affinity column that specifically captures the tag (e.g., a Ni-IDA column for the histidine tag) to isolate the protein from other impurities. The protein is then exchanged from the column using a decoupling reagent according to the specific tag (e.g., imidazole for histidine tag). This method is more specific, when compared with traditional purification methods.

[00104] Suitable further tags include c-myc domain EQKLISEEDL (SEQ ID NO: 24), hemagglutinin tag YPYDVPDYA (SEQ ID NO: 25), maltose-binding protein, glutathione-S-transferase, FLAG tag peptide, biotin acceptor peptide, streptavidin-binding peptide and calmodulin-binding peptide, as presented in Chatterjee (2006) *Curr. Opin. Biotech.* 17: 353 - 358. Methods for employing these tags are known in the art and may be used for purifying Cas proteins.

[00105] Methods for expression proteins in *E. coli* are known in the art and can be used for expression and purification of the Cas-proteins.

[00106] In a preferred method, Cas proteins are expressed in *E. coli* from a codon-optimized expression construct. Said construct is placed in a bicistronic expression plasmid containing a Strep-tag and amino-acid sequence Glu-Asn-Leu-Tyr-Phe-Gln-(Gly/Ser) at the N-terminus, which amino acid sequence is recognized by a Tobacco Etch Virus (TEV) protease. The expression plasmid is transformed into *E. coli*, for example in strain B121(DE3). Following growth at 37 °C in a desired culture volume, until GD600 of ~0.6, the culture is placed on ice for 1 hour after which isopropyl B-D-1-thiogalactopyranoside is added to a final concentration of 0.1 mM. The culture is then incubated at 18 °C for ~16 hours (overnight). The cells are harvested and lysed in Buffer A (100 mM Tris-HCl, 150 mM NaCl) by sonication and subsequently spun down at 30.000g for 45 min. The clarified lysate is filtered and run over a pre-equilibrated StrepTrap FPLC column (GE Healthcare, Chicago, IL). After washing the column with Buffer A until no

more protein is present in the flow through, the protein of interest is eluted using Buffer B (100 mM Tris-HCl, 150 mM NaCl & 2.5 mM D-desthiobiotin). The protein is cleaved from the affinity tag by addition of TEV protease and left to incubate overnight at 4 °C. The protein of interest is separated from the mixture by a HisTrap and StrepTrap affinity chromatography step, from which the flow through is collected. If required, an additional size exclusion chromatography is added to achieve higher purity.

[00107] Cas proteins of use in the invention comprise Cas10 and may comprise Csm or Cmr proteins, at least Cmr 1 and Cmr 4, preferably Cmr 1-6, or at least Csm 2, preferably Csm 2 - 5, more preferably Csm 1 - 6.

[00108] For any of the proteins described herein, mutants of said proteins, including insertion mutants, deletion mutants, chimeric proteins and amino acid substituted proteins which retain the respective functions required for the system and method described herein may also be used according to the invention.

[00109] To increase sensitivity of the Type III CRISPR/Cas detection method, catalytically dead mutant Cmr and/or Csm complexes are preferably created and used in a detection system of the invention. The term “catalytically dead” refers to the target RNA-digesting activity of the CRISPR-based ribonucleic acid system according to the invention. These mutants are referred to as dCmr and dCsm. The mutations are introduced in the Cmr4 and Csm3 subunits that are responsible for target binding and cleaving and are selected for abolishment of target cleavage, while maintaining target binding. A number of mutations in the Cmr4 protein (H15A,D26A,E277A), have been described, with the strongest catalytic impairment being observed in Cmr4 D26A and D26N mutations (Benda *et al.*, (2014) Molecular Cell 56: 43-54; Ramia *et al.*, (2014) Cell Reports 9: 1610-1617). This inactivation mutation is experimentally proven to work in *Pyrococcus furiosus*, and sequence alignments of Cmr4 orthologs shows that this amino acid is highly conserved (data not shown). In addition, crystallography data shows that this particular amino acid is located in a groove of the complex where target RNA is expected to bind (data not shown). Together, these results indicate that the D26 amino acid residue is important for the catalytic activity of Cmr4. Alteration of this residue will result in a *Thermus thermophilus* dCmr4 mutant. Next to this, alignment of the Pf_Cmr4 amino acid sequence with orthologs of Csm3 also show that D26 is one of the few amino acids that is conserved between the two Type III systems and could therefore also be used to create a dCsm3 mutant. Further cleavage-dead mutations include a E227A and E228 double mutant of Cmr4, and Cmr4 D86A (Ramia *et al.*, (2014) Cell Reports 9: 1610 - 1617; Zhu and Ye, (2015) Nucleic Acids Res 43: 1257 - 1267). In addition, a Csm3 D32A is a good candidate for a cleavage-dead Type IIIA Csm mutant (Samai *et al.*, (2015) Cell 161: 1164-1174).

[00110] Additionally, Jia *et al.* (2019) Mol Cell 73: 264 – 277 and Park *et al.* (2017) EMBO reports 18: 826 - 840 describe a dCsm3 mutant created by the D36A substitution. A double mutant, K56A and R60A, was also mentioned, but did not fully abolish target cleavage in Csm3.

- 5 [00111] The methods of the invention for detecting specific RNA sequences may be used in human healthcare, veterinary diagnostics, detection of plant pathogens, detection of water contaminants and the detection of food and feed contaminants.

[00112] In addition, the methods of the invention for detecting specific RNA sequences may be used for detecting beneficial organisms. In general, the methods of the invention
10 can be used for detection of bacterial, fungal, archaeal, protest, protozoal, eukaryotic, viral and viroidal pathogens. In addition, the methods of the invention may also be used for detection and diagnosis of genetic alterations or traits that are expressed as RNA molecules, in human, animals and plants.

[00113] For human healthcare and veterinary diagnostics, nucleic acid material, including
15 RNA, is preferably isolated from a biological fluid, preferably from cerebrospinal fluid, saliva, nasopharyngeal secretion, oropharyngeal secretion, sweat, urine stool, or blood. The term “blood” includes blood plasma, which is prepared by removing red and white blood cells, for example by centrifugation, and blood serum, which is prepared by formation of a blood clot, and removal of the clot using, for example, a centrifuge. A
20 preferred biological fluid is blood. Methods and compositions for isolation of nucleic acid material from biological fluids, particularly blood, preferably employ aqueous solvents without use of organic solvents and chaotropic salts.

[00114] If necessary, nucleic acid material, including RNA, may be purified from a sample using, for instance, a combination of physical and chemical methods. Commercially
25 available systems for nucleic acid isolation are preferably used, such as the NucliSENS® easyMAG® or NucliSENS® miniMAG® nucleic acid extraction system (bioMérieux, Marcy l'Etoile, France), or a MagNA Pure 96 System (Roche Diagnostics, Almere, The Netherlands). Also, RNA may be isolated from a sample by any technique known in the art, including but not limited to suitable commercial RNA isolation kits include Trizol
30 (Invitrogen; Carlsbad, California), RNAqueous® (Applied Biosystems/Ambion, Austin, Tx), Qiazol® (Qiagen, Hilden, Germany), Agilent Total RNA Isolation Kits (Agilent; Santa Clara, California), RNA-Bee® (Tel-Test. Friendswood, Texas), the RNeasy mini kit (Qiagen, Venlo, The Netherlands), and Maxwell™ 16 Total RNA Purification Kit (Promega; Madison, Wisconsin). The isolated RNA, preferably mRNA, is preferably reverse transcribed with
35 the aid of a RNA-dependent DNA polymerase into single or double stranded cDNA.

[00115] Use of Type III CRISPR/Cas according to the invention for diagnostic purposes include, but is not limited to, medical diagnostics such as detection of urinary tract infection, respiratory tract infection, (for example SARS-CoV-2 and respiratory syncytial virus (RSV)), blood infection (sepsis), expression of antibiotic resistance markers such as methicillin-resistant *Staphylococcus aureus* markers and extended spectrum beta - lactamases markers, gastrointestinal infection, skin infection, odontogenic infection, vaginal infection such as Candidiasis, *Trichomonas vaginalis*, and Gardnerella, male reproductive system infection, tropical infectious disease such as malaria, trypanosoma, dengue fever, Zika fever, chikungunya fever, detection of sexually transmissible diseases caused by *Chlamydia* spp., gonorrhea, human immunodeficiency virus, *Herpes* spp., syphilis, and of genetic defects including cancer and autoimmune disease.

[00116] The Type III CRISPR/Cas methods and systems according to the invention may further be used for veterinary diagnostics including detection of cattle infectious diseases such as mastitis, bluetongue, foot and mouth disease, *Salmonella* spp., *Klebsiella* spp., *Campylobacter* spp., pig infectious diseases such as respiratory diseases, dermatitis, diarrhea, and infections by porcine parvovirus; sheep and goat infectious diseases such as Clostridial diseases, soremouth, pneumonia and infections with Rift Valley Disease Virus; poultry infectious diseases such as infectious bronchitis, *Salmonella* spp.; feline infectious diseases such as infections with feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV), respiratory infections; canine infectious diseases such as rabies, and infections with *Bordetella*, *Leptospira*, and *Borellia*.

[00117] The Type III CRISPR/Cas detection method or system according to the invention may further be used for detection of plant pathogens such as detection of certain fungi such as Ascomycetes species and Basidiomycetes species, certain fungi-like organisms such as oomycetes and phytomyxea, certain bacteria such as *Burkholderia*, proteobacteria, and *Pseudomonas* species; viruses, viroids and virus- like organisms such as tobacco mosaic virus, cauliflower mosaic virus; nematodes such as *Meloidogyne chitwoodii* and *M. fallax*; and protozoa and algae such as *Phytophthora* and *Cephaleuro*.

[00118] The Type III CRISPR/Cas detection system or methods according to the invention may further be used for detection of water contaminants, including detection of bacterial contamination such as *Vibrio cholerae*, *E. coli*, *Shigella* spp., *Legionella* spp., *Salmonella* spp.; viral contamination such as hepatitis A, hepatitis E, poliovirus; algae contamination such as presence of *Desmodesmus* spp; and parasitic contamination such as presence of *Dracunculiasis* spp.

[00119] The Type III CRISPR/Cas detection systems or methods according to the invention may further be used for detection of food and feed contaminants including

bacterial contamination such as presence of *Clostridium botulinum*, *E. coli*, *Listeria* spp., *Salmonella* spp., *Vibrio cholera*, viral contamination such as presence of *Enterovirus* spp., hepatitis A, norovirus spp., and rotavirus spp.; parasitic contamination such as presence of *Giardia* spp. and *Trichinella* spp.; and fungal contamination.

- 5 **[00120]** More specifically, the Type III CRISPR/Cas detection system according to the invention may further be used for detection of any organism, as all organisms generate RNA during infection. Said organisms include bacteria such as *Bacillus* species, *Clostridium* species, *Enterobacter* species, *Escherichia* species, *Enterococcus* species, *Klebsiella* species, *Listeria* species, *Legionella* species, *Salmonella* species,
- 10 *Staphylococcus* species, *Streptococcus* species, and combinations thereof; viruses including DNA viruses such as hepatitis B virus, adenovirus, human papilloma virus; RNA viruses such as Influenza virus, Hepatitis A/C/D/E, polio virus, tobacco mosaic virus, Coronavirus, and HIV; viroids; Archaea; fungi such as *Aspergillus* species, Ascomycetes species, *Candida* species; protozoa; and parasites such as *Trypanosoma* species.
- 15 **[00121]** The use of the Type III CRISPR/Cas detection systems or methods according to the invention for detection of RNA will find explicit benefit in a diagnostic setting. Two such diagnostic settings are explained herein below. However, a person skilled in the art will without undue effort be able to apply the invention to a range of possible further diagnostic settings that will benefit from the Type III CRISPR/Cas detection system according to the
- 20 invention for detection of RNA.

- [00122]** If necessary, for example to increase detection levels, the methods of the invention for detecting specific RNA sequences can be preceded by amplification of target sequences. Amplification may be performed by any suitable amplification system including, for example, ligase chain reaction (LCR), isothermal ribonucleic acid
- 25 amplification systems such as nucleic acid sequence-based amplification (NASBA) and cleavage-based signal amplification of RNA, transcription mediated amplification, strand displacement amplification and, polymerase chain reaction (PCR). As is known to a person skilled in the art, RNA preferably is reverse transcribed prior to, or during the amplification reaction.

- 30 **[00123]** A preferred amplification reaction is a single tube, isothermal reaction such as NASBA, loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA) reaction, and nicking enzyme amplification reaction (NEAR). A preferred single tube, isothermal reaction is a RPA reaction (TwistDx Ltd., Cambridge, UK). Said single tube, isothermal reaction such as
- 35 RPA preferably is integrated with the CRISPR-based ribonucleic acid detection system of the invention as a “one pot” reaction system. An advantage of such one pot, or single

tube, system is a reduced risk for contamination of the samples, or cross-contamination of different samples.

[00124] Methods for the isolation of nucleic acid material include, but are not limited to organic extraction, chelex extraction, solid phase extraction, magnetic beads, and/or anion exchange. In addition to the reduction of endogenous P_i levels, sample preparation steps are also required to solve readout interference by other factors including, but not limited to, proteases in patient samples, salt concentrations, and pH. Specific qPCR primers as described in van der Zee *et al.* (2016) PLOS ONE 11: e0150755) may be used for detection of UTI-causing pathogens.

[00125] The CRISPR/Cas-based ribonucleic acid detection method and system according to the invention may be manifest as a device. Said device preferably comprises one or more detection systems that target one or more specific RNA sequences. Said device may comprise openings such as inlet and outlet ports, for the introduction and extraction of fluids into and from the device. Said openings may be connected to valves, tubes, channels, chambers, syringes and/or pumps. The devices may be connected to fluid flow actuators that allow directional movement of fluids within the microfluidic device. Example actuators include, but are not limited to, syringe pumps, mechanically actuated recirculating pumps, electroosmotic pumps, bulbs, bellows, diaphragms, or bubbles intended to force movement of fluids. In certain example embodiments, the devices are connected to controllers with programmable valves that work together to move fluids through the device. Additionally, a temperature control mechanism may be provided for incubation of the reaction mixture at a desired temperature.

[00126] Said CRISPR/Cas-based ribonucleic acid detection system preferably is present in a biosensor, preferably by using a disposable cartridge. A preferred biosensor provides methods and means for detecting interaction of the CRISPR/Cas-based ribonucleic acid detection system with a target nucleic acid. A preferred biosensor comprises a reusable hand-held reader capable of simple push-button operation for automated analysis of samples, and cost-effective disposable cartridges, preferably disposable microfluidic sensor cartridges, that have been functionalized to provide optimal detection and/or quantification of multiple clinically relevant agents such as pathogens. A possible biosensor is a lateral flow test device, for example based on the accumulation of quantifiable substances such as magnetic particles.

[00127] In an embodiment, a device or biosensor in accordance with the invention is a Point of Care (POC) testing device, which is a transportable, portable, and handheld instrument or test kit that allows to collect a sample and obtain the results in a very short period of time at or near the location of the patient so that the treatment plan can be

adjusted as necessary. Said device preferably comprises means that allow a rapid, low-cost, and reliable determination of the presence or absence of a target nucleic acid and, preferably also, a quantification of said target nucleic acid. A POC comprising a CRISPR/Cas-based ribonucleic acid detection system preferably is directed to detecting a limited number of target nucleic acid molecules, including 5 or less target nucleic acid molecules, 4 or less target nucleic acid molecules, 3 or less target nucleic acid molecules, such as 2 target nucleic acid molecules and 1 target nucleic acid molecule. For this, the crRNA ribonucleoprotein complexes are preferably present at discrete positions ribonucleic acid detection system, allowing to determine a level of cOA for each of the individual crRNA ribonucleoprotein complexes.

[00128] An array comprising a CRISPR/Cas-based ribonucleic acid detection system of the invention may target at least 5 different target nucleic acid molecules, preferably at least different 10 target nucleic acid molecules, preferably at least different 20 target nucleic acid molecules, preferably at least different 50 target nucleic acid molecules, preferably at least different 100 target nucleic acid molecules; or any number of target nucleic acid molecules from 2 to as many as 12,000 target nucleic acid molecules.

[00129] As will be clear to a person skilled in the art, said different target nucleic acid molecules may all be directed to different organisms such that each of the crRNA molecules is derived from, and is used to detect, a different target organism such as different bacteria, viruses, fungi, protozoa, and/or parasites. Said different crRNA molecules may also be chosen such that a subset of the different crRNA molecules are directed to the same organisms. Said subset may comprise 2 different crRNA molecules, 3 different crRNA molecules, 4 different crRNA molecules, 5 different crRNA molecules. The number of different crRNA molecules that are directed to the same organism preferably is limited to a maximum of 10. It will be clear that detection of a target organism by all different crRNA molecules provides confirmation that the identification of the target organism is correct.

[00130] In addition, a specific crRNA molecule may be present in multiple copies in a CRISPR/Cas-based ribonucleic acid detection system according to the invention, for example in multiple wells of a microtiter plate such as a 48 well plate, a 96 well plate, a 192 well plate, a 384 well plate or a 768 well plate. Detection of a target organism by multiple copies of a crRNA molecule provides confirmation that the identification of the target organism is correct.

BRIEF DESCRIPTION OF THE DRAWINGS

[00131] Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

[00132] Figure 1A is a schematic diagram showing the operon for the type III-B CRISPR system from *Haliangium ochraceum* DSM 14365, together with associated proteins.

[00133] Figure 1B is a schematic diagram showing the arrangement of components in the type III-B protein complex.

5 [00134] Figure 1C is a schematic diagram of the interaction between the SAVED-CHAT protein when activated by coA₃ and the PCaspase protein.

[00135] Figure 2a is schematic representation of the *H. ochraceum* type III-B CRISPR-Cas operon containing the Cmr1-6 genes encoding the crRNA-guided type III protein complex and its associated genes: *SAVED-CHAT*, *PCc-σ*, *PCi*, *PCK* and *PCaspase* (locus tags Hoch_1313-1323).

[00136] Figure 2b is a genomic neighborhoods diagram for *SAVED-CHAT* and *PCaspase*.

[00137] Figure 2c shows the domain architecture of *SAVED-CHAT*.

[00138] Figure 2d shows *SAVED* domain phylogenetic midpoint-rooted tree.

15 [00139] Figure 2e shows cyclic oligonucleotide-based antiphage signaling system (CBASS) type operons containing *Haliangium* *SAVED* clade domains with associated proteins containing cyclase, protein kinase, Sigma-70 and transposase domains.

[00140] Figure 3a is a schematic representation of the second *H. ochraceum* type III CRISPR-Cas operon and its associated genes: *SAVED-CHAT*, *PCc-σ*, *PCi*, *PCK*, and *PCaspase* (locus tags Hoch_5578-5588).

20 [00141] Figure 3b are *CHAT* domain general and individual focal clade phylogenetic trees.

[00142] Figure 3c is a phylogenetic tree of caspase domain focal clade.

[00143] Figures 3d and 3e are phylogenetic trees of *PCK* and *PCc-σ*, respectively. All trees are midpoint-rooted, scale bars indicate substitutions per position in the alignment.

[00144] Figure 4a is an amino acid sequence alignment of certain *CHAT* domain-containing proteins known in the art. The shading corresponds to the category of amino acid (i.e. polar, basic, etc.,) in accordance with the Clustal coding scheme.

25 WP_015211748.1/1-592 [SEQ ID NO: 2] is the *SAVED* domain-containing protein from *Oscillatoria nigro-viridis*; WP_046712262.1/1-650 [SEQ ID NO: 3] is the *SAVED* domain-containing protein from *Myxococcus fulvus*; WP_095983898.1/1-651 [SEQ ID NO: 4] is the *SAVED* domain containing-protein from *Cystobacter fuscus*; WP_082175316.1 [SEQ ID NO: 5] is the *SAVED* domain-containing protein from *Archangium gephyra*;
30 WP_012830652.1 [SEQ ID NO: 6] is the *CHAT* domain-containing protein from *Haliangium ochraceum*; WP_012826486.1 [SEQ ID NO: 1] is the *CHAT* domain-

containing protein from *Haliangium ochraceum*. The shading in Figure 2 is in grey-scale yet in original colour version follows the Clustal default colour coding as follows:

Category	Colour	Residue at position
Hydrophobic	BLUE	A,I,L,M,F,W,V
		C
Positive charge	RED	K,R
Negative charge	MAGENTA	E
		D
Polar	GREEN	N
		Q
		S,T
Cysteines	PINK	C
Glycines	ORANGE	G
Prolines	YELLOW	P
Aromatic	CYAN	H,Y
Unconserved	WHITE	any / gap

[00145] Figure 4b is an alignment of SAVED-CHAT protein sequences known in the art.

5 The SAVED and CHAT domains are indicated. The residues involved in cOA binding are shown as solid triangles and protease activity shown as open triangles.

[00146] Figure 4c is an alignment of CHAT-SAVED protein sequences known in the art.

[00147] Figure 5 is evolutionary tree built using a neighbourhood-joining algorithm from the sequence-alignment presented in Figure 4a.

10 **[00148]** Figure 6a is a photograph of an SDS-PAGE gel showing the results of a SAVED-CHAT cleavage assay showing its dependency on coA₃ for cleaving PCaspase.

[00149] Figure 6b is a photograph of an SDS-PAGE gel showing the results of cleavage activity of SAVED-CHAT on PCc-σ and PCi.

[00150] Figure 6c is a photograph of an SDS-PAGE gel showing the results of cleavage activity of activated PCaspase on PCc- σ and PCi.

[00151] Figure 6d is a photograph of a native SDS-PAGE gel showing oligomerization of SAVED-CHAT upon coA₃ addition.

5 **[00152]** Figure 7 is a photograph of an SDS-PAGE gel wherein comparison with various controls shows that only through coA₃ activation of SAVED-CHAT, PCaspase is cleaved. Plus and minus above each well indicate presence and absence of coA₃ respectively. A catalytically dead version H375A/C422A of SAVED-CHAT (dSAVED-CHAT) does not cleave PCaspase.

10 **[00153]** Figure 8 is a photograph of an SDS-PAGE gel showing the co-factor requirements of PCaspase cleavage by SAVED-CHAT.

[00154] Figure 9 is a photograph of an SDS-PAGE gel showing how PCi inhibits PCc- σ cleavage by PCaspase. An increasing concentration of PCi reduces the ability PCaspase to cleave PCc- σ , cleavage of PCi is also incomplete. Molar ratios of PCi:PCaspase range
15 from 1:20 – 2:1.

[00155] Figure 10 is a photograph of an SDS-PAGE gel showing various control that cleaved PCaspase, by cA₃ activated SAVED-CHAT, leads to the cleavage of a reporter protein (casein). A catalytically dead version of caspase-like (dPCaspase) does not cleave casein.

20 **[00156]** Figure 11 is a photograph of an SDS-PAGE gel showing co-factor requirements PCaspase cleavage of casein. SDS-PAGE protein cleavage assays with SAVED-CHAT, PCaspase and casein, demonstrating that the activity of PCaspase does not require co-factors.

[00157] Figure 12 is a photograph of an SDS-PAGE gel showing how PCi inhibits casein
25 cleavage by PCaspase. An increasing concentration of PCi reduces the ability PCaspase to cleave casein and complete cleavage of PCi. Molar ratios of PCi:PCaspase range from 1:20 – 2:1.

[00158] Figure 13a a real-time fluorescence assay plot of various combinations of proteins. The transparent bands represent the standard error of the mean (technical
30 replicates, n=3). The plot shows how FAM-peptide is specifically cleaved by activated PCaspase

[00159] Figure 13b is a real-time fluorescence assay plot of increasing molar ratio (PCi:PCaspase) of PCi, which reduces the cleavage activity of a FAM-peptide substrate by activated PCaspase. The faded colors represent the standard error of the mean (n=3).

[00160] Figure 13c is a real-time fluorescence assay plot for increasing cOA₃ concentrations. The sensitivity of the FAM-peptide visualization method for cOA₃ is 15.6 nM. The faded colours represent the standard error of the mean (technical replicates, n=3).

5 **[00161]** Figure 14 is a plasmid map of expression vector pJS-BCD.

[00162] Figure 15a is a 2D class averages of SAVED-CHAT bound to cOA₃, showing side and top views. In the side view, the filament curvature is evident, with the CHAT domain at the periphery of the arch. In the top view, singlet filaments form a partial inter-filament doublet, with two singlets running in opposite polarities forming cross-fiber contacts
10 spanning ~3 monomers.

[00163] Figures 15b and 15c are 3.1 Å-resolution cryo-EM reconstruction and model of the cOA₃-bound SAVED-CHAT filament. In some monomers, the CHAT domain is disordered and absent from the reconstruction (see diagrammatic solid tone areas).

[00164] Figure 15d is a close-up view of the CHAT-CHAT singlet intra-filament interface,
15 consisting of a four-helix bundle.

[00165] Figure 15e is a close-up view of the CHAT-CHAT doublet inter-filament interface.

[00166] Figure 15f shows cOA₃ binding site, at the interface between adjacent SAVED domains.

[00167] Figure 15g shows the CHAT domain active site. The residues H375 and C422
20 that comprise catalytic dyad (central dark portion) are 3.8 Å apart and located within the substrate-binding channel beneath two unstructured gating loops.

[00168] Figure 16a shows flexibility of SAVED-CHAT monomer. Four AlphaFold2 models were aligned based on the SAVED domain. Solid spheres correspond to the same residue in all four models, highlighting the flexibility.

25 **[00169]** Figure 16b shows alignment of a single SAVED-CHAT proteolytic active site with TPR-CHAT (Csx29) bound to type III-E CRISPR effector in complex with activating non-self target RNA and substrate peptide Csx30. TPR-CHAT and SAVED-CHAT His-Cys active site catalytic dyad aligned in both structures, poised for cleavage of Csx30, confirming that the structure of SAVED-CHAT is in an active conformation.

30 **[00170]** Figure 17 is a diagram of a model of the type III-B CRISPR-Cas system and its associated genes in *H. ochraceum*. Binding of coA₃ to the SAVED domain of SAVED-CHAT induces oligomerization that activates its CHAT domain. Activated SAVED-CHAT cleaves PCaspase, which subsequently mediates further downstream events by cleaving PCi (inhibitor of PCaspase), PCc-σ (a transcriptional response) and potentially also PCK

(likely phosphorylation / toxicity) and/or other (host) proteins. Broken lines indicate hypothesized events.

[00171] Figure 18 shows how PCaspase activation reduces the transformation efficiency of a target plasmid. (A) is a schematic overview of the experimental setup in *E. coli* BL21-AI. (B) is data of transformation efficiencies (relative to the non-target control) of target or non-target plasmid in *E. coli* co-expressing *H. ochraceum* type III CRISPR-Cas complex and different combinations of effector proteins. (C) is data of transformation efficiencies (relative to the non-target control) when SAVED-CHAT and PCaspase are co-expressed with PCi and PC- σ . Error bars represent the standard deviation of the mean. Statistical significance was calculated using one-sided unpaired Welch's t-test. * - $p < 0.05$, ** - $p < 0.005$, *** - $p < 0.0005$ ($n = 3$ biological replicates).

[00172] Figure 19 shows linearized plasmid maps and transformation efficiencies with repressed protein expression for the plasmid challenge assay. (A) is a map of the *H. ochraceum* type III CRISPR-Cas expression plasmid pHochTypeIII, with *cmr1-cmr6* from the SAVED-CHAT genomic neighbourhood, *csb2* from a co-occurring type I-G system, and the associated CRISPR array with a single spacer sequence targeting a protospacer on pTarget. (B) are maps of the effector expression plasmids, showing the different combinations of effectors used in the study. pEcNucC and pEmpty were used as positive and negative controls respectively. (C) are maps of the pTarget and pNon-target, having identical backbones except for a protospacer on pTarget. (D) is data showing transformation efficiencies (relative to the non-target) of pTarget or pNon-target in *E. coli* with repressed expression of *H. ochraceum* type III CRISPR-Cas complex and different combinations of SAVED-CHAT and PCaspase. (E) is data for transformation efficiencies (relative to the non-target) with repressed expression of different combinations of SAVED-CHAT, PCaspase, PCi, and PC- σ . Statistical significance was calculated using one-sided unpaired Welch's t-test. * - $p < 0.05$, ** - $p < 0.005$, *** - $p < 0.0005$ ($n=3$).

[00173] Figure 20 shows expression of the post-cleaved PCaspase fragments in *E. coli*. Transformation efficiencies of plasmids constitutively expressing PCaspase ("WT"), or the N- (aa 1-153) and/or C-terminal (aa 154-666) PCaspase fragment(s).

[00174] The inventors have discovered a novel class of allosterically-activated proteases associated with type III CRISPR-Cas systems. Various aspects of the invention are as hereinbefore defined.

[00175] Where the cA_x -dependent protease is a SAVED-CHAT or CHAT-SAVED protein, it may be as hereinbefore defined with reference to any of the reference sequences SEQ ID NO: 14 or SEQ ID NO: 15 or any variant sequence of at least 70% identity therewith,

including functional fragments thereof. As further described herein in more detail, a variant sequence may have any percentage identity from 70% to 100%.

[00176] Additionally, or alternatively, to the amino acid structure of the SAVED-CHAT as hereinbefore defined, the SAVED-CHAT protein may comprise one or more of the

5 following amino acid (peptide) motifs, wherein X is any amino acid:

GX(V/A)X(I/L)PXXX(H/Y)LGXXL **[SEQ ID NO: 16]**

(P/N)XAXXXDXXXL(R/E)XXXPX(I/V)(V/L)HXX(S/G)HG **[SEQ ID NO: 17]**

DXX(A/S)XXF(S/A)XX(F/L)YXXLXXXXX(I/V)XXAF **[SEQ ID NO: 18]**

[00177] Additionally, or alternatively to (a) the amino acid structure of the SAVED-CHAT protein and/or (b) the one or more amino acid (peptide) motifs, as hereinbefore defined, the SAVED-CHAT protein may comprise one or more of the amino acids R106, K121, Q122, N129, Q184, R185, R243, Q274, R276, Y285, F286, H375 or C422. This amino acid position numbering is referenced to SEQ ID NO: 14 (see Figure 4b) but will be adjustable to the equivalent position of any other particular SAVED-CHAT amino acid sequence if it is of a different overall length.

[00178] Additionally, or alternatively, to the amino acid structure of the CHAT-SAVED protein as hereinbefore defined, the SAVED-CHAT may comprise one or more of the following amino acid (peptide) motifs, wherein X is any amino acid and wherein the underlined amino acid is not subject to substitution or change, but any of the other specified amino acids may be substituted conservatively as elsewhere herein defined:

LRLVVLXACX **[SEQ ID NO: 19]**

IPAVIASQMPLS **[SEQ ID NO: 20]**

XPLPLAXXXGX **[SEQ ID NO: 21]**

IHLFLAXPXSXAX **[SEQ ID NO: 22]**

25 **[00179]** Further possible SAVED-CHAT proteins are described in Makarova K. S. *et al.*, (2020) Nucleic Acids Research 16: 8828 – 8847 and/or EMBL-EBI Interpro Classification of protein Families: SAVED-CHAT
<https://www.ebi.ac.uk/interpro/protein/UniProt/?ida=79522f25ef21f197fad0c9e4c4def3ad4bb6d23b#tableas>

30 as set forth in Table 1 below. As well as the proteins listed in Table 1 are variants of these proteins having at least 70% identity to the amino acid sequences thereof, or functional fragments of these proteins or sequence variants.

Table 1

Accession No.	Domains	Entry Name	Protein names	Gene Name	Organism
A0A1Q3GUM1	SAVED-CHAT	A0A1Q3GUM1_9BACT	CHAT domain-containing protein	BKI52_15820	marine bacterium AO1-C
A0A538PUU4	SAVED-CHAT	A0A538PUU4_9DELT	SAVED domain-containing protein	E6J90_50190 E6J91_35800	<i>Deltaproteobacteria</i> bacterium
A1ZW70	SAVED-CHAT	A1ZW70_9BACT	CHAT domain-containing protein	M23134_06692	<i>Microscilla marina</i> ATCC 23134
D0LG35	SAVED-CHAT	D0LG35_HALO1	CHAT domain-containing protein	Hoch_5578	<i>Haliangium ochraceum</i> (strain DSM 14365 / JCM 11303 / SMP-2)
D0LT12	SAVED-CHAT	D0LT12_HALO1	CHAT domain-containing protein	Hoch_1319	<i>Haliangium ochraceum</i> (strain DSM 14365 / JCM 11303 / SMP-2)

[00180] An alignment of sequences is shown in Figure 4.

[00181] Further possible CHAT-SAVED proteins are set forth in Table 2 below are described in EMBL-EBI Interpro Classification of protein Families: CHAT-SAVED

5 <https://www.ebi.ac.uk/interpro/protein/UniProt/?ida=fe89a97dba89c15dd1e7455158a2e6552cb831ac#table>

as set forth in Table 1 below. As well as the proteins listed in Table 1 are variants of these proteins having at least 70% identity to the amino acid sequences thereof, or functional fragments of these proteins or sequence variants. As well as the proteins listed in Table 2 are variants of these sequences of at least 70% identity therewith, or functional fragments thereof.

Table 2

Accession No.	Domains	Entry Name	Protein name	Gene Name	Organism
A0A085WRI0	CHAT- SAVED	A0A085WRI0_9DELT	High-affinity carbon uptake protein Hat/HatR	DB31_5335	<i>Hyalangium minutum</i>
A0A0G2ZWP7	CHAT- SAVED	A0A0G2ZWP7_9DELT	CHAT domain-containing protein	AA314_04845 ATI61_118112	<i>Archangium gephyra</i>
A0A150NZE3	CHAT- SAVED	A0A150NZE3_SORCE	CHAT domain-containing protein	BE08_26600	<i>Sorangium cellulosum</i> (<i>Polyangium cellulosum</i>)
A0A150PTC0	CHAT- SAVED	A0A150PTC0_SORCE	CHAT domain-containing protein	BE04_43580	<i>Sorangium cellulosum</i> (<i>Polyangium cellulosum</i>)
A0A1L9AVB4	CHAT- SAVED	A0A1L9AVB4_9DELT	SAVED domain-containing protein	BON30_46110	<i>Cystobacter ferrugineus</i>
A0A1Q3HNP2	CHAT- SAVED	A0A1Q3HNP2_9DELT	CHAT domain-containing protein	BO221_16505	<i>Archangium</i> sp. Cb G35
A0A250IU23	CHAT- SAVED	A0A250IU23_9DELT	CHAT domain-containing protein	CYFUS_000656	<i>Cystobacter fuscus</i>
A0A2S9XEX7	CHAT- SAVED	A0A2S9XEX7_9DELT	CHAT domain protein	ENSA5_55320	<i>Enhygromyxa salina</i>

A0A2T4V0U2	CHAT- SAVED	A0A2T4V0U2_VITXG	CHAT domain- containing protein	DAT35 _5475 5	<i>Vitiosangium</i> sp. (strain GDMCC 1.1324)
A0A2T4V8T9	CHAT- SAVED	A0A2T4V8T9_VITXG	SAVED domain- containing protein	DAT35 _3974 5	<i>Vitiosangium</i> sp. (strain GDMCC 1.1324)
A0A2W4TP91	CHAT- SAVED	A0A2W4TP91_9CYA N	SAVED domain- containing protein	DCF25 _2021 5	<i>Leptolyngbya foveolarum</i>
A0A321LDF2	CHAT- SAVED	A0A321LDF2_9BACT	SAVED domain- containing protein	DMF6 1_186 00	<i>Blastocatellia bacterium</i> AA13
A0A355ETU9	CHAT- SAVED	A0A355ETU9_9BACT	CHAT domain- containing protein	DD490 _0091 5	<i>Acidobacteria bacterium</i>
A0A355F9L6	CHAT- SAVED	A0A355F9L6_9BACT	CHAT domain- containing protein	DD490 _3253 5	<i>Acidobacteria bacterium</i>
A0A3A8JIK6	CHAT- SAVED	A0A3A8JIK6_9DELT	SAVED domain- containing protein	D7V88 _1287 5	<i>Corallococcus terminator</i>
A0A3A8JP65	CHAT- SAVED	A0A3A8JP65_9DELT	SAVED domain- containing protein	D7W8 2_007 20	<i>Corallococcus</i> sp. CA049B
A0A3A8PG37	CHAT- SAVED	A0A3A8PG37_9DELT	SAVED domain- containing protein	D7V93 _2326 0	<i>Corallococcus llansteffanensis</i>
A0A3A8QUY7	CHAT- SAVED	A0A3A8QUY7_9DELT	SAVED domain- containing protein	D7W8 1_056 95	<i>Corallococcus aberystwythensis</i>
A0A3A8RJB2	CHAT- SAVED	A0A3A8RJB2_9DELT	SAVED domain- containing protein	D7X99 _2262 5	<i>Corallococcus</i> sp. AB032C
A0A3A8RL35	CHAT- SAVED	A0A3A8RL35_9DELT	SAVED domain- containing protein	D7X99 _1865 0	<i>Corallococcus</i> sp. AB032C

A0A3A8TCN7	CHAT- SAVED	A0A3A8TCN7_9DELT	CHAT domain- containing protein	D7Y15 _4039 0	<i>Corallococcus</i> sp. AB030
A0A3A8UW57	CHAT- SAVED	A0A3A8UW57_9DELT	SAVED domain- containing protein	D7Y15 _1129 5	<i>Corallococcus</i> sp. AB030
A0A3M9YX02	CHAT- SAVED	A0A3M9YX02_9CYAN	SAVED domain- containing protein	EDM0 5_336 85	<i>Leptolyngbya</i> sp. IPPAS B-1204
A0A4Q5ZXK4	CHAT- SAVED	A0A4Q5ZXK4_9DELT	SAVED domain- containing protein	EOO71 _1269 5	<i>Myxococcaceae</i> bacterium
A0A554GQQ6	CHAT- SAVED	A0A554GQQ6_9DELT	SAVED domain- containing protein	FOF48 _0247 0	<i>Corallococcus</i> sp. Z5C101001
A0A6G9SFC3	CHAT- SAVED	A0A6G9SFC3_9CYAN	SAVED domain- containing protein	HCG51 _1135 5	<i>Tolypothrix</i> sp. PCC 7910
A0A7Y4IS77	CHAT- SAVED	A0A7Y4IS77_CORCK	SAVED domain- containing protein	HMI51 _0170 0	<i>Corallococcus coralloides (Myxococcus coralloides)</i>
A0A7Y4N8A0	CHAT- SAVED	A0A7Y4N8A0_MYXXA	SAVED domain- containing protein	HNV27 _2074 0	<i>Myxococcus xanthus</i>
A0A838RJD3	CHAT- SAVED	A0A838RJD3_9CHLR	CHAT domain- containing protein	H0T73 _0993 0	<i>Ardenticatenales</i> bacterium
A0A844MA26	CHAT- SAVED	A0A844MA26_9CYAN	SAVED domain- containing protein	F7734 _1160 0	<i>Scytonema</i> sp. UIC 10036
A0A848LLT7	CHAT- SAVED	A0A848LLT7_9DELT	CHAT domain- containing protein	HG543 _2849 5	<i>Pyxidicoccus fallax</i>
A0A8A4TJN5	CHAT- SAVED	A0A8A4TJN5_9BACT	CHAT domain- containing protein	J3U87 _2960 5	<i>Sulfidibacter corallicola</i>

A0A8A4TNQ9	CHAT- SAVED	A0A8A4TNQ9_9BAC T	SAVED domain- containing protein	J3U87 _3352 5	<i>Sulfidibacter corallicola</i>
A0A8D6NLI3	CHAT- SAVED	A0A8D6NLI3_9CYAN	SAVED domain- containing protein	CAL77 16_04 8530	<i>Calothrix</i> sp. PCC 7716
A0A8E4WWM0	CHAT- SAVED	A0A8E4WWM0_MYX XA	SAVED domain- containing protein	I5Q59 _3586 5	<i>Myxococcus xanthus</i>
A0A8J7E4Q2	CHAT- SAVED	A0A8J7E4Q2_9CYAN	SAVED domain- containing protein	IQ254 _1347 0	<i>Nodosilinea</i> sp. LEGE 07088
A0A8J7Q9E5	CHAT- SAVED	A0A8J7Q9E5_9BACT	SAVED domain- containing protein	J3U88 _1775 5	<i>Acanthopleuribacter pedis</i>
A0A8J7QDT5	CHAT- SAVED	A0A8J7QDT5_9BACT	SAVED domain- containing protein	J3U88 _2601 5	<i>Acanthopleuribacter pedis</i>
A0A8J7U1Z9	CHAT- SAVED	A0A8J7U1Z9_9BACT	CHAT domain- containing protein	J3U88 _0634 5	<i>Acanthopleuribacter pedis</i>
A0A8S9T9D2	CHAT- SAVED	A0A8S9T9D2_9CYAN	SAVED domain- containing protein	DA73_ 04000 29135	<i>Tolypothrix bouteillei</i> VB521301
Q1CW20	CHAT- SAVED	Q1CW20_MYXXD	CHAT domain- containing protein	MXAN _7290	<i>Myxococcus xanthus</i> (strain DK1622)
WP_015211748.1	CHAT- SAVED	K9VRM4_9CYAN	n/a	n/a	<i>Oscillatoria nigro- viridis</i>
WP_074960092.1	CHAT- SAVED	n/a	n/a	n/a	<i>Myxococcus xanthus</i>
WP_046712262.1	CHAT- SAVED	A0A0F7BS87_MYXFU	n/a	n/a	<i>Myxococcus fulvus</i>
WP_082175316.1	CHAT- SAVED	n/a	n/a	n/a	<i>Archangium gephyra</i>

[00182] Mutations may be tolerated in any of the referenced CHAT-**SAVED** proteins or **SAVED**-CHAT proteins of tables 1 or table 2, or any of the reference sequences SEQ ID Nos 1 – 6, 14 and 15, or the consensus sequence motifs SEQ ID NOS: 16 - 22, and may

include deletions or substitutions or combinations thereof. For example, the mutations may be conservative or non-conservative amino acid substitutions.

[00183] “Conservative amino acid substitutions” refer to the interchangeability of residues having similar side chains, and thus typically involves substitution of an amino acid in a polypeptide with amino acids within the same or similar defined class of amino acids. By way of example, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid, e.g., alanine, valine, leucine, and isoleucine; an amino acid with a hydroxyl side chain may be substituted with another amino acid with a hydroxyl side chain, e.g., serine and threonine; amino acids having aromatic side chains may be substituted with another amino acid having an aromatic side chain, e.g., phenylalanine, tyrosine, tryptophan, and histidine; an amino acid with a basic side chain may be substituted with another amino acid with a basic side chain, e.g., lysine and arginine; an amino acid with an acidic side chain may be substituted with another amino acid with an acidic side chain, e.g., aspartic acid or glutamic acid; and a hydrophobic or hydrophilic amino acid may be substituted with another hydrophobic or hydrophilic amino acid, respectively. Exemplary conservative substitutions are provided below:

Residue	Possible Conservative Substitutions
A, L, V, I	Other aliphatic (A, L, V, I)
G, M	Other non-polar (A, L, V, I, G, M)
D, E	Other acidic (D, E)
K, R	Other basic (K, R)
N, Q, S, T	Other polar
H, Y, W, F	Other aromatic (H, Y, W, F)
C	None
P	None

[00184] “Non-conservative substitution” refers to substitution of an amino acid in a polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and may affect (a) the structure of the peptide backbone in the area of the substitution (e.g., proline for glycine) (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[00185] "Deletion" refers to modification of a polypeptide by removal of one or more amino acids in comparison to a wild-type or control polypeptide. Deletions can comprise removal of 1 or more amino acids, 2 or more amino acids, or 3 or more amino acids of the polypeptide while retaining enzymatic activity. Deletions can comprise a continuous segment or can be discontinuous.

[00186] The inventors have discovered a type III-B system in *Haliangium ochraceum* DSM 14365 that is flanked by a second messenger oligonucleotide or dinucleotide synthetase-associated and fused to various effector domains (SAVED) -Caspase HetF associated with TPR (CHAT) protein and a Caspase-like gene (see Figures 1A and 2a). The type III CRISPR-Cas system from *Haliangium ochraceum* DSM 14365 comprises a multi-subunit type III-B protein complex and this, together with suitably complementary guide/crRNA, targets RNA molecules for cleavage (see Figure 1B). Base pairing of the target RNA triggers the target RNA cleavage, but also activates the production of cyclic oligoadenylate (cOA) second messenger molecules, made from ATP. These cOA messenger molecules activate the SAVED-CHAT protein (see Figure 1C).

[00187] More particularly, the inventors identify a set of genes that resides close to an operon encoding a type III-B CRISPR-Cas crRNA-guided protein complex (Cmr1-6) in the *Haliangium ochraceum* DSM 14365 genome. These are shown in Figures 1A and 2a. The inventors observed a SAVED-CHAT gene, which encodes a fusion protein of a SAVED sensory domain and a CHAT domain (related to cysteine proteases that include the caspases, known to be involved in controlled cell death in eukaryotes). Further downstream, the inventors observe a gene encoding a caspase-like protein, which is named PCaspase (Prokaryotic Caspase). In between these genes, a set of three genes encode: a sigma-factor, which is named PCc- σ (Prokaryotic Caspase-controlled sigma factor), a hypothetical gene, which is named PCi (Prokaryotic Caspase inhibitor, due to predicted structural homology with the CI-2 family of serine protease inhibitors), and a gene encoding a serine/threonine protein kinase, which is named PCk (Prokaryotic Caspase-controlled kinase). A similar operon appears to be encoded elsewhere on the chromosome of *H. ochraceum* (see Figure 3a).

[00188] The inventors identified six additional bacterial genomes encoding SAVED-CHAT and PCaspase genes within the same gene cluster from the phyla Myxococcota (n=2), Bacterioidota (n=2) and Cyanobacteria (n=1) (Fig. 1b). Only the gene cluster of *Enhygromyxa salina_A* contained an identical gene set as *H. ochraceum*, with the exception of PCi, where homologs could not be identified in other prokaryotes. Type III CRISPR-Cas systems were present in the neighboring regions of two of the operons. In the remaining four operons CRISPR-Cas was missing in the neighboring genes, but genes

encoding SMODS cyclase domains were found to cluster with SAVED-CHAT, indicative of CBASS defense systems.

[00189] Sensory domains like SAVED are often fused to different types of effector domains, implying a separate evolutionary origin of the SAVED and CHAT domains in SAVED-CHAT (Figure 2c). Phylogenetic trees were calculated for respective domains from representative prokaryotic and eukaryotic proteomes. The CHAT domains of the two SAVED-CHAT variants were only distantly related, indicating independent acquisition events (see Figure 3b). The *H. ochraceum* SAVED domains, however, formed a monophyletic clade (Figure. 3d) and were likely acquired once and duplicated, leading to the two SAVED copies to which CHAT domains of different origin were added subsequently. This scenario is further supported by phylogenies of PCaspase, PCc- σ , and PCk (see Figures 2b - 2e), which all support monophyly of the two respective copies. The SAVED domains are most closely related to those of other Myxococcota and may be part of a conserved system in these bacteria. All but one gene neighborhoods of SAVED-CHAT relatives contain a setup reminiscent of a cyclic oligonucleotide-based antiphage signaling system (CBASS) defense system (see Figure 2e) composed of two to three putative cyclases, a protein kinase homolog of PCk, a Sigma-70 like sigma factor and a ubiquitin activating ThiF gene. Therefore coA sensory and effector components of a CBASS system appear co-opted in *H. ochraceum* to work in tandem with a Type-III CRISPR-Cas system.

[00190] The inventors have cloned and purified the various proteins in *E. coli* and discovered that SAVED-CHAT is activated by coA₃ signaling molecules. The coA₃-activated SAVED-CHAT subsequently cleaves the PCaspase protein at a particular site.

Example 1: SAVED-CHAT & PCaspase cleavage activity

[00191] All type III-associated proteins were purified from *H. ochraceum* (except for PCk, which could not be cloned either individually or in combination with PCc- σ and PCi, likely due to toxicity) and conducted *in vitro* cleavage assays with SAVED-CHAT incubated with any of the three other proteins.

[00192] Protein cleavage assays were also performed with SAVED-CHAT and PCaspase in combination with either PCc- σ or PCi alone or with PCc- σ and PCi together.

[00193] Protein purification.

For recombinant expression and purification of SAVED-CHAT, PCaspase (and mutants hereof), PCc- σ and PCi, the expression vector pJS-BCD (Figure 14 and SEQ ID NO: 23) was used. Particular plasmids used were:

Plasmid name	Cloned gene
pJS-BCD-Strep-SAVED-CHAT	N-terminal Strep-tagged SAVED-CHAT
pJS-BCD-Strep-dSAVED-CHAT	N-terminal Strep-tagged dSAVED-CHAT (H375A / C422A)
pJS-BCD-Strep-PCaspase	N-terminal Strep-tagged PCaspase
pJS-BCD-Strep-dPCaspase	N-terminal Strep-tagged dPCaspase (H78A / C145A)
pJS-BCD-Strep-PCc- σ	N-terminal Strep-tagged PCc- σ
pJS-BCD-Strep-PCi	N-terminal Strep-tagged PCi

E. coli BL21 (DE3) cells transformed with the expression vector were inoculated in 1.5 L lysogeny broth (LB) and grown at 37 °C with 150 rpm shaking. After an optical density (O.D. 600 nm) of 0.6-0.7 was reached, a cold shock on ice for 30-60 min was performed.

- 5 Protein expression was induced by adding 0.5 mM IPTG (isopropyl β -D-1-thiogalactopyranoside). Subsequently, cells were grown at 16 °C for 18-24 hours and harvested via centrifugation. Pelleted cells were resuspended in 20-30 mL Wash Buffer (150 mM NaCl, 100 mM Tris, pH 8.0) and a complete protease inhibitor tablet (Roche) was added. Cells were lysed by sonication and lysate was clarified by centrifugation and
- 10 subsequent filtration (0.45 μ M). The supernatant was applied to a StrepTrap XT 5 mL column (Cytiva) and the bound protein was eluted with Elution Buffer (Wash Buffer + 50 mM biotin). Finally, a Superdex 200 (Cytiva) size exclusion chromatography column was used with Wash Buffer as eluate. After confirmation with SDS-PAGE analysis, protein fractions were pooled and concentrated to a working stock of 5 μ M.

15 Protein cleavage activity assay.

- [00194]** All cleavage assays were performed in a buffer containing 125 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM DTT. For SAVED-CHAT, PCaspase (and mutants thereof), PCc- σ , and PCi, a final protein concentration of 0.5 μ M was added, unless otherwise specified. Depending on the assay, a final concentration of 1 μ M coA₃ (Biolog.de), 5 mM
- 20 EDTA, 5 mM EGTA, 2 mM MgCl₂, 2 mM CaCl₂, 1 mM ATP and 0.1 mg/mL casein was added. After incubation for 1h at 35 °C, SDS loading dye was added and incubated for a further 5 min at 95 °C. SDS-PAGE analysis using Coomassie blue staining was utilized to visualize the results (Bio-Rad Gel Doc XR).

SAVED-CHAT complex formation on native PAGE.

[00195] For native PAGE analysis of SAVED-CHAT complex formation, 1 μ M SAVED-CHAT was incubated with or without 1 μ M coA₃ for 1h at 35 °C in a buffer containing 125 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM DTT. Afterwards, the reaction was run on
5 a native 4-20% polyacrylamide gel, stained with Coomassie blue, and visualized (Biorad Gel Doc XR).

[00196] Figure 6a, 7 and 8 show that SAVED-CHAT specifically cleaves PCaspase, in a coA₃-dependent and co-factor independent manner, whereas no cleavage was observed for PCc- σ and PCi (Fig. 6b). SAVED-CHAT cleaved PCaspase into at least three defined
10 fragments in addition to a myriad of products. Figure 7 shows how a catalytically dead version of SAVED-CHAT (dSAVED-CHAT, H375A, C422A) abrogated this activity.

[00197] Figure 6c shows cleavage of PCaspase by coA₃-induced SAVED-CHAT resulted in the subsequent cleavage of PCc- σ and PCi into defined cleavage products.

[00198] These results indicate that PCaspase cleavage by SAVED-CHAT activates its
15 proteolytic activity, similar to other eukaryotic caspases. Interestingly, when PCi was present, one of the cleaved PCaspase fragments was stabilized and the smear of degradation products of PCaspase was absent. Furthermore, the efficiency of PCc- σ cleavage by PCaspase was decreased when PCi was included in the reaction, indicating that PCi acts as an inhibitor for PCaspase activity. Indeed, similar PCaspase-mediated
20 PCc- σ cleavage reactions with increasing PCi concentrations showed increasing rates of inhibition (Figure 9). Lastly, addition of coA₃ prevented the migration of SAVED-CHAT in a native gel, indicating that SAVED-CHAT oligomerizes to form large complexes in the presence of coA₃ (Figure 6d).

Example 2: Activated PCaspase protein has general protease activity

[00199] Biologically unrelated casein protein was used as a substrate for activated
25 PCaspase in an *in vitro* cleavage assay. Activated PCaspase leads to the degradation of casein in a co-factor independent manner, while SAVED-CHAT by itself does not (see Figures 10a and 11). A catalytic mutant of PCaspase (dPCaspase, H78A, C145A) abrogates this activity completely. A PCi titration experiment demonstrates that PCi
30 inhibits PCaspase activity on casein, demonstrating this inhibitory effect is independent from PCc- σ (Figure 12).

Example 3: Prototype fluorescent *in vitro* diagnostic assay

[00200] To monitor PCaspase activity in real-time in an *in vitro* diagnostic assay format, a
small fluorophore-quencher peptide was provided as a substrate, which is specifically
35 cleaved by activated PCaspase (Figure 13a).

[00201] Real-time PCaspase activity assay. SAVED-CHAT/PCaspase activity assays were conducted in vitro in activity buffer (125 mM NaCl, 10 mM Tris, 1 mM DTT, pH 8.0) to which different combinations of component were added: SAVED-CHAT (0.5 μ M), PCaspase (0.5 μ M), coA₃ (15.6 nM to 1 μ M), and/or FAM-peptide substrate (5 μ M) (Eurogentec AS-60579-01). Assays were incubated for one hour at 37 °C with a FAM channel measurement at 1 min intervals in a Thermo Scientific Quantstudio 1 RT-qPCR instrument running Quantstudio Design & Analysis software (v1.5.2). Data was visualized using Graphpad Prism 9 (n=3) with standard error depiction.

[00202] Using the assay, the inhibitory effect of PCi on PCaspase is monitored in real-time, which shows a clear, inverse relationship between reaction kinetics and amount of PCi (Figure 13b). To probe the sensitivity and kinetics of this detection method to coA₃, a concentration range was used to determine near instant signal generation in the presence of >31.2 nM and a limit of detection of 15.6 nM (Figure. 13c). This fluorescence assay avoids the time and labour involved in running an SDS-PAGE gel as a read-out of SAVED-CHAT & PCaspase activity.

Example 4: Structural basis for SAVED-CHAT activation

[00203] To understand the structural basis for coA₃-induced activation, the cryo-EM structure of the activated SAVED-CHAT in complex to coA₃ was determined.

Structural analyses.

[00204] 10 μ M SAVED-CHAT was mixed with 125 μ M coA₃. 2.5 μ l of complex was immediately applied to C-flat grids (1.2/1.3, 300 mesh) which had been plasma-cleaned for 30 seconds in a Solarus 950 plasma cleaner (Gatan) with a 4:1 ratio of O₂/H₂. Grids were blotted with Vitrobot Mark IV (Thermo Fisher) for 6 seconds, blot force 0 at 4°C & 100% humidity, and plunge-frozen in liquid ethane. Data were collected on a FEI Glacios cryo-TEM equipped with a Falcon 4 detector. Data was collected in SerialEM, with a pixel size of 0.94 Å, a defocus range of -1.5 - -2.5 μ m, and a total exposure time of 15s resulting in a total accumulated dose of 40 e/Å² which was split into 60 EER fractions. Motion correction, CTF estimation and particle picking was performed on-the-fly using cryoSPARC Live v4.0.0-privatebeta. Data were collected on a FEI Glacios cryo-TEM equipped with a Falcon 4 detector, as described for the binary complex. In total, 2,550 movies were collected. All subsequent data processing was performed in cryoSPARC v3.2. 1,229,738 particle co-ordinates were picked, selected and subjected to 2D classification. Multiple rounds of ab-initio modelling and heterogeneous refinement resulted in a subset of 39,001 particles, which yielded a 3.1 Å-resolution which was used for modelling. For modelling, multiple AlphaFold2 models of the individual SAVED and CHAT domains were rigid-body fitted into the map. coA₃ was built in Coot, and other adjustments were performed in

Isolde, before running real-space refinement in Phenix. All structural figures and movies were generated using ChimeraX.

[00205] Size-exclusion chromatography (SEC) indicated that while SAVED-CHAT is monomeric in solution, coA₃ binding induces formation of large, polydisperse oligomers.

5 Prolonged incubation of coA₃ with SAVED-CHAT resulted in the formation of large, polymorphous aggregates that were not amenable to structural determination. However, by vitrifying immediately after mixing SAVED-CHAT and coA₃, a structure of the filament at a global resolution of 3.1 Å was obtained.

[00206] SAVED-CHAT oligomerization results in long, curved filaments with the SAVED domain on the inside and the CHAT domains at the periphery, with a curvature of ~10° between each monomer (Figure 15a). SAVED-CHAT monomers assemble via head-to-tail oligomerization, with a single coA₃ bound at the interface between two SAVED domains, forming 'singlet' filaments. We additionally observed partial 'doublets' where two antiparallel filaments make a cross-fiber interaction spanning up to three SAVED-CHAT monomers (Figure 15a).

[00207] In the 'singlet' regions of the map, the CHAT domains are poorly resolved and often absent due to molecular motion (Figures 15b and 15c). Analysis of AlphaFold2 models of monomeric SAVED-CHAT revealed a high degree of flexibility between the two globular domains, with an unstructured linker (residues 297-304) acting as a hinge to accommodate a maximum displacement of up to 35 Å (Figure 16). It is likely that the strain induced by the filament curvature prevents more than three successive CHAT domains from forming stable inter-fiber contacts.

[00208] The intra-filament head-to-tail CHAT-CHAT interface is comprised of a four-helix bundle with two helices from each protomer (Figure 15d). This bundle is held together through a network of predominantly electrostatic interactions. The CHAT-CHAT inter-filament doublet interface is mediated by an unusual π - π stacking interaction between two R349 residues (that is, the same residue from different monomers), reinforced by additional electrostatic contacts (Figure 15e).

[00209] The coA₃ is buried within the intra-filament interface between two SAVED domains, and participates in a plethora of hydrogen bonds, electrostatic and stacking interactions (Figure 15f). This network of contacts suggests that coA₃ acts as a molecular glue to bridge SAVED domain intra-filament interactions, which subsequently provides a platform for CHAT domain rigidification, doublet formation and ultimately substrate capture. This is a distinct mechanism of activation from the TIR-SAVED filaments which assemble a composite active site across two adjacent TIR domains within a filament which assembles upon coA₃-binding, or the CRISPR-associated Lon protease (CalpL) activation that

appears to have a concentration-dependent effect, reflecting the mechanistic diversity of cOA-responsive ancillary factors.

[00210] Within the resolved doublet CHAT domains, the active site is positioned underneath two unstructured loops which create a narrow substrate-binding channel (Figure 15g). This channel is wide enough to accommodate unstructured peptides, but would likely be inaccessible to highly structured peptides. The Cys422-His375 catalytic dyad are aligned and positioned $\sim 4\text{\AA}$ apart, confirming that the coA₃-bound filament represents the active conformation of SAVED-CHAT. Comparison with the CHAT domain of the activated type III-E protein TPR-CHAT bound to proteolytic substrate Csx30 revealed a near-identical active site configuration where the catalytic dyad is aligned, and the substrate-binding channel is exposed (Figure 16).

[00211] Without wishing to be bound by any particular theory, the model shown in Figure 17 summarises the inventors understanding of the CRISPR-Cas type III-B system in *H. ochraceum* and its effector components, where detection of target RNA results in the generation of coA₃ (not shown in Figure 17). The synthesized cOA₃ binds to the sensory domain of SAVED-CHAT. Acting as a molecular glue, cOA₃ participates in a multitude of interactions that initially stabilize SAVED:SAVED dimerization (Figures 15a - g). The subsequent multimerization of the unusual antiparallel SAVED-CHAT doublet filament results in the activation of the CHAT domains, a unique mechanism for CARF and SAVED effector proteins (Figures 15a - g). The activated CHAT domains cleave and activate PCaspase, which in turn becomes an active protease, cleaving at least PCc- σ and PCi into defined products (Figure 6a - d). In contrast to the specific cleavage of PCaspase by SAVED-CHAT, PCaspase might have a broader substrate repertoire, as demonstrated by the cleavage of the casein and FAM-peptide substrates (Figures 13b and c).

[00212] Interestingly, the inventors found that PCi is a potent inhibitor of PCaspase on all tested substrates, hinting at an auto-regulated feedback loop to specifically control PCaspase activity

[00213] This experiment demonstrates how the SAVED-CHAT/PCaspase proteins can be coupled via the coA₃ messenger to a type III CRISPR-Cas system that can be programmed to recognize an RNA (or DNA) target of choice. Once the targeted RNA or DNA is recognised, then cleavage of the nucleic acid results in a production of the coA₃ signalling molecules that in turn activated SAVED-CHAT/PCaspase proteins to cleave the fluorescently labelled casein reporter protein, thereby providing a visual readout. In this way, a read-out method is provided which is not dependent on nucleic acid reporter molecules and this has various advantages.

Example 5: SAVED-CHAT and PCaspase activation leads to a strong defense mechanism phenotype

Plasmid interference assay

[00214] To assess SAVED-CHAT/PCaspase-mediated *in vivo* response,
 5 electrocompetent *E. coli* BL21-AI (Invitrogen) carrying pHochTypeIII and one of the pEffectors (see Figures 18A, 19A and 19B) were prepared for target/non-target plasmid transformation.

[00215] For pHochTypeIII, *H. orchaceum* type III-B *cmr1-6*, *csb2*, and a minimal CRISPR array carrying one spacer sequence were placed individually under the control of T7
 10 promoters. For the pEffectors, the various effector genes (and mutants or fragments thereof) were cloned into one operon and placed under the control of a constitutively expressing lacUV5 promoter. For the target/non-target plasmids, a non-coding RNA was placed under the control of a trc promoter and lacO. This non-coding RNA carries the protospacer targeted by the spacer of the crRNA guide encoded by the CRISPR array on
 15 pHochTypeIII.

[00216] The transformations were carried out in biological triplicates, using 100 ng of target or non-target plasmid, by electroporation (BTX electroporation system). After transformation, all cells were recovered in 1 mL of LB at 37 °C for 1h. Ten-fold dilutions were plated on LB agar medium containing 0.2% arabinose, 34 µg/mL chloramphenicol,
 20 50 µg/mL carbenicillin, 50 µg/mL kanamycin, and either 1 mM IPTG or 0.2% glucose to induce or repress target RNA transcription, respectively. Finally, the plates were incubated overnight at 30 °C, and transformation efficiencies were quantified.

[00217] To assess the activity of cleaved PCaspase fragments *in vivo*, genes coding for either PCaspase residues 1-153, 154-666, and their combination, were cloned into a
 25 pEffector coding for SAVED-CHAT as well. The resulting pSAVED-CHAT_PCaspase fragments plasmids were transformed into BL21-AI in biological triplicates, using 5.25 fmol for each plasmid, and recovered in 1 mL of LB at 37 °C for 1h. Ten-fold dilutions were plated on LB agar medium containing 50 µg/mL carbenicillin. Finally, the plates were incubated overnight at 37 °C, and transformation efficiencies were quantified.

30 [00218] Data was analyzed and visualized using R version 4.3.0, with statistical significance calculated by one-sided unpaired Welch's t-test.

[00219] To test the activity of the type III complex, SAVED-CHAT and PCaspase on cell viability, the pHochTypeIII plasmid was constructed, which encodes *H. ochraceum* Cmr1 to Cmr6 (forming the type III complex), *csb2* (a cas6 homolog processing the pre-crRNA),
 35 and a minimal CRISPR array containing two repeats and a spacer (see Figure 19A). The

SAVED-CHAT and PCaspase genes were expressed in different combinations from the pEffector plasmid (see Figure 19B). NucC (a cA3-responsive nuclease that causes abortive infection) from *Escherichia coli* MS115-1 and an empty vector were used as positive and negative controls, respectively (see Figure 19B). Target and non-target plasmids encoded an isopropyl- β -D-thiogalactopyranoside (IPTG) inducible protospacer that was complementary and non complementary to the spacer, respectively (see Figure 19C).

The target or non-target plasmid was transformed into *E. coli* BL21-AI that harboured pHochTypeIII and one of the pEffector plasmids (see Figure 18A). Ten-fold serial dilutions were plated on glucose- or IPTG-containing plates to repress or induce, respectively, the target or non-target RNA to calculate transformation efficiencies. First, we demonstrated that the *H. ochraceum* type III system produces cA3 upon target RNA recognition and leads to an abortive infection phenotype in the presence of NucC (see Figures 18B and 19D). When both SAVED-CHAT and PCaspase were present, target RNA expression reduced transformation efficiency by six orders of magnitude (see Figures 18B and 19D). Additionally, we investigated the *in vivo* effect of expressing different combinations of the two expected PCaspase fragments after R153 cleavage, along with SAVED-CHAT. None yielded a strong reduction in transformation efficiency compared with expression of full-length, wildtype PCaspase, perhaps owing to changes in the structural conformations of the fragments or weaker interactions when they were expressed separately (see Figure 20). Overall, *H. ochraceum* type III-B produces (at least) cA3 and mounts a robust defence response in combination with its effector proteins SAVED-CHAT and PCaspase.

AMINO ACID SEQUENCES

[00220] CHAT domain-containing protein [*Haliangium ochraceum*] NCBI Reference Sequence: WP_012826486.1 [SEQ ID NO: 1]

MTHHADHDCQPHRESVQPDEIQCILVLDLSIDNAITACSVTPHLPRRAARRVELHLNDFGAE
RAPHYGGASDRRTWRCWMQAVDAMLADARAQLGAEVEFTHYYLAGRAALPVFAYLGLRL
GKQANITTVNRRDDGCWDVVPQCRRPASSAASGVSPSARFFDEVRLDTERSSSESGMV
AVWWSTQRDVRGLLRAFARARGDRDLGIVSLRARPAAGDDTGDMRLLLEGADGPDA
RELVNCFRSIPNQYPRSSGLMVFSVPVTLAAMVGRAINPRIHGPVWWPYFRGGYEYEP
LEYPWPLISGPPRILIATANAPEGENPTLDVEAELEKHLEEALEPRKRKLCEVQRCPAATV
SDITSALRSFKPHILHFHGHGTALGVYLRSAEHDGAQFVRGEDFQQMIATSLRQKDREMH
VVLNACCTHELAKALTEQVSCTIGTDIEVYDSASIHFAARFYDHLVHGTSVHYAFNAAVDE
CRAHSTSGQEVFCLHPAAERAPASATPPVRADELVFFSPAERHS

[00221] SAVED domain-containing protein [*Oscillatoria nigro-viridis*] NCBI Reference
Sequence: WP_015211748.1 **[SEQ ID NO: 2]**

MGQNWAITIGINGYRNLQSLNYAKFDAEQVFFRQELNIQTVYHFADDSLPIKQDYGSD
5 LSHPTYTTLNRRFFRVRFEESFLKSGDNLWFFFAGHGKRHKDRDYLPIDVDPGDVESS
GISLQYVSERLRRSGADNVVMLIDACRSGNGQRDGVGFGQEKQQGVISLFSCSPGESSY
EINELQHGSFTYALLEGKIEGESNCATVERLYQRLRQRVPSLTQQYRKVQQTPYGVVEP
PTKYHLILLPRKANLTDIVTLENDALLAEVQQNFQLAKQLWIRVLAVSPTNSKAIEGIERLAR
VVPAGYGSCKTDVQHPVGTFFAIRHQSFELLTVRISEESLPAHFRHYKIQHIEFYLSFFTN
10 GVCDPISAVQQNNNLINDFLVVFQANPEAVIGYYGIVHIPLQFCAGYAVSTWPRIALFELDR
DVNCWYELAGDDSPKLDLSVSNISRPANAVAVAIRIAISFDITKNDVDDVVPQPYEDIQIKIG
KRRIDAITHYSQVKEVCNAFRQVLDLHTRVDKSLIVHIFYSGPVSLGFSLGRRISRTIHHQ
VIVYNYTSHTSPRYAWGVKINSADSPESRVISTKLLIHDTS

15 **[00222]** SAVED domain-containing protein [*Myxococcus fulvus*] NCBI Reference
Sequence: WP_046712262.1 **[SEQ ID NO: 3]**

MSTKEPLEITLEFTGTRKEFSWKPKRYFIKPTFGEGESVSFPWSEMQRELESLKRQDPGS
DVFERFGRRLRRLAPADWASDEDAINDAFAAGQPVHLTIRSKNADEIYYLPWEALWLKG
GNRLGSMEDCLIQYESASQSSRELMLHPTGRILVAWSAKGGWVPADKHIEAISQACRDS
20 GLHFNVPEDILPDVSRKRLAEKLEETERPVTALHLLCHGGELDGPDSNAYGLVFNVPDPE
DGRPDLLDASLLRDLLFKSRRPSSLRLVILCACQGGDAGAPAHVPVGSVARMFHRQGVPA
VLASRLPLSCSGSVSLTRTLYQGLLVKRDNLRTVLSAARTQLLRDERSSDWISLQFYARS
RDEASLSPFSEPAPTTAPAATSRELVLIRHEAYSTAHGEPSEAEDAPTLFAHRSVRPLVSLD
QASALSGRRWENLEPEVARLAARDGILRRVFEERDIDIVYFGFPYVSFAVLAGYLAKTRP
25 VHVLEHDRTTQRFTWQQGAKGPAPQLRVDAASRD TGSVARVRLSISADVRLEECAAVLP
DSEVRDLHFSLEKPARGVVRREAQLLEYAQSI REAIDQHIAGNPGFNNGGVHVFAAVPVS
AFHLGRALAFGLPECFVYNYDGQDIPHYKWRLSLQAAAEGRRPAVTLFPS

30 **[00223]** SAVED domain-containing protein [*Cystobacter fuscus*] NCBI Reference
Sequence: WP_095983898.1 **[SEQ ID NO: 4]**

MARMPRTSKVEELSLEFLRKNKTHDPYNFSYEREEYIVMRSYGAPKPAFFPWDDTQLQK
DLAGLSALRPNDLAVARIGRRLREFLDTADWAADEEAEKALREKRPVHLTIRSHAAELYY
LPWELLPLRVSGTLLGELPDFLLRYQWPGTQSQDAGQDTIRILLACSAAGERVPFAEHRE
AIEDARSSRAGIISARTELSFLSNANRQSLALALRDPDRPIDVLHLLCHGKQLEGNTCGLVL
35 NSEDREEGPDYLTPSDIRRLVSTIEKPPRLIVLCVCQSSHTGTPAHLGSAIEAFHRQGIPA

VIASRLPLSGTGSILFTKAFYEELLGGSGHLRTALVAARQQFLQKSLDWASLQLYAREGD
 DAALDPFRRSTPAPRVLPTRSSELVLLCHEAYDRAYVAPGADDPALFSNREARKVVIDQ
 TLALKGRNWERLGDEVKWLSPGELLQLLTKRDTELVYYGFPYIPLAVLVGFLANTRPV
 HVVEYDREVERFTWMRDATGSFPPLRKKRSEYATGSVARLRLSISATVNVNDCEEVLPS
 5 RDVGLDLHFTVKTPQRGIVRREAQVQAYIQQIRAAALDRYVAGNSRIQALHVFASVPVSI AF
 RLGQALAATGLPACCVYNYGAQELPRYKWRLWLRADPTGSPLVDVF

[00224] SAVED domain-containing protein [*Archangium gephyra*] NCBI Reference

Sequence: WP_082175316.1 **[SEQ ID NO: 5]**

10 MEVLVEVRAMQVVLEPLKLYGGLLALHQDQFNQVTHPPVGLLRGQLQRRRELEEGARVH
 RAELPCEVRHSPRDER SAGHHEEPSEGLAQGRSRRREAAQQPPLQRADDLKQLGRQR
 PAPGAHQPGSEARTCQFRVYRFLLPNFRQRIPSRVLRPALRIGQALIALGLAIHVHERLP
 ARRDLEPGAQLALRISDELLPVLRGEHVCLGDDLVIQSVHDTPLRTTGEAIFRLLPERCLH
 HSRKHHSGGARSRSRCPKIRGEPFRARTRKKLRQDLSPESSRLCAERLMNSYREIHLRFT
 15 QNGPDSYRVAVDSTSVGSASATTPHLPLSRLRDLQLQIQAAALLQSRSCDAKIDDELARL
 GDALYEAICPPGRVREILTSLGGVLFSSDEQQQLRLFLHFDPCDSELAWLA EFPWDVL
 RIPPVSPTRHAALDHRLSIVRSLDVTQPSQQRALVRPLRVLLVRAGARGCGGLQFESEQD
 SITDALSSIVGVECESLDMPTRLDLRRKL RDFDPHVLHFMGHGKVDENTGNGWYLRDE
 KGAPVLLRAQDVAELFSSRFAPRLVVLNACQSARGTLSVTGHGSVAGALVSQGVA AVIA
 20 MQFAISDGAARAFTA EFYTCLAKGSSIDDAVTEGRLAMRSKVPDSFEWCTPALYMRAGV
 AGDLLQRELQEPADRVDSTPVVSGGAPAGDLVLLCHEAYAETHSAPEEEDAPALFAQRR
 TRKVLIDQKKHLQKREWAYVEPAVYALVDPEGGLQRAISEQGTTLLYYGFPLIPLAALAGH
 LVTTTRPVHVIEHDRESGRFTWSKATGEAIP ELKLDQLLHSTGSAVRVRLSISSEVELDDC
 QEVLPDERVRLDLHFRLEDPGRGAVRLEKQALEYARKLRATFDRLVAGKRALDSVHLFAA
 25 VPVSVAFLLGRELASTGLPPCFVYNFASRDSPRYRWRLCLQAAVEGRPSVDILGE

[00225] CHAT domain-containing protein [*Haliangium ochraceum*] NCBI Reference

Sequence: WP_012830652.1 **[SEQ ID NO: 6]**

[00226] MAGDYPRTMAITSRDSNRTPEPVQCLLILDFDENTYSERSFAEHLPPSERRRC
 30 YRATGFDKRRPHDQALPSRDWRAWQRAIDDMVDAARGELGDDEELAHYYYVTGRAPLP
 AFAHLGMRLSAFARISIVHLREGQACTLTPCTPPLGAKLAEPPFFDRVTGLERVEAYATG
 RVAVWISTQSEVDRAAIRAQLGEVPIVSLFATPAEQSSDNPQRHLTPADGPRVAAELFKR
 MRQLHNCYPRRSGITMFLSGPNILATMVGRALNRRLHCPVAWPNNYYRAYQGYRPAMMS
 PWPRVEGKARLLLIVAHTADSSRRRLNSDEELRNMYQSLLSKPMRDRCELRLLPAAARLSD
 35 FTSALRDFRPHIVHLIGHGAHGGLYFVDERQDGQFVSSEALCELLEVAELNDLQLVLLNAC

NSEYTAELISKSLPCTTIGTKLKVPDGTATSFSTRFYDDLAAGSSVAHAFHRALGEIKAGPS
EHADSYLLCPAATREGAELVIFAPEEAT

[00227] PCaspase Peptidase C14 caspase catalytic subunit p20 *Haliangium ochraceum*

5 (strain DSM 14365 / JCM 11303 / SMP-2) **[SEQ ID NO: 7]**

MMKRALLIGAPLAHSATHALAGVANDIDRAGEILRLYGFCDDVLFGERATRDGIVARLEA
LIAETQADDAVVIYFSGHGGRVVNTGIVKDLPGGDYRPGAHQFLVPEDYQPKAQTFTGIL
DFELRYLVARLAARTENVSVILDCCHSGGAIRAPGRPTPKALDEKSLKPHTVHLNRVIRER
LVQLRESLAASRAPRLTQDAHPHLVRIEAAHEFRLAYETRINNRRQQGVLTAAWADTLWRY
10 RGLAVSWQALASEIQARARAFVPMQYVHIDGPTERRLFLSLAREPARHSLAIVQERDGTW
LDGGRFQGVASGARFAVLPTSTESDIDSALAEVRAVEVMPERTRVELATAADDIPRWQ
GLHAWPLHARGAWTGVAVRAQTAAVCAALLRAIEASQRMHLCPDSDGPVATVIELSSDE
GSRIAVLDGHGVCVSPPVATPERALALVERLQRAALVRMLGSGTDEHALPIPLALRLERA
DGIGGTCTRHAEGRTEPPSELLLASAGAQVHAIVENHGSAIATDTQDVYLTMFCAADGRV
15 ERVSKQQSSGIRLCGGAKHRLEYELGAGGASLPEYRAADSSTSGYGLHSLIVVATDRQQ
ELRNLETWSGEGSAAKLQPGVLDLTASKRAPARRGSASASGPLRYAVLRVDLRVAAAVA
GRDAPTL

[00228] cmr1 type III-B CRISPR module RAMP protein Cmr1 *Haliangium ochraceum*

20 DSM 14365 **[SEQ ID NO: 8]**

MDAFCHTDGFPHPFEAHMIRMVADYELVTPAFIGGADNQTGFPELRVPSIKGALRFWWR
ALRWRQGISVAQLREQEARLFGSADSTIGRSRVSLALTGMTSSGAKVSKRGAKVSELF
DAAGARTTQQRQPAPGLTYLGYGLVTAKDGKLERGCLRPPLAFQLSLMFDPRERQDAK
ALTEQLAQAMQALGLLGGLGARSRRGFGSVALRALRIDGENAWEAPKTPGELVKRIRALH
25 GNDTRSSDSGPTPLPEWTALSARTRHLVLVGRPDAQDGTPTHLLEQLGQELVRFRSYGR
NNKILGNETPERRFADDHHLFKDVLKGKRPQTHPRRAVFGLPHNYYSSSDRKGMQVQP
RAHKLDRRGSPLLHGHRCQDQTPVLVLSFLPARFLPERRNKISVARHAVPLRPDEELWRP
ISEFLDRVRAPSRRDHLSITDAYEVPAP

30 **[00229]** Type III-B CRISPR-associated protein Cas10/Cmr2 *Haliangium ochraceum* DSM
14365 **[SEQ ID NO: 9]**

MTVKLHLSLGPVQAFIAESRRTRDLWWSYLLSYLAGRALYAAQHGIEVLPRVHDDVLA
LYAPGRTDRARLPAHASLPNRFVLECADQAAAVQAAEAATSALRAAWAHAGTVRKQFID
PVSGSDDDETQRIWRRQVESFWHVWWIGDDPALLDQRKHWRAAPALAPDPEPGEHCTM

MGRYQELSGFLRGQRGLEEFWIDVRARLGGAMNLDLRPDERLCAIALIKRLLPRVSGQAL
 GRRLDEEQVAWPSTLYMAARPWIGTVCEAQPEPAARYAKQVLRARASARGERKAGQTL
 LDALTGTSASTASAGAFPLLDGNFSFIGALENERATPLDREDERRGLVKALKALHARQGT
 GPSPYYAFLMDGDSLGTLLSRAEPRITDCLSDFTERVDPDIVCARGGATVYAGGDDVLA
 5 LLPVEGALPTALALARCYEQRFADSQLDRELLPAATISGAIVFAHYHLPLRYITARAHELLO
 HVAKDQTGRASLAISLHQSSGETARFSPVWSYLRTEDEDDRTTSIDPLLADIQAGRLGKSL
 YRLRALLGRISGAGEVGPVPLDLSTLHQAGAESGASDPVLDLFAAEIRSTRGDAERTPA
 QVRELAHILQAACRVVRRVAGSKHQIERGHLCLD GARLAYF MATGG SNEDEI

10 **[00230]** Type III-B CRISPR-associated protein Cmr3 *Haliangium ochraceum* DSM 14365
[SEQ ID NO: 10]

MTTRAYLLQPTDVWFFRDGRPYDRYEASQTAVKSLFPPSPLTVLGALRAGLARALGWRD
 GPWPAEICAVLGDGIEDLAELSLRGPYLARSVDPERPEPWWPLPVHLVGIVQNGVRHAQ
 ALGHREDEQSLPWRARALLRPSQEPIRCDLGEVHLPVPSSDKPAPPSERLSARPRYWWN
 15 TAGLDAILAGRLPKPEDVIAPPWQHQMVRVGIHRDETTRTTSDRAHALYSPLMVSLRPDFG
 LLAEMRGVPDTPDDPAPVLPLGGESRLAACQRVASPRAPSCPSNLIRKSRRRCVVVHLSP
 ARLSSLPRPGETLPDLPGARVVTACLRPLEQIGGWDGRDRAKARPRPLNPVVAAGSVWF
 CELDGDVDATLNMHDGRIGDDTRCGFGHLALGTWPA

20 **[00231]** Type III-B CRISPR-associated protein Cmr4 *Haliangium ochraceum* DSM 14365
[SEQ ID NO: 11]

MKAQLLGMLAESPIHSGSGQSTSFVDLPVSREAATDHPSIPGSSLKGALLDLARRRWQC
 RPDASVAADTGASDSATDNAPSGATPELLGDKNAAGSASRPRAQRVFGTAGHAGDLVV
 TDARLLLLPVRSLSVYAWWTCPIIERLVRDRARARLPAISNLDMAPLDNIKRGEALFADD
 25 GELFLEERQFTHRGRLPAGLLDLVEPLVAHETTRARLSAQIAVLSDHDFAWFARYGLAVS
 ARNQLDPEKKTSEN LWYEE SLPPDSVFYTL LCARGGDSL DLVSELFEEMPYLQVGGNET
 VGQGWFAIAQVRPGRADANAAGEPAGGAR

30 **[00232]** Type III-B CRISPR-associated protein Cmr5 *Haliangium ochraceum* DSM 14365
[SEQ ID NO: 12]

MSDSPCTIEQQRAADALSRIHEL RDQ RDCGNYSYAERLPAQIVTNGLGQALAMLR SRYT
 EDDQPPRQARDGARRQRGRDHDDPLADREPSNARDEVAYRYLFQHVGGWLCRDHASA
 PYRGVALNALLMKLSTCDQDTYVRAHAEALAYLNWLKKFARAFLSASKDEP

[00233] Type III-B CRISPR-associated protein Cmr5 *Haliangium ochraceum* DSM 14365
[SEQ ID NO: 13]

MSADKELFLHADAARHPTTSRRSTDTHAGLWYDKYYRWPASENQSSSVSKDRGQREWL
 QTLTTGDPIARADRLDEFAGRRLERLAQFHGGRTLYARTTSRFVTGLGRAHPVENGF
 5 HSLGTPYLPGSGIKGLVRAWAGRAQYSAEDIDALLGKAPGRNLAAEVGRVIFLDAVPTVP
 PQLEADVMTPHYGAYYQQTEAPGDWLSVPVIVFLVAAAGLEFQFPVLP
 SARNSATAASD
 DDLETVIDWMQHALRDVGAGAKTAVGYGRFADAWSGRLRYPLWWKDLTERREAQRPPLE
 RAIHAVQDMPEQEALTLVRVLSQGGSEHSDMEREHLRAALCQVYLEAWSQRGGMGNTG
 EKKRKLYLRWLRGET

10

[00234] Consensus amino acid sequence of SAVED-CHAT proteins **[SEQ ID NO: 14]**

MQELDLISAVMAGAIPVVNSGRASPDPPADAACAPHDVDHLSALLMEHGAPAMAIKCILIL
 DLIHNEISEAEVAEHLPRAAQRECLHLERFDAEVRPYDQEEIKDFGEPDRDWRAWKRAID
 ALFDAARQPLGRDDEDTHYYFAGRAPLPFAHLGSRLSDQARITKAFTHHRDDKEWYLD
 15 APCKPPASAKASALADAPFFDEVFGLLPDEASSATGGVAVRLSTQRPVDPADIRAFADA
 RGDADAEAGIVSLAAGPADGSVTNPQHDLSTEADGPDAAAEKLLRKLCNLYPRRSGFH
 VFAAGPTGLAFLVGRAINPRMHAPVQTPQYDGAAQGEYEPALPWLVAQTNEDLPKK
 VLVITACAADQENPRLNVEAEADEIEEALAEKAKRRDACEVIFCPAATLSDITSALREFKPHI
 VHIAGHGAAGGLYLG DGRGDGAQFVPS EDLQQEIEESWERLFRAYDMDLQLVILNACCS
 20 EELAEIAIEKIDCTVIGFDGEVADDDAIRFSARFYDSL GAGYSVADAFNAAKDEIRLGSSDA
 AGCFCLCPAAEREGAAAGDHANVEAAELVEFAPAEAEVFLVRRSTP

[00235] Consensus amino acid sequence of CHAT-*SAVED* proteins **[SEQ ID NO: 15]**

MNGRFMERPSMMKPMMGPLEFTLEFTRTESPEAPYAFDWKPQQYSARVAAGEVGSAS
 25 ACEPVELPWSLKKLRELLNEIDALLFLPDARGIEPKAEEDLGKPAVEPEELLRAAPVSDAR
 ESSEEPEAVLERLGHLLREFLEPAEKELGGWARYEAKIRTFLEGGVLFSSDALKQRLGRP
 VHLTIRSFANADELFYLPWELLPLPGNGRPLGAALDHCLSIVRYEWPKEAGQPRRPPLAL
 PPPGRILFASASPAGGGGKEVPLDAHLDAIREACLQDARLRFD FELDVLCAHVTLRSLREK
 LRLAKEEDRPPHVLHFLGHGGPVGGGGELEDLLAYDELGAYGLVLEPPDRPTGRPDRVD
 30 ARALADLLGLFSHADEDLRLVVLNACQGGDPKAGEPGHMLHAGGVAHDLHRQGNLIPAV
 IASQMPLSDDGSIAFTRAFYDALAQEGRSLDTALQAARAALSLAGIDQRPSSLDWASLQLF
 ARAPDAAALRLPPAQEPAAQTDES GGRPPGQRTPFSEPPPLPTAPASFRRLVLIAHEANF
 RKTPQVPTTPPTVELDAPELFAGRLGRPPDV LIDQPLALGERRDGWPNLEREVQRLLAPE
 AGKLRALGEKETGHRD TTVDLFGFAPLPLAFLLGWLLKDTRGYKVRVYQRDRGRSGR
 35 FEWWYDSGEPSPAEGEPFLRVEAWPTLEAGHEAGGRLAVRVSISAPVRSEDCAEVHLP

DEGDPSVGLALAEFPDPLDVVVRLVPLRETGPSRGAVRGPEDAAALVQEIRECLDRIRAE
LPDPDYRAKEIHLFAAVPVSLAFALGRALNAAKGLPELYEFNFRAEPEGYPKPRYKWRLG
LQAAKARRPERPSIKIFKSPSQGSNANHG

5 **[00236]** Plasmid JS-BCD-Strep-PCaspase **[SEQ ID NO: 23]**

TAATACGACTCACTATAGGGGCCCAAGTTCACCTTAAAAAGGAGATCAACAATGAAAGC
AATTTTCGTA CTGAAACATCTTAATCATGCAGGGGAGGGTTTCTAATGTGGAGCCACC
CGCAGTTCGAAAAAAGCGCGATGAAAAGGGCTCTGCTGATAGGTGCACCGCTGGCA
CATAGCGCAACCCATGCACTGGCAGGTGTTGCAAACGATATCGACCGTGCAGGTGAA
10 ATCCTGCGTCTGTATGGTTTTTCATTGTGATGACGTTCTCTTTGGTGAACGTGCAACTA
GAGATGGTATCGTTGCACGTCTGGAAGCACTGATCGCTGAAACCCAGGCTGATGATG
CTGTTGTTATCTATTTTAGCGGCCATGGTGGCCGTGTTGTTAACACCGGGATCGTTAA
AGATCTACCGGGTGGTGACTATCGTCCCGGCGCTCACCAGTTCCTGGTTCCGGAAGA
CTACCAACCGAAAAGCACAGACCTTTACCGGTATCCTGGACTTCGAGCTGAGGTATCT
15 AGTTGCACGTCTGGCAGCACGTACCGAAAACGTTAGCGTTATCCTGGATTGTTGTCAT
AGCGGTGGTGCAATAAGAGCACCGGGTAGACCGACCCCGAAAGCACTGGATGAAAA
AAGCCTGAAGCCGCATACTGTTACCTGAACCGTGTTATCCGTGAACGTCTGGTTCAA
CTGCGTGAATCGCTGGCAGCTAGCCGTGCACCGCGTCTGACCCAGGATGCTCATCC
GCATCTGGTTTCGTATCGAAGCAGCACACGAATTCCGTCTCGCATATGAAACCAGGAT
20 CAACAACCGTCAGCAGGGGGTTCTGACCGCAGCATGGGCAGACACCCTGTGGAGAT
ACCGTGGGCTGGCAGTTAGCTGGCAGGCACTGGCAAGCGAGATCCAGGCAAGGGCA
CGTGCAATTTGTTCCCATGCAGTATGTTACATCGATGGCCCGACCGAACGTCGTCTG
TTCAGCCTGGCTCGTGAGCCGGCACGTATAGCCTGGCAATCGTTCAGGAAAGGGA
CGGTACCCTGTGGCTGGACGGTGGCAGATTTAGGGTGTGCAAGCGGTGCACGTT
25 TCGCAGTTCTGCCGCCGACCAGCACCGAAAAGCGACATCGATAGCGCACTGGCAGAA
GTTTCGTGCAGTTGAAGTTATGCCGGAACGTACCCGTGTTGAACTGGCTACCGCAGCA
GATGACATCCCGAGATGGCAAGGCCTCCATGCATGGCCGCTGCATGCACGTGGCGC
TTGGACCGGTGTAGCAGTTTCGTGCTCAGACCGCAGCAGTTTGTGCTGCACTCCTGAG
AGCTATCGAAGCAAGCCAGCGTATGCATCTGTGTCCGATAGCGACGGGCCGGTTG
30 CTACCGTTATCGAACTATCGAGCGATGAGGGCAGCAGGATCGCAGTTCTGGATGGGC
ACGGTGTGTTGTGTTAGCCCCCGGTTGCAACCCCGAACGTGCACTCGCACTGGTTG
AAAGGCTCCAGAGAGCTGCACTCGTTCGTATGCTGGGGAGCGGTACCGATGAGCAT
GCACTACCGATCCCGCTCGCACTCAGACTGGAACGTGCAGACGGTATCGGTGGTAC
CTGCCGTCATCTAGCAGAAGGGCGTACCGAACC GCCGAGCGAACTGCTACTCGCAA
35 GCGCTGGGGCACAGGTACATGCAATCGTAGAGAACCATGGGAGCGCTATCGCAACC
GATACCCAGGATGTTTATCTGACCATGTTCTGTATCGCAGCAGACGGTCGTGTTGAAA

GAGTTAGCAAGCAGCAGAGCAGCGGGATCCGTCTGTGTGGGGGTGCAAAGCATAGG
CTCGAGTATGAACTAGGTGCAGGGGGTGCAAGCCTGCCGGAATACAGGGCAGCAGA
TTCGAGCACCAGCGGCTATGGGCTGCATAGCCTCATAGTTGTTGCAACCGACCGTCA
GCAGGAGCTGCGTAACCTGGAAACCTGGTCGGGTGAAGGTAGCGCTGCAAAACTGC
5 AGCCGGGTGTTCTGGATCTGACCGCTAGCAAACGTGCACCGGCTAGACGTGGCTCG
GCTAGCGCAAGCGGGCCGCTGAGATATGCTGTTCTACGTGTAGATCTGCGTGTAGCT
GCAGCAGTAGCAGGGCGTGATGCACCGACCCTGTAATGCCGACTCAGTTGCTGCTTC
TACTGGGCGCCCCGCTTCGGCGGGGTTTTTTTGAAGGGATCGGTTGTGAGTAAG
GATCTCCAGGCATCCTGTTGTTTGTGCGGTGAACGCTCTCTACTAGAGTCACACTGGCT
10 CACCTTCGGGTGGGCCTTTCTGCGTTTATAGGATCCTAACTCGAGCCTAGGGATATAT
TCCGCTTCCTCGCTCACTGACTCGCTACGCTCGGTCGTTGACTGCGGCGAGCGGA
AATGGCTTACGAACGGGGCGGAGATTTCTGGAAGATGCCAGGAAGATACTTAACAG
GGAAGTGAGAGGGCCGCGGCAAAGCCGTTTTTTCATAGGCTCCGCCCCCCTGACAA
GCATCACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAG
15 ATACCAGGCGTTTCCCCCTGGCGGCTCCCTCGTGCGCTCTCCTGTTCCCTGCCTTTCG
GTTTACCGGTGTCATTCCGCTGTTATGGCCGCGTTTGTCTCATTCCACGCCTGACACT
CAGTTCCGGGTAGGCAGTTGCTCCAAGCTGGACTGTATGCACGAACCCCCCGTTCA
GTCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGAAAGACA
TGCAAAAGCACCCTGGCAGCAGCCACTGGTAATTGATTTAGAGGAGTTAGTCTTGAA
20 GTCATGCGCCGGTTAAGGCTAAACTGAAAGGACAAGTTTTTGGTGAAGTGCCTCCTCC
AAGCCAGTTACCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAACCGCC
CTGCAAGGCGGTTTTTTTCGTTTTTCAGAGCAAGAGATTACGCGCAGACCAAAACGATCT
CAAGAAGATCATCTTATTAATCAGATAAAATATTTCTAGATTTAGTGCAATTTATCTCT
TCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTTACTAGTGCTTGGAT
25 TCTACCAATAAAAAACGCCCCGGCGGCAACCGAGCGTTCTGAACAAATCCAGATGGA
GTTCTGAGGTCATTACTGGATCTATCAACAGGAGTCCAAGCGAGCTCTCGAACCCCA
GAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAAT
CGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTGCGCGCCAAGC
TCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCC
30 AGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGC
AAGCAGGCATCGCCATGGGTACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTT
GAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCAT
CCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCG
CTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCA
35 TCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGC
CCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTGAGC
ACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTC

CTGCAGTTCATT CAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCC
 CCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCC
 CAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCA
 TCTTGTTCAATCATGCGAAACGATCCTCATCCTGTCTCTTGATCAGATCATGATCCCCT
 5 GCGCCATCAGATCCTTGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGGCTTCCC
 AACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGACGTCTGAATTCAAAAGATCTTA
 AGTAAGTAAGAGTATACGTATATCGGCTAATAACGTATTAAGGCGCTTCGGCGCCTTT
 TTTTATGGGGGTATTTTCATCCCAATCCACACGTCCAACGCACAGCAAACACCACGTC
 GACCCTATCAGCTGCGTGCTTTCTATGAGTCGTTGCTGCATAAC

10

[00237] PCk (GenBank: ACY13880.1) [SEQ ID NO: 42]

MDERDEDQENTRHPADAADGERMPADSPRPAGQSSDQGDSDSDSGDSGDSDEFGIGA
 LKLLDALSESDKDRVRARTLELPVPDRICGGRYELRERLGGGGMGTVYAGYDRQLERAV
 AIKRLHKRFAQDSSEASERLKREAIAMAQFASEPNVVQVYDIVADHDQAFLIMELISGTTLE
 15 KVQRKHSPSQAEIIDIYLGAGRGLAAIHRAHLVHRDFKPANVMIADNPASGHQRVIVCDLG
 LAITRALATSSSDSEPPSEPRPRALGEQFTATRALAGTPVYMAPEQLRGERDLDRCDQF
 AFCVALYEALSGTRPF AEAEAGAKSEPLLAIPAGPPPLPKRDGGAVPVRVEQALRRGLA
 FEPGERFPDMDALLVALAPPERFPWSVWLSLGLALALGATLLLLGRDAAPSCESQAQSK
 AAGLVDASALQRSEQRIPAQHRHAWLALRDVTTQRVNTWREEMSASCD AERRNDDDNA
 20 APITARKQACLENAAVLRTAGSYLTQEENESAPMFELADALRRMPSCIHLREEPKLVPPA
 DAASQSIMDEFHEVLAESSELREYEGRYDSAVELAGEALRQSEAMSFPYKDVLA AKARFRL
 SRAHAYAGDHNDAAENFDQAARAAAGFQLGAESLEGALFHAKYLLADLEKSTLAWEQLV
 RAELLLGWLGVDCEADDAEMPMDANWRIWACA EYDEANGLLASRLGELEQAIAYHEQ
 ALQWRERLSPAPPALDAFLHSKSLNNLANAEANLARQWMDEGEEDTATRYWDSAAAHY
 25 QAALRLRGEALGNDHPLVDRIQLNIRLLQVARGQEIGDDLRLGLRVLAQNLARSEVQLES
 LPGWLVLVIDAALGRYYGPSDPKDRDTAHLQSAAEAAAFIRSIHAQMPPSFMQHRRRVSE
 YLALAGVSEAKGQWSEALAELEHA FEILDEHDAATTCDQYLQNVYRSTLFS AASIVCAKPE
 SEPEQARSYLKRAFAPLADCAAADAVIDAIYEQTLMEPQDGGIPANCHP

[00238] PC-σ (GenBank: ACY13878.1) [SEQ ID NO: 43]

MDHDHRDDDEGKPALAEDSEYIDENVLGDADVLEDDDERGESDGDEVSDAELFASWAA
 GDKHAADVLRIRYMDKLSGYFAAKLSQGDSVADLVQQTLEGCHRAAPSFRGECSFRFTL
 YRVAGNKL RDFLRGKARRPRAEDIDEISVADLNPGPSTIRRQNHQAVLLDALRSLPLQM
 QELLEFRYQQDLSIREIAEILGIEVSATKSRLRRAKEALRRALGTSGADAL

[00239] Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of them mean “including but not limited to”, and they are not intended to (and do not) exclude other moieties, additives, components, integers or steps. Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

[00240] Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive. The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

[00241] The reader's attention is directed to all papers and documents which are filed concurrently with or previous to this specification in connection with this application and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

CLAIMS

1. A clustered regularly interspaced short palindromic repeats (CRISPR) based nucleic acid sequence detection system comprising:

5 (a) a type III CRISPR-associated effector protein complex which when comprised with a targeting RNA substantially complementary to a desired target polynucleotide sequence recognises that target sequence in a nucleic acid molecule and cleaves the nucleic acid molecule and produces a cyclic oligoadenylate (cA_x) messenger;

10 (b) a cyclic oligoadenylate (cA_x)-dependent protease; and

(c) a protein or polypeptide susceptible to cleavage by the protease of (b) and from which a detectable signal is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (b).

2. A detection system as claimed in claim 1, further comprising the targeting RNA.

3. A detection system as claimed in claim 1 or claim 2, wherein the cA_x-dependent protease is a second messenger oligonucleotide or dinucleotide synthetase-associated and fused to various effector domains-caspase HetF associated with Tetratricopeptide repeat (TPR) (SAVED-CHAT) protein; or a

20 CHAT-SAVED protein.

4. A detection system as claimed in claim 3, wherein the SAVED-CHAT protein has an amino acid sequence of SEQ ID NO: 14, or a sequence of at least 70% identity therewith, or a functional fragment thereof.

5. A detection system as claimed in claim 3 or claim 4, wherein the SAVED-CHAT protein comprises one or more of the following peptide motifs:

25 GX(V/A)X(I/L)PXXX(H/Y)LGXXL [SEQ ID NO: 16];

(P/N)XAXXXDXXXXL(R/E)XXXPX(I/V)(V/L)HXX(S/G)HG [SEQ ID NO: 17]; or

DXX(A/S)XXF(S/A)XX(F/L)YXXLXXXXX(I/V)XXAF [SEQ ID NO: 18].

6. A detection system as claimed in claim 3, wherein the CHAT-SAVED protein

30 has an amino acid sequence of SEQ ID NO: 15, or a sequence of at least 70% identity therewith, or a functional fragment thereof.

7. A detection system as claimed in claim 3 or claim 6, wherein the CHAT-
SAVED protein comprises one or more of the following peptide motifs:
LRLVVLXACX [SEQ ID NO: 19]; IPAVIASQMPLS [SEQ ID NO: 20];
XPLPLAXXXGX [SEQ ID NO: 21]; or IHLFLAXPX SXAX [SEQ ID NO: 22], wherein
5 X is any amino acid, wherein underlined amino acids are not substituted, and
wherein specified amino acids may independently be substituted conservatively
with another amino acid, as herein defined.
8. A detection system as claimed in any of claims 1 to 7, wherein the x in cA_x is
selected from 2, 3, 4, 5 or 6; preferably wherein the cA_x is cA₃.
- 10 9. A detection system as claimed in any preceding claim, wherein the protein
or polypeptide of (c) comprises an amino acid sequence susceptible to cleavage at
a site therein by the cA_x-dependent protease so as to generate at least two peptide
portions.
- 15 10. A detection system as claimed in claim 9, wherein at least one peptide
portion comprises a detectable marker.
11. A detection system as claimed in claim 10, wherein the detectable marker is
a fluorescent moiety; optionally wherein the protein or polypeptide prior to cleavage
has a quencher moiety, preferably not attached to the same peptide portion as the
fluorescent moiety.
- 20 12. A detection system as claimed in any preceding claim, wherein the protein
or polypeptide (c) is a PCaspase protein comprising an amino acid sequence of
SEQ ID NO: 7 or a sequence of at least 70% identity therewith, or polypeptide
fragment thereof, and wherein protease activity of CASPASE-like protein is
activated by the action of the cA_x-dependent protease.
- 25 13. A detection system as claimed in claim 12, wherein (d) a protein or
polypeptide substrate for the PCaspase protein or polypeptide is provided and
which when cleaved provides the detectable signal.
14. A detection system as claimed in claim 13, wherein the protein or
polypeptide substrate is cleaved into at least two peptide portions, and wherein at
30 least one peptide portion comprises a detectable label.
15. A detection system as claimed in claim 14, wherein the detectable label is a
fluorescent moiety; optionally wherein the protein substrate also comprises a

quencher moiety which on cleavage is not comprised in the cleavage portion comprising the fluorescent moiety.

16. A detection system as claimed in any of claims 13 to 15, wherein the protein substrate (d) comprises a portion, optionally an unstructured portion, comprising a positively charged amino acid, e.g. lysine (K) or arginine (R) amino acid; preferably the protein substrate is casein.

17. A detection system as claimed in any preceding claim, wherein the type III CRISPR-associated effector protein (Cas) is Cas10 with an amino acid sequence of SEQ ID NO: 9 or a sequence of at least 70% identity therewith.

18. A detection system as claimed in claim 17, wherein the type III CRISPR complex is the type III-B system comprising proteins with amino acid sequences of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 or sequences of at least 70% identity therewith; preferably wherein the type III-B system is from *Haliangium ochraceum* DSM 14365 and comprises proteins of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13.

19. A detection system as claimed in any preceding claim, further comprising a sample of nucleic acid; preferably wherein the sample comprises RNA.

20. A method of detecting a target polynucleotide sequence in a sample of nucleic acid, comprising combining in a reaction mixture:

- (a) a type III CRISPR-associated protein (Cas);
- (b) a targeting RNA substantially complementary to a desired target polynucleotide sequence; which with (a) above provides an effector complex;
- (c) a cyclic oligoadenylate (cA_x)-dependent protease; and
- (d) a protein or polypeptide susceptible to cleavage by the protease of (c) and from which a detectable signal is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (c).

21. A method as claimed in claim 20, wherein the cA_x-dependent protease is a second messenger oligonucleotide or dinucleotide synthetase-associated and

fused to various effector domains-caspase HetF associated with Tetratricopeptide repeat (TPR) (SAVED-CHAT) protein as set forth in claim 4 or claim 5, or a CHAT-SAVED protein as set forth in claim 6 or claim 7..

22. A method as claimed in claim 20 or claim 21, wherein the x in cA_x is
5 selected from 2, 3, 4, 5 or 6; preferably wherein the cA_x is cA₃.

23. A method as claimed in any of claims 20 to 22, wherein the protein or polypeptide of (d) comprises an amino acid sequence susceptible to cleavage at a site therein by the cA_x-dependent protease so as to generate at least two peptide portions.

10 24. A method as claimed in any of claim 23, wherein at least one peptide portion comprises a detectable marker.

25. A method as claimed in claim 24, wherein the detectable marker is a fluorescent moiety; optionally wherein the protein or polypeptide prior to cleavage has a quencher moiety, preferably not attached to the same peptide portion as the
15 fluorescent moiety.

26. A method as claimed in any preceding claim, wherein the protein or polypeptide (d) is a PCaspase protein comprising an amino acid sequence of SEQ ID NO: 7 or a sequence of at least 70% identity therewith, or polypeptide fragment thereof, and wherein protease activity of PCaspase protein is activated by the
20 action of the cA_x-dependent protease.

27. A method as claimed in claim 26, wherein (e) a protein or polypeptide substrate for the PCaspase protein or polypeptide is provided and which when cleaved provides the detectable signal.

28. A method as claimed in claim 27, wherein the protein or polypeptide
25 substrate is cleaved into at least two peptide portions, and wherein at least one peptide portion comprises a detectable label.

29. A method as claimed in claim 28, wherein the detectable label is a fluorescent moiety; optionally wherein the protein substrate also comprises a quencher moiety which on cleavage is not comprised in the cleavage portion
30 comprising the fluorescent moiety.

30. A method as claimed in any of claims 27 to 29, wherein the protein substrate (e) comprises a portion, optionally an unstructured portion, comprising a positively charged amino acid, e.g. lysine (K) or arginine (R); preferably the protein substrate is casein.

5 31. A method as claimed in any preceding claim, wherein the type III CRISPR-associated effector protein (Cas) is Cas10 with an amino acid sequence of SEQ ID NO: 9 or a sequence of at least 70% identity therewith.

32. A method as claimed in claim 31, wherein the type III CRISPR complex is the type III-B system comprising proteins with amino acid sequences of SEQ ID
10 NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 or sequences of at least 70% identity therewith; preferably wherein the type III-B system is from *Haliangium ochraceum* DSM 14365 and comprises proteins of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13.

15 33. A method as claimed in any of claims 20 to 32, further combining (g) a sample comprising nucleic acid; preferably comprising RNA, into the reaction mixture.

34. A method as claimed in any of claims 20 to 33, wherein the type III CRISPR-associated effector protein (Cas) complex (a) is combined with the targeting RNA
20 (b) prior to combining with the other components of the reaction mixture.

35. A method as claimed in any of claims 20 to 34, wherein the sequential order of combining of reaction components is: (g), then (a)/(b), then (c), then (d), then (e).

36. A method as claimed in any of claims 20 to 33, wherein the sequential order
25 of combining of reaction components is:

- (g), then (a), then (b), then (c), then (d), then (e); or

- (g), then (a), then (b), then (c), then (d), then (e).

37. A polynucleotide comprising a nucleotide sequence encoding one or more of:

30 (i) a type III CRISPR complex comprising proteins with amino acid sequences of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID

NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, or sequences of at least 70% identity therewith;

- 5 (ii) a SAVED-CHAT protein with an amino acid sequence of SEQ ID NO: 14 or a sequence of at least 70% identity therewith, or a functional fragment thereof;
- (iii) a SAVED-CHAT protein with an amino acid sequence comprising one or more of the following peptide motifs:
 GX(V/A)X(I/L)PXXX(H/Y)LGXXL [SEQ ID NO: 16];
 (P/N)XAXXXDXXXXL(R/E)XXXPX(I/V)(V/L)HXX(S/G)HG [SEQ ID
 10 NO: 17]; or DXX(A/S)XXF(S/A)XX(F/L)YXXLXXXXX(I/V)XXAF [SEQ ID NO: 18], wherein X is any amino acid;
- (iv) a CHAT-SAVED protein with an amino acid sequence of SEQ ID NO: 15, or a sequence of at least 70% identity therewith, or a functional fragment thereof;
- 15 (v) a CHAT-SAVED protein with an amino acid sequence comprising one or more of the following peptide motifs: LRLVVLXACX [SEQ ID NO: 19]; IPAVIASQMPLS [SEQ ID NO: 20]; XPLPLAXXXGX [SEQ ID NO: 21]; or IHLFLAXPXSXAX [SEQ ID NO: 22], wherein X is any amino acid, wherein underlined amino acids are not substituted, and
 20 wherein specified amino acids may independently conservatively substituted;
- (vi) a PCaspase with an amino acid sequence of SEQ ID NO: 7, or a sequence of at least 70% identity therewith;
- 25 (vii) a PCK with an amino acid sequence of SEQ ID NO: 24, or a sequence of at least 70% identity therewith;
- (viii) a PC- σ with an amino acid sequence of SEQ ID NO: 25, or a sequence of at least 70% identity therewith.

38. A polynucleotide as claimed in claim 37 which comprises a combination of nucleotide sequences selected from:

- 30 a. (i) and (ii), optionally further comprising (vi) and/or (vii) and/or (viii);
 b. (i) and (iii); optionally further comprising (vi) and/or (vii) and/or (viii);

- c. (i) and (iv) optionally further comprising (vi) and/or (vii) and/or (viii);
- d. (i) and (v) optionally further comprising (vi) and/or (vii) and/or (viii).

39. A polynucleotide as claimed in claim 37 or claim 38, further comprising a nucleotide sequence encoding a guide RNA with a sequence specific for a desired target nucleic acid sequence.

40. A plasmid comprising a polynucleotide of claim 37 or claim 38; optionally wherein the plasmid is a native conjugative plasmid, or an expression plasmid.

41. A viral vector comprising a polynucleotide of claim 37 or claim 38, or a plasmid of claim 40; optionally wherein the viral vector is a bacteriophage.

42. A composition comprising a polynucleotide of claim 37 or claim 38, or a plasmid of claim 40, and a nanoparticle.

43. A composition comprising a polynucleotide of claim 37 or claim 38, a plasmid of claim 40, a viral vector of claim 41, or a nanoparticle composition of claim 42.

44. A plasmid comprising a polynucleotide of claim 39; optionally wherein the plasmid is a native conjugative plasmid, or an expression plasmid.

45. A viral vector comprising a polynucleotide of claim 39 or a plasmid of claim 44; optionally wherein the viral vector is a bacteriophage.

46. A composition comprising a polynucleotide of claim 39 or a plasmid of claim 44, and a nanoparticle.

47. A composition comprising a polynucleotide of claim 39, a plasmid of claim 44, a viral vector of claim 45, or a nanoparticle composition of claim 46.

48. A kit comprising (a) a plasmid of claim 40, viral vector of claim 41, nanoparticle composition of claim 42, or a composition of claim 43; and (b) a separate polynucleotide, plasmid, vector or nanoparticle composition comprising a nucleotide sequence encoding a guide RNA with a sequence specific for a desired target nucleic acid sequence.

49. A composition comprising:

- (a) a type III CRISPR-associated protein (Cas);

(b) a targeting RNA substantially complementary to a desired target polynucleotide sequence; which with (a) above provides an effector complex; and

(c) a cyclic oligoadenylate (cA_x)-dependent protease.

5 50. A composition as claimed in claim 49, further comprising (d) a protein or polypeptide susceptible to cleavage by the protease of (c) and from which a detectable signal is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (c).

51. A composition as claimed in claim 50, wherein the cA_x-dependent protease
10 (c) is a second messenger oligonucleotide or dinucleotide synthetase-associated and fused to various effector domains-caspase HetF associated with TPR (SAVED-CHAT) protein as set forth in claim 4 or claim 5, or a CHAT-SAVED protein as set forth in claim 6 or claim 7.

52. A composition as claimed in claim 51, wherein the protein or polypeptide (d)
15 is a PCaspase protein comprising an amino acid sequence of SEQ ID NO: 7 or a sequence of at least 70% identity therewith, or polypeptide fragment thereof, and wherein protease activity of PCaspase protein is activated by the action of the cA_x-dependent protease.

53. A composition as claimed in claim 52, further comprising (e) a protein or
20 polypeptide substrate for the PCaspase protein or polypeptide, which when cleaved provides the detectable signal.

54. A composition as claimed in claim 53, wherein the protein or polypeptide substrate is cleaved into at least two peptide portions, and wherein at least one peptide portion comprises a detectable label.

25 55. A composition as claimed in claim 54, wherein the detectable label is a fluorescent moiety; optionally wherein the protein substrate also comprises a quencher moiety which on cleavage is not comprised in the cleavage portion comprising the fluorescent moiety.

56. A composition as claimed in any of claims 54 to 55, wherein the protein
30 substrate (e) comprises a portion, optionally an unstructured portion, comprising a positively charged amino acid, e.g. lysine (K) or arginine (R); preferably the protein substrate is casein.

57. A composition as claimed in any of claims 49 to 56, wherein the type III CRISPR-associated effector protein (Cas) is Cas10 with an amino acid sequence of SEQ ID NO: 9 or a sequence of at least 70% identity therewith.

58. A composition as claimed in claim 57, wherein the type III CRISPR complex
5 is the type III-B system comprising proteins with amino acid sequences of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 or sequences of at least 70% identity therewith; preferably wherein the type III-B system is from *Haliangium ochraceum* DSM 14365 and comprises proteins of
10 SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13.

59. A composition as claimed in any of claims 49 to 58, further comprising (f) a sample comprising nucleic acid; preferably wherein the nucleic acid is RNA.

60. A device for detecting a polynucleotide molecule comprising a defined nucleic acid sequence in a sample, comprising a container, wherein the container
15 comprises (i) a type III CRISPR-associated protein (Cas), (ii) a target RNA substantially complementary to the defined RNA sequence, which together with (i) provides an effector complex; (iii) a cyclic oligoadenylate (cA_x)-dependent protease; and (iv) a protein or polypeptide susceptible to cleavage by the protease
20 of (v) and from which a detectable signal moiety is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (iii).

61. A detection device as claimed in claim 60, further comprising the sample.

62. A device for detecting an polynucleotide molecule comprising a defined sequence in a sample, comprising a lateral flow substrate comprising (a) a sample receiving portion, (b) a flow through portion, and (c) a capture portion, wherein the
25 flow through portion comprises (i) an effector complex comprising a type III CRISPR-associated effector protein (Cas), (ii) a targeting RNA substantially complementary to the defined RNA sequence, (iii) a cyclic oligoadenylate (cA_x)-dependent protease; and (iv) a protein or polypeptide susceptible to cleavage by the protease of (iii) and from which a detectable signal moiety is generated whether
30 directly or indirectly by cleavage of said protein or polypeptide by the protease of (iii); and wherein the capture portion retains the detectable signal moiety.

63. A kit for the detecting of a polynucleotide molecule comprising a defined nucleic acid sequence in a sample, comprising at least one container, (i) a type III CRISPR-associated protein (Cas); (ii) a cyclic oligoadenylate (cA_x)-dependent protease; and (iii) a protein or polypeptide susceptible to cleavage by the protease of (ii) and from which a detectable signal moiety is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (ii).
64. A kit as claimed in claim 63, further comprising (iv) a targeting RNA substantially complementary to the defined RNA sequence, so that in combination with (i) an effector complex is provided.
65. A kit as claimed in claim 63 or claim 64, wherein each of (i), (ii), (iii) or (iv) is comprised in a separate container.

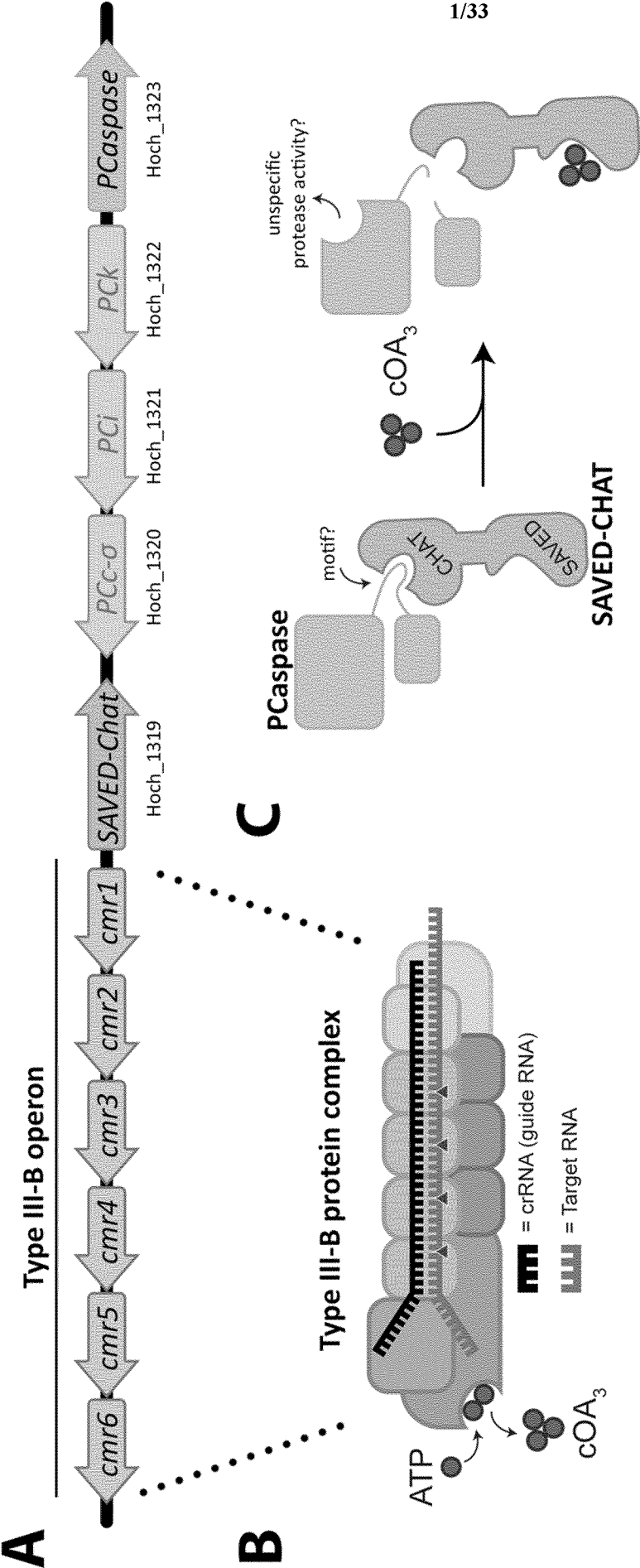
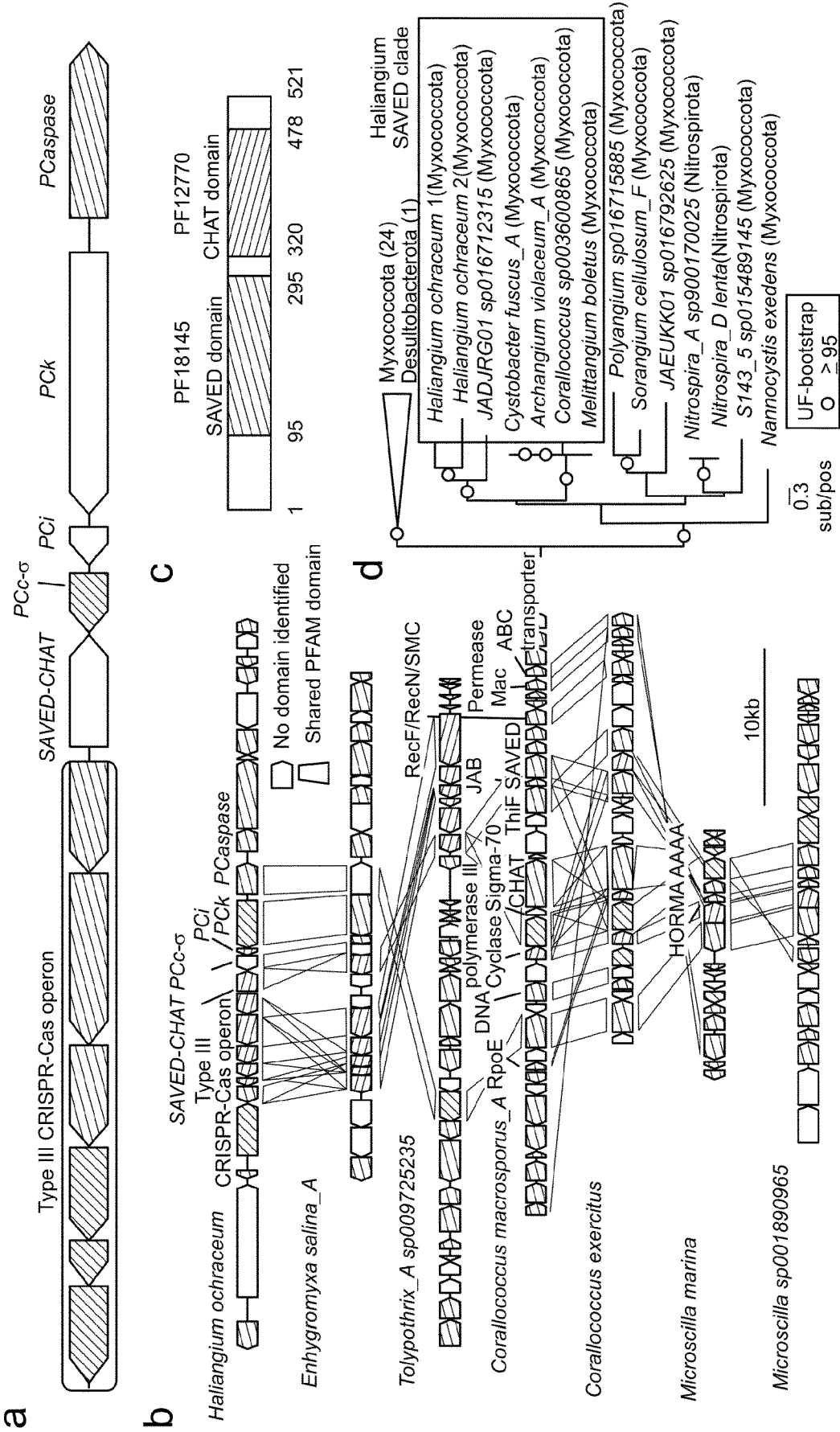


FIGURE 1



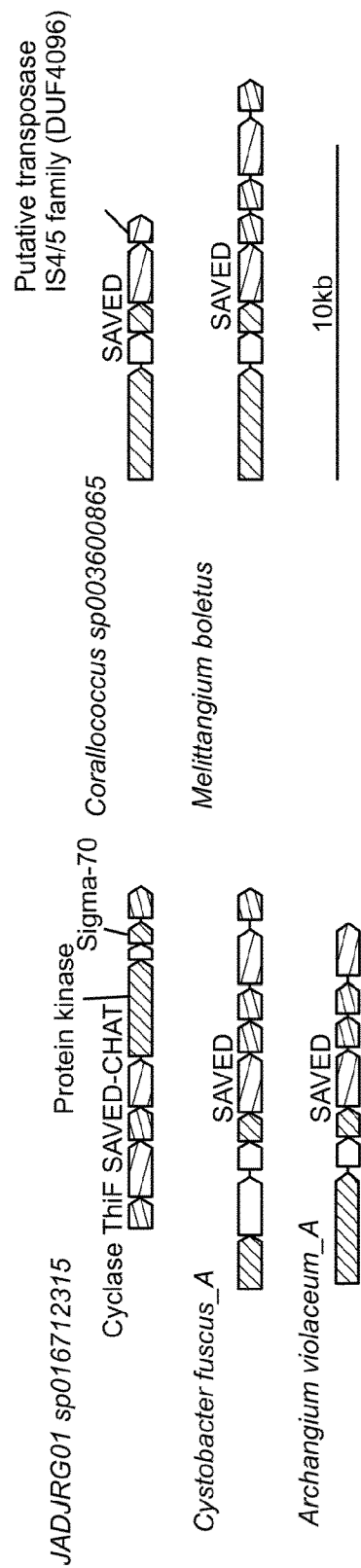
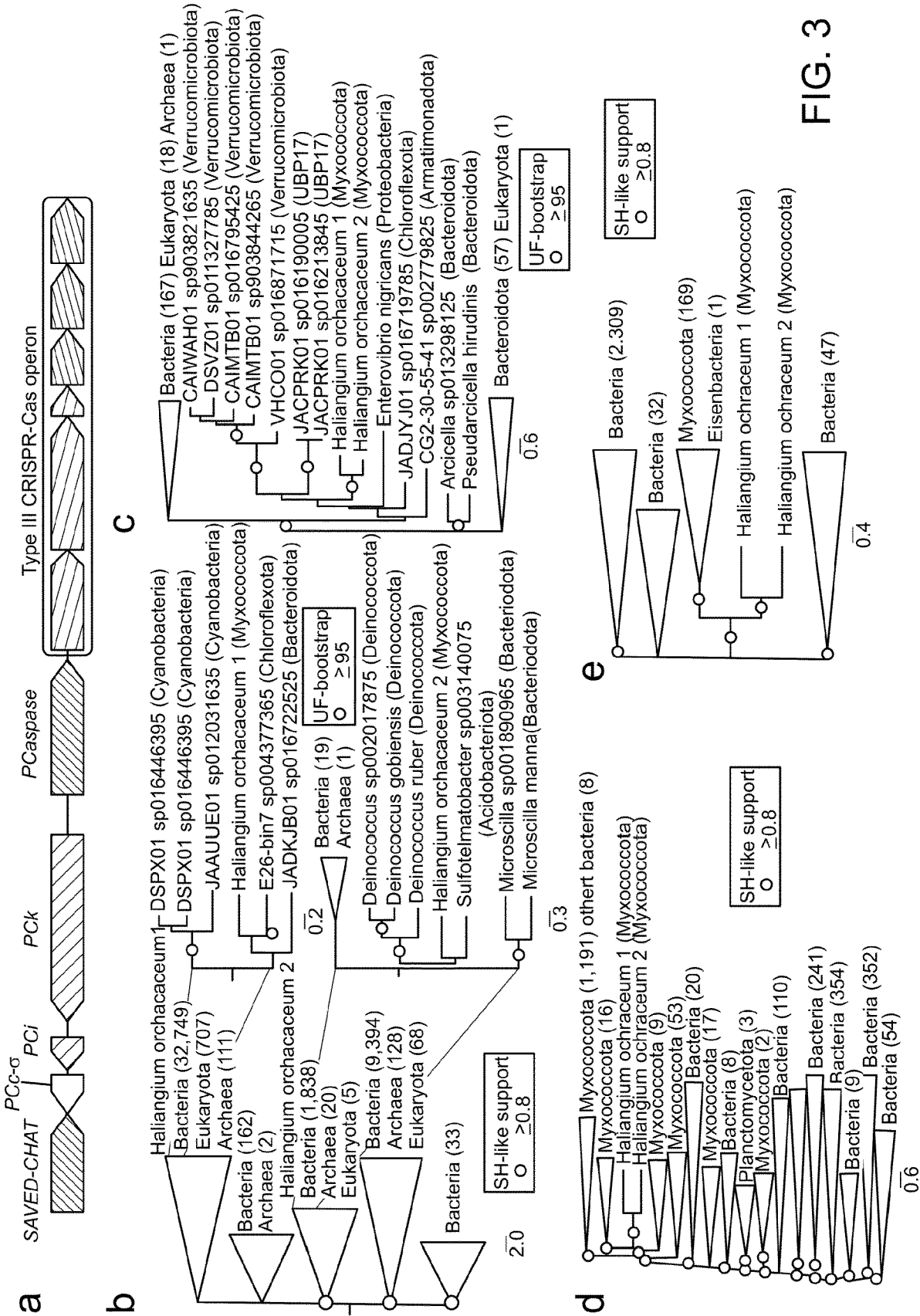


FIG. 2
(continued)



WP_01521748.1/1-592
WP_04671262.1/1-650
WP_095983898.1/1-651
WP_082175316.1/1-951 1 MEVLVEVRAMQVVLEPLKLYGGLLALHQDQFNQVTHPPVGLLRGLQRRRELEEGARVHRAELPCEVRHSPRDERSAQHHEEPSEG LAQGRSRRREAQ 88
WP_012830652.1/1-502 1 MAGDYPRMTMAITSR DSIRT 19
WP_012826486.1/1-521 1 MTHH..ADHDCQPH RESVQ 17

WP_01521748.1/1-592
WP_04671262.1/1-650
WP_095983898.1/1-651
WP_082175316.1/1-951 100 QPPLQRADDLKQL...GRRPAPGAHQPGSEARTCQFRVYRF..LLPNFRQRIPSRVLRPALRI....GQALIALGLAHVVHERLPARRDLEPGAL 188
WP_012830652.1/1-502 20 PERVQCLLILDLFDENTYSERSFAEHLPPSERRCY.RATGFDAKRRPHDQALPSRDWRWQRAIDDMVDAARGELGDDELAHYVVTGRAPLPATAHL 117
WP_012826486.1/1-521 18 PDEIQCILVLDLSIDNAITACSVTPHLPRARRVEL.HLNDFAERAPYGGASDRRTWRCWMQAVDAMLADARAQLGAEVEFTHYYLAGRAALPVFAYL 115

WP_01521748.1/1-592
WP_04671262.1/1-650
WP_095983898.1/1-651
WP_082175316.1/1-951 188 ALRISDELLPVLRGEHVCLGDDLVIQSVHDTPLRTTGEAIFRLLPERCLHHSRKHHSGGARSRCPKIR.....GEFFRARTKKLRQLSPES 278
WP_012830652.1/1-502 118 QHRLSAF.....ARISIVHLR.....EGQACTLTPTPPLGAK...LAEEPRFFDRVTGLERVEA.YAT 172
WP_012826486.1/1-521 116 QLRLGKQ.....ANITTVNRR.....DDGCDVVVFQCRPASSAASGVSPSARFFDEVRLDTERSSSES 174

FIGURE 4a

WP_015211748.1/1-592
 WP_046712262.1/1-650 1 MSTKE·PLEIITL·EFT.....GTRKEFSWKPKRYFIKPTFGEGESVSFPWSE...M...QREIESL.....KRQDPG..... 50
 WP_095983898.1/1-651 1 MARMPTSK·VEELS·EFLRKNKTHDPYNFSYEREEY|VMRS|GAPKPAFFPWDD...TQLQDLAQL.....SALRPN..... 70
 WP_082175316.1/1-951 279 SRLCAERLM·NS·YREIHL·RFTQNGPDS...YRVAVDS...TSVGSASATPLHLPLSRLDLQLQIQALLQS.....RSCDAKI..... 351
 WP_012830652.1/1-502 173 GRVA·VWISTQSEVDRAA|RAQL.....GEVP|VS...LFATPAEQ.....SS...DNQPHLTPADGPRVAAELFKMRQLHNCYPRRSGL|247
 WP_012826486.1/1-521 175 GMVA·VWVSTQRDVDRLRAFARAR...GORDLAG|VS...LRARPAAG.....DD...TGDMRLLEGADGPDARELVNCFRS|PNQYPRSSGL 255
 WP_015211748.1/1-592 1 MGQNWAITI...GNGYRNQLSLNYAKFDAEMQVFF.....ROELNIQTVYHFADDSLP|KQDYGSOLD... 82
 WP_046712262.1/1-650 80 SDVFERGRRRLRLAPADW.....ASDEDA|NDAFAGQPVHLTIRSKNADEIYYLPWEALWLK·GG·NRLGSME 128
 WP_095983898.1/1-651 71 DLAVAR|GRRLREFLDIADW.....AADEEALAKALREKRPVHLTIRSH·AAELYYLPWELLPLRVSG·TLLGELP 139
 WP_082175316.1/1-951 352 DDELARLGDALYEA|CPGQVRREI|TSLGGVL.....F·SSDEQQQ....RLRFLHFDPDDESELAWLAEPWDVLR|PPVSPTRHAALD 432
 WP_012830652.1/1-502 248 TMFLSGPN|LATMVGRALNRR|HCPVAMPNY.....RAYQGYRPAMMSPWPRV.....EG..... 288
 WP_012826486.1/1-521 258 MVFVSGPVTLAAMVGRA|NPR|HGPVWMPYFR.....G...GEYEPALEYWPPL|.....SG..... 304
 WP_015211748.1/1-592 63 SHPTYTT|NRRFFRVRFEESFLKS·GDNLWFFFAHGK 98
 WP_046712262.1/1-650 129 DCL...IQ·YESASQSSRELMLHPTGR|LVANSK·GGWVP...ADKHIEA|SQACRDSG...LHFNPVED|LPDVSRRKRLAEKLEETERPVTALHLLCHGG 219
 WP_095983898.1/1-651 140 DFL...LR·YQWPGTQSDA·GQDTIR|LLACSAA·GERVP...FAEHREA|EDARSSRAGI|SARTELSFSNANRQSLALALRDPDRP|DVLHLLCHGK 231
 WP_082175316.1/1-951 433 HRLS|VRS|DVTQPSQQRALVRPLRVLLVRAGARGCGGLQ...FESEQDS|DALSS|VGVECE.....SLDMPTRLDLRRKL RD...FDPHVLHFMGHGK 522
 WP_012830652.1/1-502 289 KARLL|VAHTADSSRRRLNSDEELRNWYQSLSKP...MRDRCELRL|PAARLSDFTSALRD...FRPH|VHL|GHGA 371
 WP_012826486.1/1-521 305 PPRI|IATANAPEGENPTLDVEALKHLEEA|AEPR...KRKLCEVQRCPAATVSD|ITSALRS...FKPH|LHF|IGHGT 377

FIGURE 4a ctd.

WP_015211748.1/1-592 90 RHKRDY.....LMPIDVDPG...DVESGISLQYVUSERLRRSGADNVVMLIDACRSGNGQDGVGFQE.....KQQGVISLFCSPGESSVEINELQ 184
WP_046712262.1/1-650 220 ELGGPDSNAYGLVFNVPVDPEDGRPOLLDASLLRD.LLFKS..RRPSSLRLVILCACQGGAGAPAHVPVGSVARMFHRQGVPAVLAS.....RLPLSCSG 310
WP_095933898.1/1-651 232 QLEGN...TGLVLNSEDREEG.PDYLTPSDIRR.LVSTI...EK..PPRLIVLCVQCQSSHTGTPAHLGSAEAFHRQGIPIAVIAS.....RLPLSGTG 316
WP_082175316.1/1-951 523 VDENT...GNGWV...YLRDEKGAPEVLLRAQDVAE.LFSSR.....FAPRLVVLNACQSAQGTLSVTGHSVAGALVSGVAAVIAM.....QFAISDGA 605
WP_012830652.1/1-502 372 HGG.....LYFV..DERQDG.QFVSSEALCE.LLEV...AELNDQLVLLNACNSEYTAE.....L.....ISKSLPCTTIGTKLKVPDGT 440
WP_012826486.1/1-521 378 ALG.....VYLR..SAEHGGAQFVRGEDFQQ.MIATSLRQKQDREMHVVLNACCTHELAK.....A.....LTEQVSC.TIGTDIEVYDSA 449

WP_015211748.1/1-592 185 HGSFYALLEGKIEGESNCATVERLYQRLRQRPVSLTQQYRKVQQTTPYGVVEPTKYHLILLPRKANLTDIVTLENDALLAEVQQNFQLAKQLWIRVL 283
WP_046712262.1/1-650 311 SVSLTRTLVQGLLVKRDN.....LRTVLSAARTQLLRDERS.....LRTVLSAARTQLLRDERS.....DWISLQ 353
WP_095933898.1/1-651 317 SILFTKAFYEELGGSGH.....LRTALVAARQQFL.KKSL.....DWASLQ 358
WP_082175316.1/1-951 608 ARAFTAEFYTCLAKGSSI.....DDAVTEARLAMRSKVPODSF.....EWCTPA 648
WP_012830652.1/1-502 441 ATSF SFRFYDDLAAGSSVAHAFHRL.....GEIKAGPSEHADSYLC.....P 484
WP_012826486.1/1-521 450 SIHFAARFYQHLVHGTSVHYAFNAV.....DEGRAHSTSGQEVFCLH.....P 493

WP_015211748.1/1-592 284 AVSPTNSKATIEGIERLARVVPAGY..GSGKTDVQHPVGTFFAIRHQSFEELLTVRISEESLPAHFRHYKIQHIEFLSSFFTG.....VCDPISAVQQN 375
WP_046712262.1/1-650 354 FYARSRDE..ASLSPFSEPAPTAP.....AATSRELVLIRHEAYSTAHGEPSEAEDAPTIFAHRSVRPLVSLDOASALSGRRWENLEPEVARLAAR 442
WP_095933898.1/1-651 359 LYAREGDD..AALDPFRRSTPAPRV.....LPTRSGELVLLCHEAYDRAYVAPGADDVPALFSNREARKV.VIDQTLALKGRNWERLGDEVKWLSP 447
WP_082175316.1/1-951 640 LYMRAGVA..QDLLQRELQEPADRVDSIPVVGGAAGADLVLLCHEAYAEATHSAPEEEDAPALFAQRRTKRV.LIDQKKHLQKREWAYNEPAPYALVDP 744
WP_012830652.1/1-502 485 AATREG.....AELVIFAPEEA...T.....502
WP_012826486.1/1-521 404 AAERAPAS.....AT.....PPVRADELVFFSPAER...HS.....521

FIGURE 4a ctd.

WP_01521748.1/1-592 378 NNILNDFLVVFQANPEAVIGYGVHPLQFCAGYAVSTWPRIALCFELDRDVN..CNYELAGDDSPKLDLSVSNISRPANAVAVAIRIASFDITKNDV 472
WP_04671262.1/1-650 443 DGILRRVFEERDIDVYFGFPYVSFAVLGYLA.KTRPVHVEHDPRTTQRFTWQQGAKGRAPQLRVDAASR...DTGSAVRVRLSISADVRLLEC 533
WP_095983898.1/1-651 448 EGELLQLLTRDTELVYGFYPLAVLYGFLA.NTRPVHVEHDPREVERFTWMRDATGSPPLRKKRSEY...ATGSAVRVRLSISATVNVNDC 538
WP_082175316.1/1-951 745 EGCLQRAISEQGITLLYVGFPLPLAALAGHLVTTTRPVHVEHDPRESGRFTWSKATGEAIPELKLDQLH...STGSAVRVRLSISSEVELDDC 836
WP_012830652.1/1-502
WP_012826486.1/1-521
WP_01521748.1/1-592 473 DDVVPQPYEDIQTK..IGKRRIDAITHYSQVKEVCNAFRQVLDDLHTRVDK.SLIVHIFXSGPVSLGFSLGRRSRIHHQVIVYNYTSHTSPRVANGV 588
WP_04671262.1/1-650 534 AAVLPDSEVRDLHFSLEKPARGVVRREADLLEYAQSIREATDQHAGNPGFNGGVHVFAAVPVSI AFHLGRALAFGLPECFVYNYDQDIPHYKWRL 632
WP_095983898.1/1-651 538 EEVLPSRDVGLDHFTHKTPQGIIVRREACVQANIQITRAALDRYVAGNSRI.QALHVFASVPVSI AFRLGQALAAATGLPACCVYNYGAQELPRYKWRL 636
WP_082175316.1/1-951 837 QEVLPDERVRDLHFRUDEPGRGAVRLEKDALEYARKLRATFDRLYAGKRAL.DSVHLFAAVPVSVAFLLGRELASTGLPPCFVYNYFASRDSPRYRWRL 934
WP_012830652.1/1-502
WP_012826486.1/1-521
WP_01521748.1/1-592 588 KINSADSP...SRVISTKLLIHTSE 592
WP_04671262.1/1-650 633 SQAAGRRPAVTLFPS..... 650
WP_095983898.1/1-651 637 WLRADPTGS.PLVDVF..... 651
WP_082175316.1/1-951 935 CQAAVEGR.PSVDLGE..... 951
WP_012830652.1/1-502
WP_012826486.1/1-521

FIGURE 4a ctd.

1	MKKKRLIITHIHITIRDEPLDGGTAE...QWVIEVPPLOENVDFGQDFENEMKRR	
1	1 MQEELDLSAVTRGAIPIVNSGRASPPPHGANCARTIVDHSALLMHPGAMITDIVLMDLAAANQMTEATVRASTENAO...LRLTKHVEPYGE...GTP.PAMPVPIITA	
1	MARHIIIAHIIHREISEECLEALEEMONK...EVEFTEVRYDK...IKPLGEVEDVALKEO	
1	SNRTPEVOCILIIDLFENITSESTFHEPSEPCYRAGDARERDO...AIPSRDRAWQRA	
1	ESVQDEICILIIDLISDNATICSIPHLERARSVELHLNDCARAPGCG...ASPRRIACMMQA	
	P.m.g.illdi...nei.e...ip...g...evry.g...g...dn...a	
	N23	Y65
62	QOKIFQDEIKBHLKDNPEIVYFSGVPIETLHKGSLNDPOORLVKAYTHROTRKRYETPLKKKKDKMT...K...[...FQLEPVGSSDTGGVIRLSLPIVPODTRG...	
109	IDRLVREAORLGRQDPDCRWVVTGRAGLPEFFHGHGSLHQAATFLQPDSDSPLVLEPIER...ASADAPPLOVESGTLPRDPRCPAVLAVSRRPRVEAIIKALHD	
162	OKOLFENQVOPVLRIGVTIAYFAKAPIEMNLHGLSLHGYVKVKVFTVHTERTENYDASORNEOSILV...N...[...HHLKNEQGNVIRLSUSAPLPTDIYI...	
83	IDCMVNAARGELGDDELAHXYVTGNPIEPFAHGMKRSFARISIVILREGCACTTCPPPLGAK...LAEEPPEFDRVGLERVENVATGRVAMWITQSEURRAIRNOL	
81	VAMLLADARAQAGAEVEFTHYLAGRAALPEAYHGTGACANISIVUNRRDSCNDVFCQFPASSAGSVSPSARFDEVGLDIERSEAGMAWVSTQDURGLLEAFAR	
	id.1.dga...ig.q.e...yy.gaplpaf.hlg.r.l.a...f.a.rdd.w...p...p...d.d...g.p.a...d.g.vai.s.a.pid...ra...	
	R06	Q184 R185
	K121 Q122	
	N129	
166	VVRNCCEFFLTVQDPFEDILSEASVVEFTNAFFELLSKISKEYENAOHVEFAAMEITGTEFLIGERNENWMPATQYOKESARKEAAILLTDVYRAQINDLPMK	
219	RRSRA...SGITVYHSP...HDEDEASRSAPCOLHRLDIDCAHPPCANLIVIRTPTEFLIGRAINPNVCRDQIFGDC...GPAIAYEIPYPP...PD.RNK	
163	LSEPPLEID...K...HDEDEASRSAPCOLHRLDIDCAHPPCANLIVIRTPTEFLIGRAINPNVCRDQIFGDC...GPAIAYEIPYPP...PD.RNK	
195	SEVPISLFAFAEQSDNPKHII...TPADSPRAVAREFKMRQNCYPRAGTMEFISGNNIMVGRALNRLHCPMPNNAICQARPMNSFPR...EG.KAR	
198	ARGDRLAGIVSLRAPAAGDGTGELL...EGADGPDARRELVNTEFSFQYPSGQVVEVSCGVITAMVGRALNRLHCPMPNNAICQARPMNSFPR...EG.KAR	
	iv...p.d...np.d...ea...a.el...li...n.yps...vi...gt.lai\$igrainpm...vq.yq...y.pa...p.p.v...	
	N243	Q274R276
	Y285 F286	
276	VLVITLADKQ...QDDHVTAREICVLLMRAKLRDQCYKVTTEPATTMEIARQROIQHVHIAHSHCRLSPHIVGVRGENYSCTVYSTAETKAVVRLFNKYSMVECVINAC	
317	VVFVAPSPADI...PREIEGTDEIREOSU...VTRALDVKHPPPOPYVYK...EEOHOGVILSCGCSGCEMFLDCHGVSRILSISDILV...EIL...RIVGSLRLAVSAC	
264	VLVLTICLPHQ...QFINVK...KAKIKXVLTGNAREQYQIVKNTSYCALLEHETIVASHEDETGVLCGIDTSRQTPSSSTQNLINSEETVEVMYQNNOCVILNAC	
302	LLIVAHIAQSRRRNSDEENMKQSLSNPMRD...KCEKLLPKRLTISRRDFHIVLIGHCARGGTHFVDECDGQFVSCALCELEV...AELNDICVLINAC	
308	ILIATIANPEGENPTDVEAKELALDEKLAERKRRLCEVORCEPAIVITISRRDFHIVLIGHCARGGTHFVDECDGQFVSCALCELEV...AELNDICVLINAC	
	lave.E.eie.l...r...ev...p.a...d...abre...phivh.ghg...g.l.d...g...v...d...g.i...nlglvlnac	
	N35	G27
391	VSEIKCKIAEKTRYVIGFPHGVAANALISHSSTRSSADDVYNKKAFTDGNVATGIPCGNATCKIYIOGKMTGE...[...DIAREVFLVRSIP	
424	HCESPSTILSHVDCVIVAMGPIRADDARSAVITYRSLIDSDSDHARHMRIRQSDCAC...[...DIAREVFLVRSIP	
382	QSEKARVAVAIQQVVFSGSILHDAINESEVRSNLEIHNAEAKRSVRLNSGQSLCFHGGQIHE...[...DIAREVFLVRSIP	
413	NSEITBELISLSPCTIGIKLVPCGSIATSEFRNDDLAGSSVAHARALGKAGSEHADSIYITPAAIRREG...[...DIAREVFLVRSIP	
423	CUHELKALTEQVSCITIGTIDIEVDSASISAAARDHREHVTSTSVHFAVADVCRAHUSQGVFCFPAEAPASA...TPVRADEVFFSPAEHS...[...DIAREVFLVRSIP	
	s.elaq.i.e.i.c.v.ig...i.d.da.fs...y.L.g.si...Afd.a...iri...n...p.a.e...el...p...	

FIG. 4b

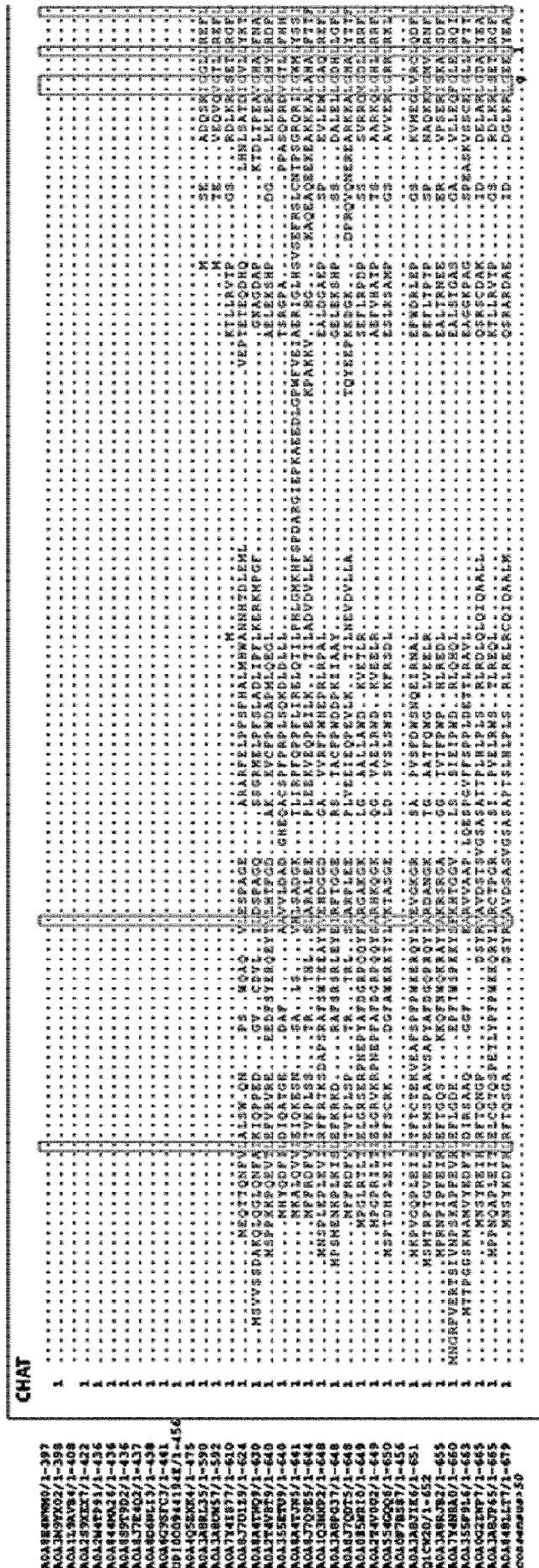
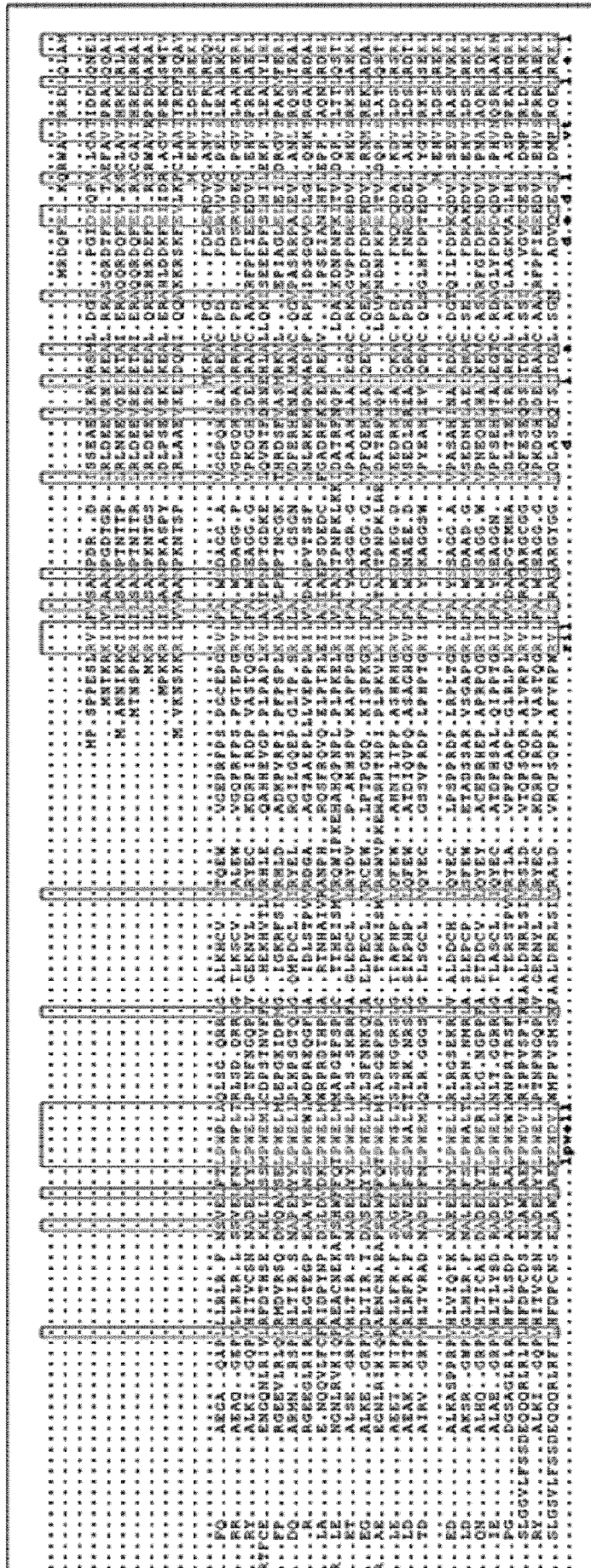


FIGURE 4c



1	ADARF49000/1-1397	ADYICROPH	ADYICROPH
2	ADARF49000/1-1398	ADYICROPH	ADYICROPH
3	ADARF49000/1-1399	ADYICROPH	ADYICROPH
4	ADARF49000/1-1400	ADYICROPH	ADYICROPH
5	ADARF49000/1-1401	ADYICROPH	ADYICROPH
6	ADARF49000/1-1402	ADYICROPH	ADYICROPH
7	ADARF49000/1-1403	ADYICROPH	ADYICROPH
8	ADARF49000/1-1404	ADYICROPH	ADYICROPH
9	ADARF49000/1-1405	ADYICROPH	ADYICROPH
10	ADARF49000/1-1406	ADYICROPH	ADYICROPH
11	ADARF49000/1-1407	ADYICROPH	ADYICROPH
12	ADARF49000/1-1408	ADYICROPH	ADYICROPH
13	ADARF49000/1-1409	ADYICROPH	ADYICROPH
14	ADARF49000/1-1410	ADYICROPH	ADYICROPH
15	ADARF49000/1-1411	ADYICROPH	ADYICROPH
16	ADARF49000/1-1412	ADYICROPH	ADYICROPH
17	ADARF49000/1-1413	ADYICROPH	ADYICROPH
18	ADARF49000/1-1414	ADYICROPH	ADYICROPH
19	ADARF49000/1-1415	ADYICROPH	ADYICROPH
20	ADARF49000/1-1416	ADYICROPH	ADYICROPH
21	ADARF49000/1-1417	ADYICROPH	ADYICROPH
22	ADARF49000/1-1418	ADYICROPH	ADYICROPH
23	ADARF49000/1-1419	ADYICROPH	ADYICROPH
24	ADARF49000/1-1420	ADYICROPH	ADYICROPH
25	ADARF49000/1-1421	ADYICROPH	ADYICROPH
26	ADARF49000/1-1422	ADYICROPH	ADYICROPH
27	ADARF49000/1-1423	ADYICROPH	ADYICROPH
28	ADARF49000/1-1424	ADYICROPH	ADYICROPH
29	ADARF49000/1-1425	ADYICROPH	ADYICROPH
30	ADARF49000/1-1426	ADYICROPH	ADYICROPH
31	ADARF49000/1-1427	ADYICROPH	ADYICROPH
32	ADARF49000/1-1428	ADYICROPH	ADYICROPH
33	ADARF49000/1-1429	ADYICROPH	ADYICROPH
34	ADARF49000/1-1430	ADYICROPH	ADYICROPH
35	ADARF49000/1-1431	ADYICROPH	ADYICROPH
36	ADARF49000/1-1432	ADYICROPH	ADYICROPH
37	ADARF49000/1-1433	ADYICROPH	ADYICROPH
38	ADARF49000/1-1434	ADYICROPH	ADYICROPH
39	ADARF49000/1-1435	ADYICROPH	ADYICROPH
40	ADARF49000/1-1436	ADYICROPH	ADYICROPH
41	ADARF49000/1-1437	ADYICROPH	ADYICROPH
42	ADARF49000/1-1438	ADYICROPH	ADYICROPH
43	ADARF49000/1-1439	ADYICROPH	ADYICROPH
44	ADARF49000/1-1440	ADYICROPH	ADYICROPH
45	ADARF49000/1-1441	ADYICROPH	ADYICROPH
46	ADARF49000/1-1442	ADYICROPH	ADYICROPH
47	ADARF49000/1-1443	ADYICROPH	ADYICROPH
48	ADARF49000/1-1444	ADYICROPH	ADYICROPH
49	ADARF49000/1-1445	ADYICROPH	ADYICROPH
50	ADARF49000/1-1446	ADYICROPH	ADYICROPH
51	ADARF49000/1-1447	ADYICROPH	ADYICROPH
52	ADARF49000/1-1448	ADYICROPH	ADYICROPH
53	ADARF49000/1-1449	ADYICROPH	ADYICROPH
54	ADARF49000/1-1450	ADYICROPH	ADYICROPH
55	ADARF49000/1-1451	ADYICROPH	ADYICROPH
56	ADARF49000/1-1452	ADYICROPH	ADYICROPH
57	ADARF49000/1-1453	ADYICROPH	ADYICROPH
58	ADARF49000/1-1454	ADYICROPH	ADYICROPH
59	ADARF49000/1-1455	ADYICROPH	ADYICROPH
60	ADARF49000/1-1456	ADYICROPH	ADYICROPH
61	ADARF49000/1-1457	ADYICROPH	ADYICROPH
62	ADARF49000/1-1458	ADYICROPH	ADYICROPH
63	ADARF49000/1-1459	ADYICROPH	ADYICROPH
64	ADARF49000/1-1460	ADYICROPH	ADYICROPH
65	ADARF49000/1-1461	ADYICROPH	ADYICROPH
66	ADARF49000/1-1462	ADYICROPH	ADYICROPH
67	ADARF49000/1-1463	ADYICROPH	ADYICROPH
68	ADARF49000/1-1464	ADYICROPH	ADYICROPH
69	ADARF49000/1-1465	ADYICROPH	ADYICROPH
70	ADARF49000/1-1466	ADYICROPH	ADYICROPH
71	ADARF49000/1-1467	ADYICROPH	ADYICROPH
72	ADARF49000/1-1468	ADYICROPH	ADYICROPH
73	ADARF49000/1-1469	ADYICROPH	ADYICROPH
74	ADARF49000/1-1470	ADYICROPH	ADYICROPH
75	ADARF49000/1-1471	ADYICROPH	ADYICROPH
76	ADARF49000/1-1472	ADYICROPH	ADYICROPH
77	ADARF49000/1-1473	ADYICROPH	ADYICROPH
78	ADARF49000/1-1474	ADYICROPH	ADYICROPH
79	ADARF49000/1-1475	ADYICROPH	ADY

FIGURE 4c ctd.

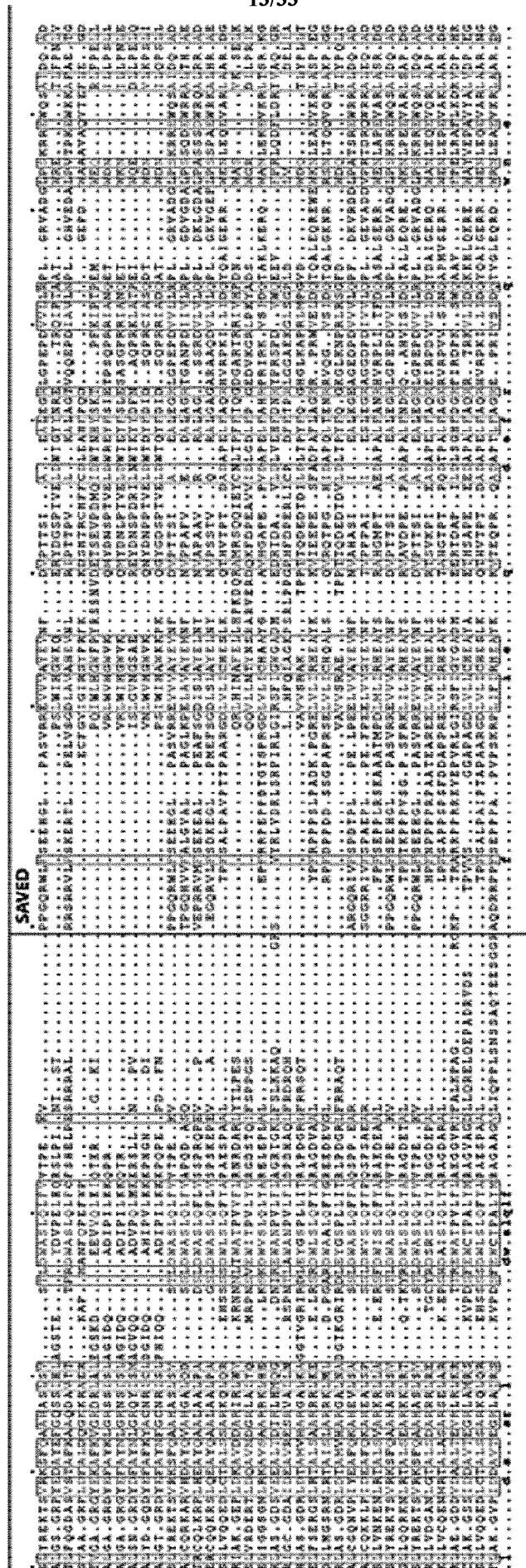


FIGURE 4c ctd.

[illegible]

FIGURE 4c ctd.

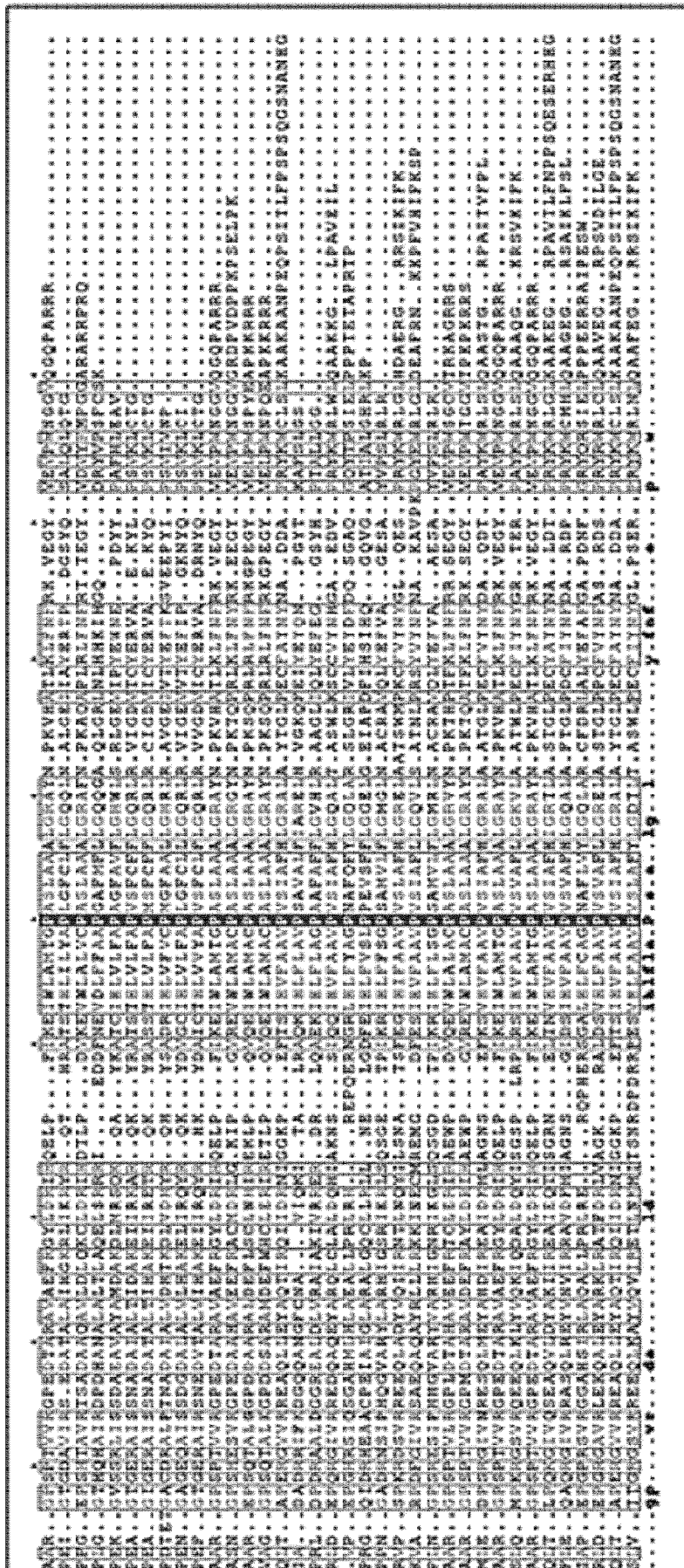


FIGURE 4c ctd.

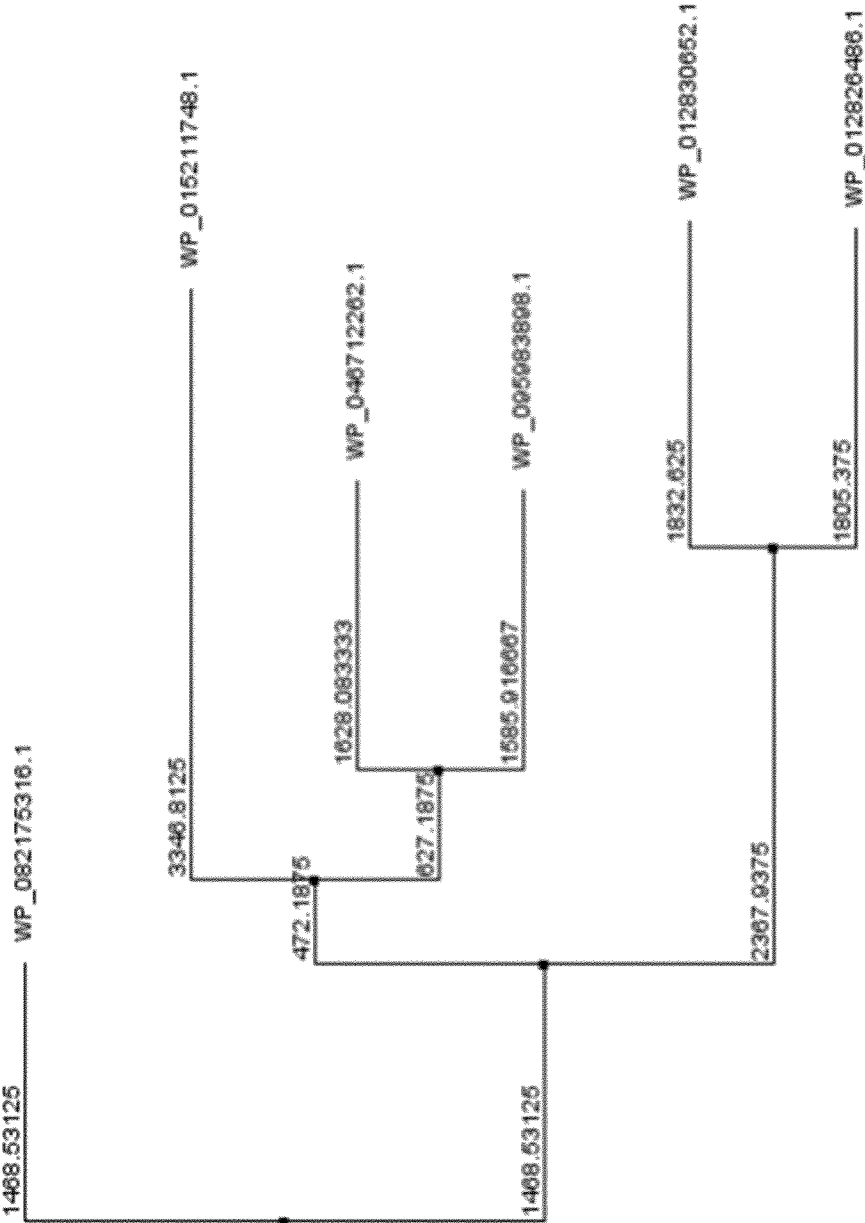


FIGURE 5

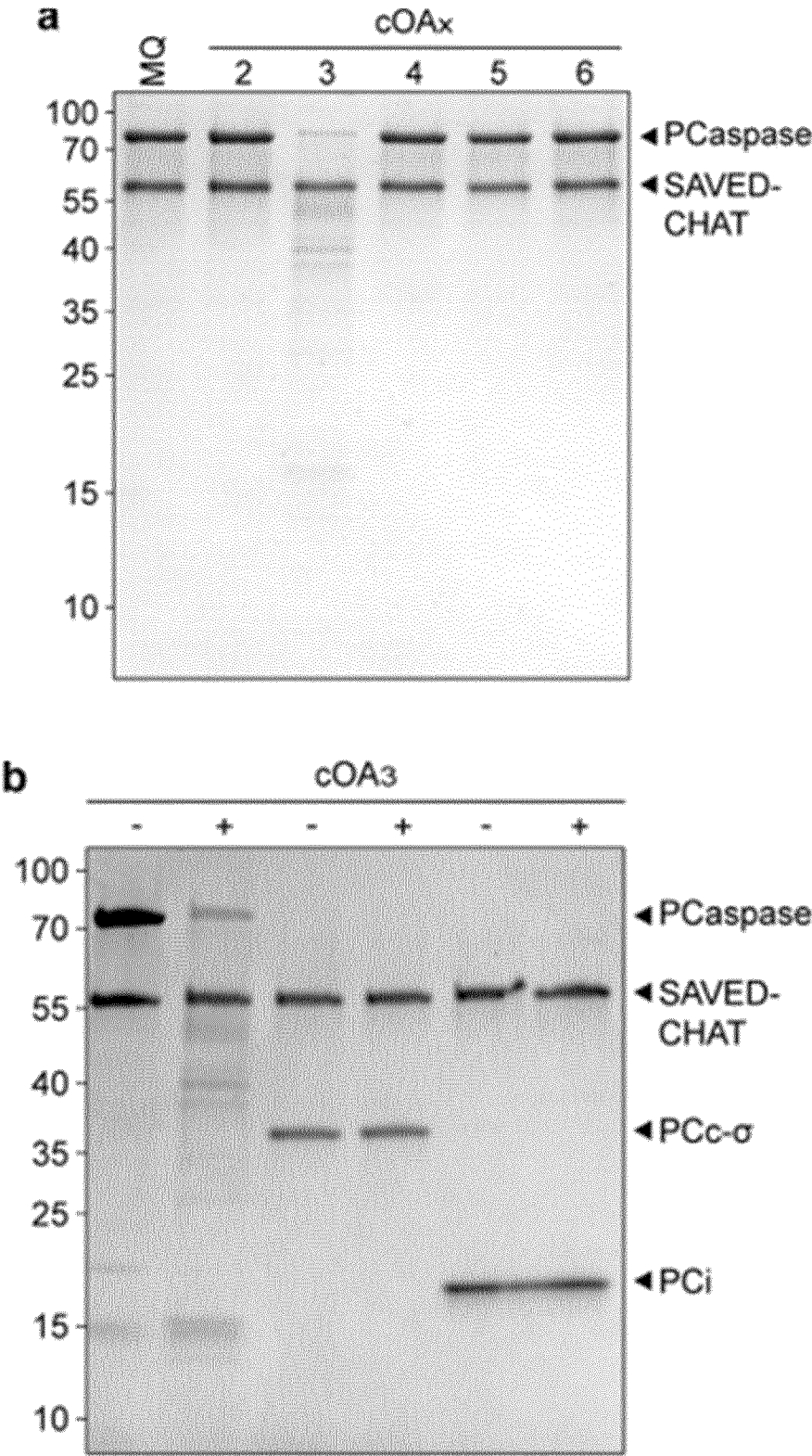


FIGURE 6

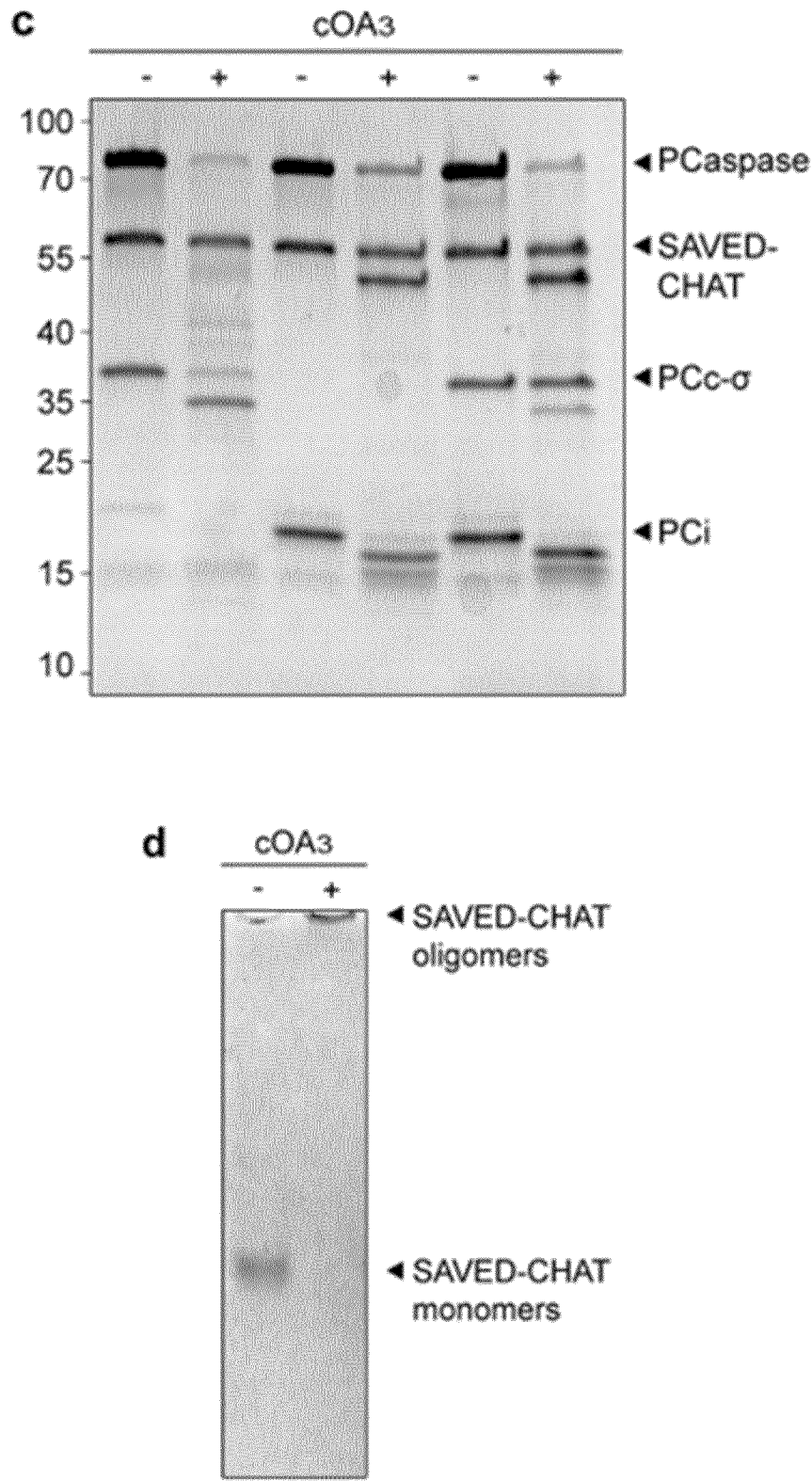


FIGURE 6 ctd.

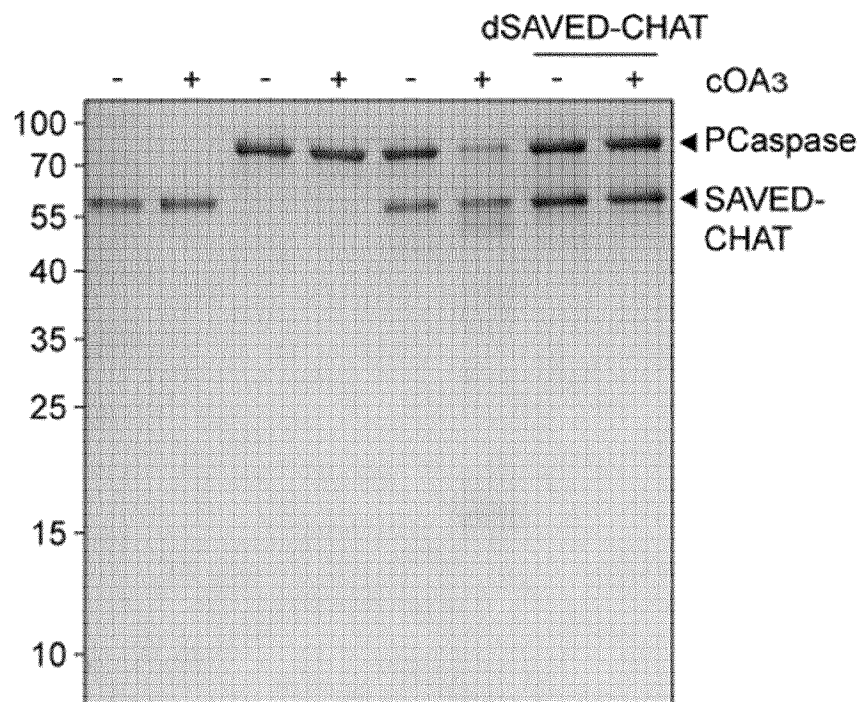


FIGURE 7

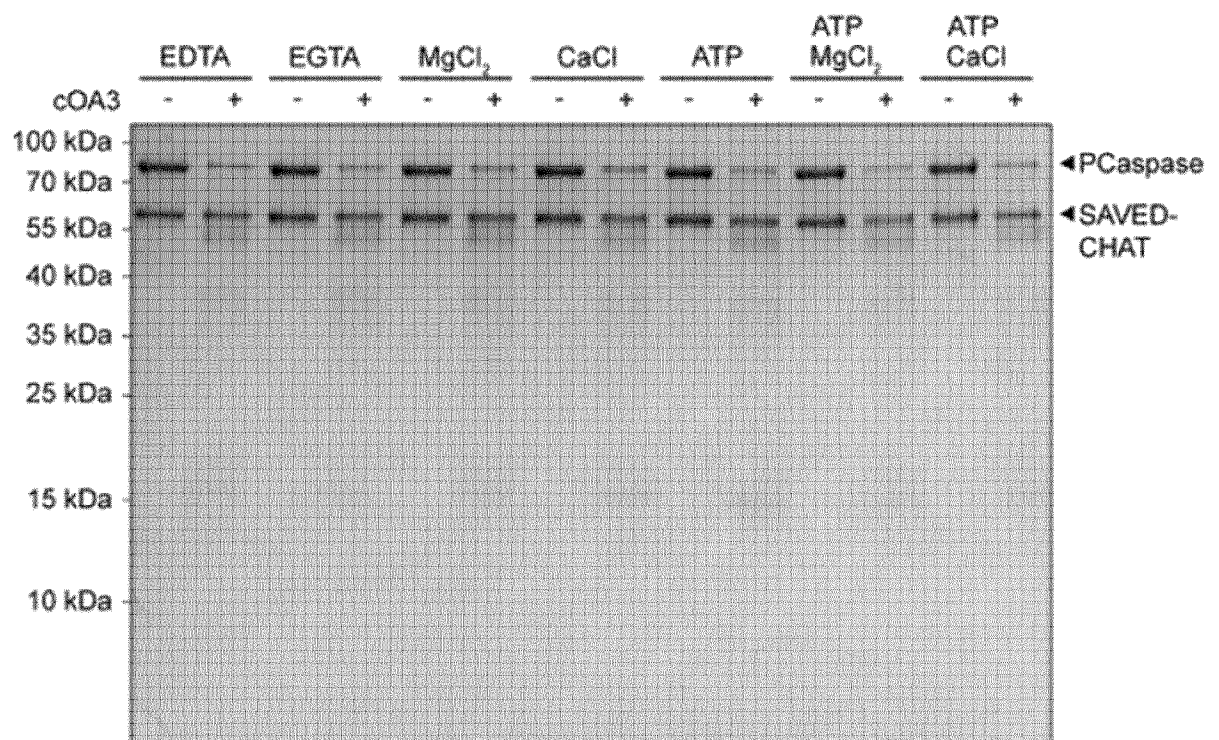


FIGURE 8

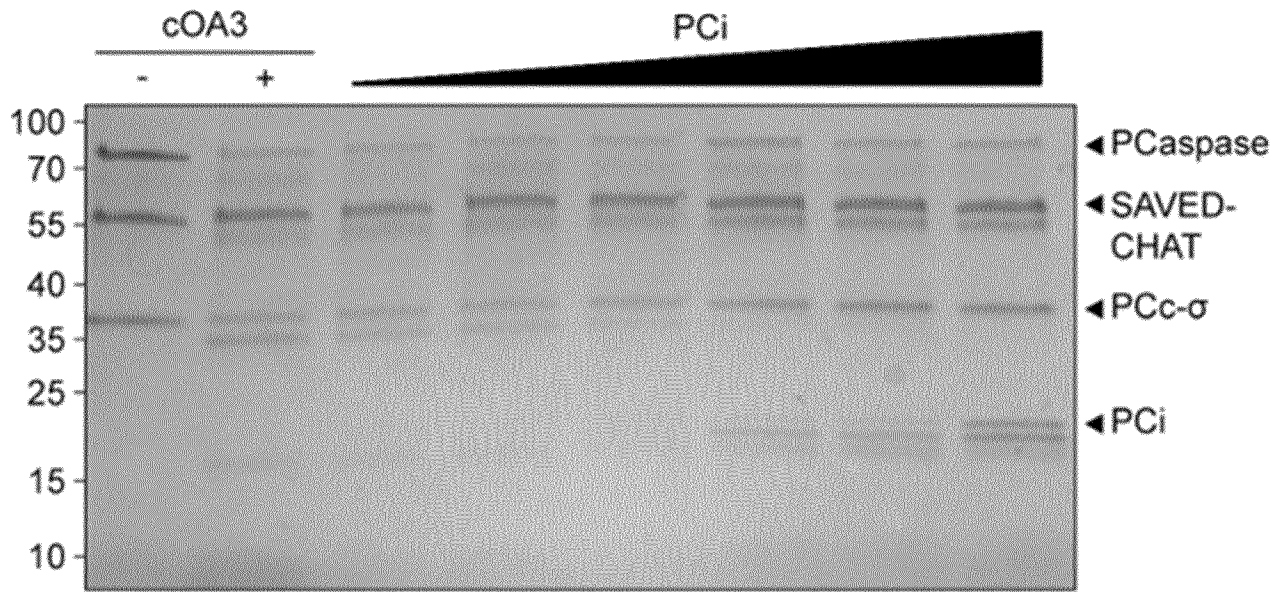


FIGURE 9

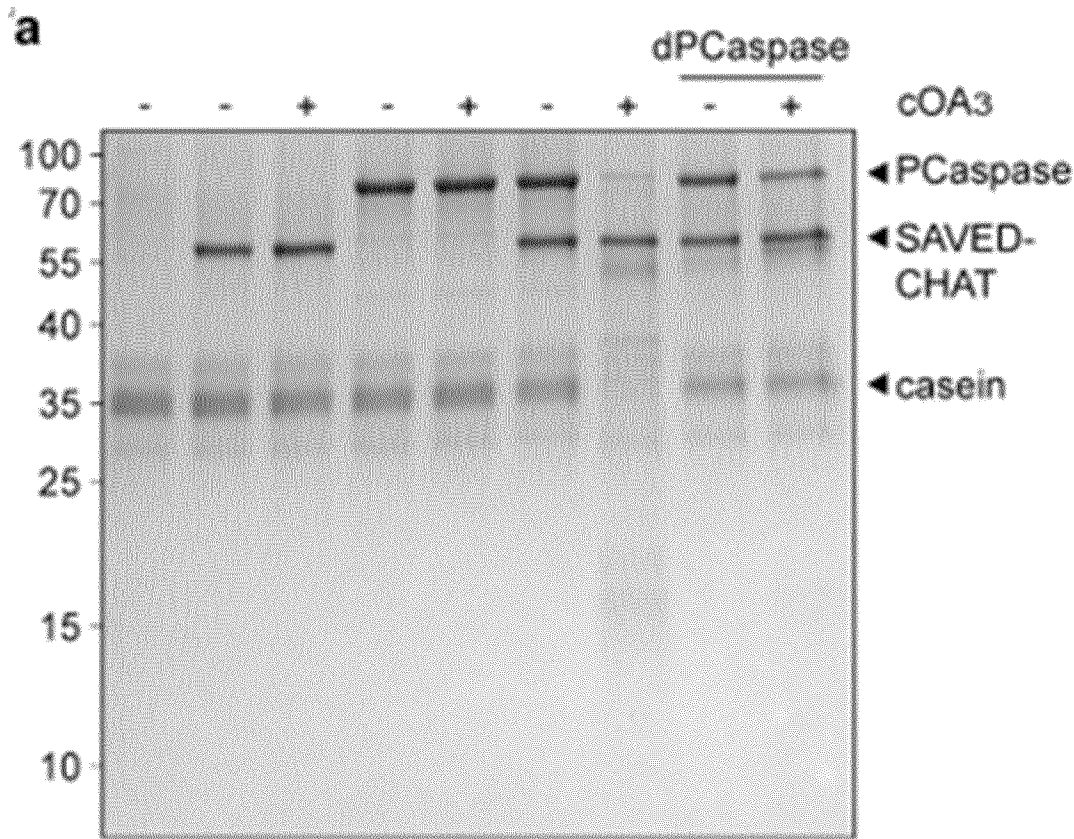


FIGURE 10

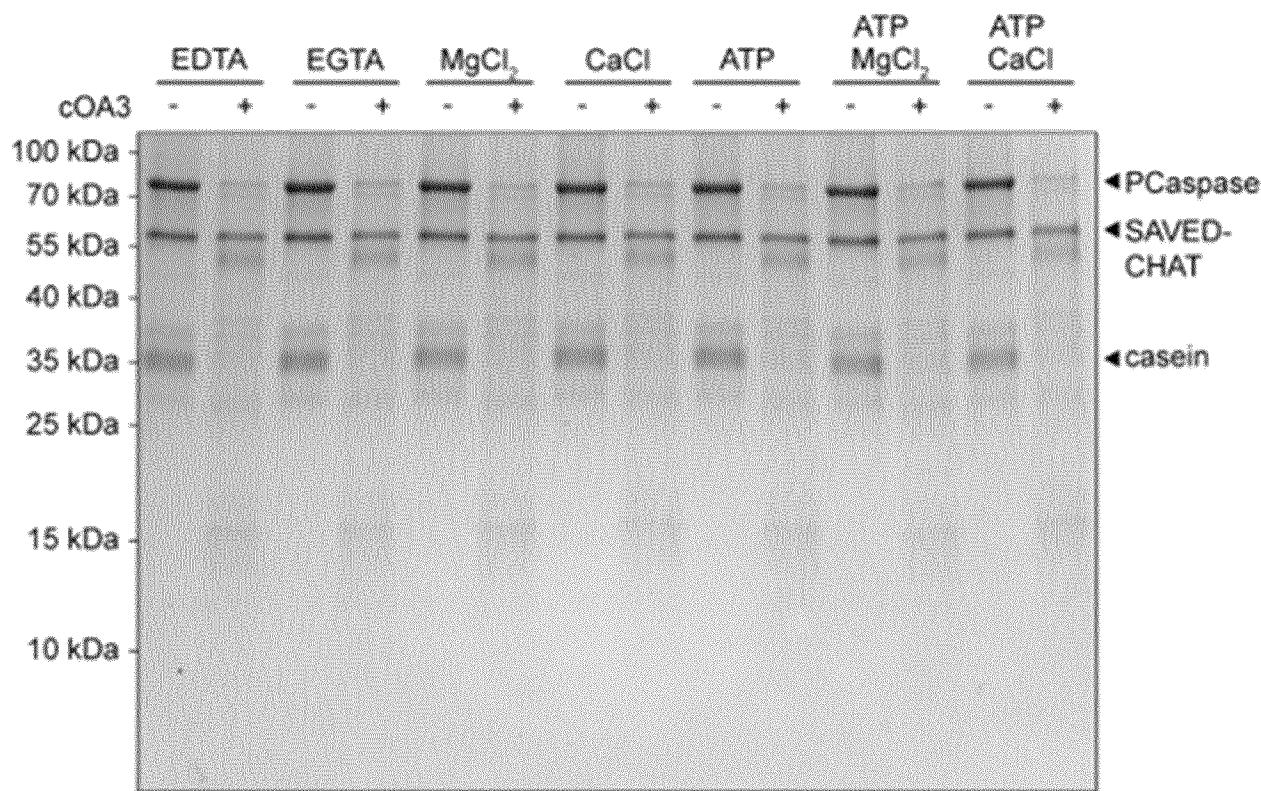


FIGURE 11

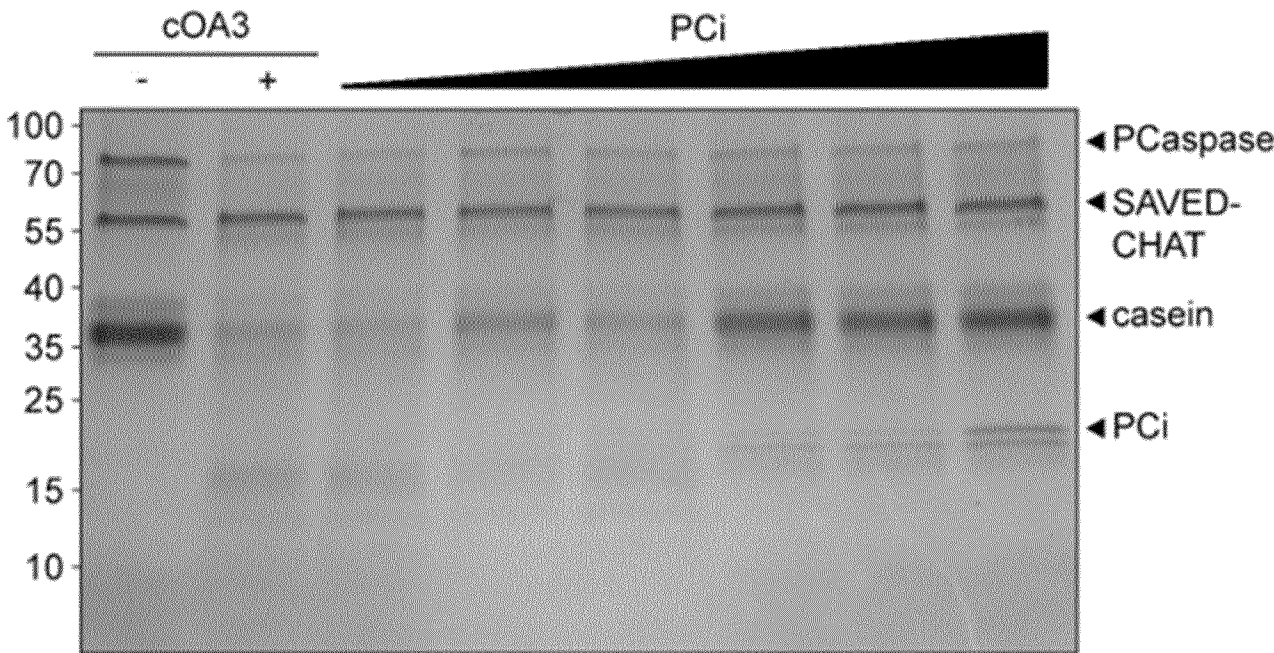


FIGURE 12

a

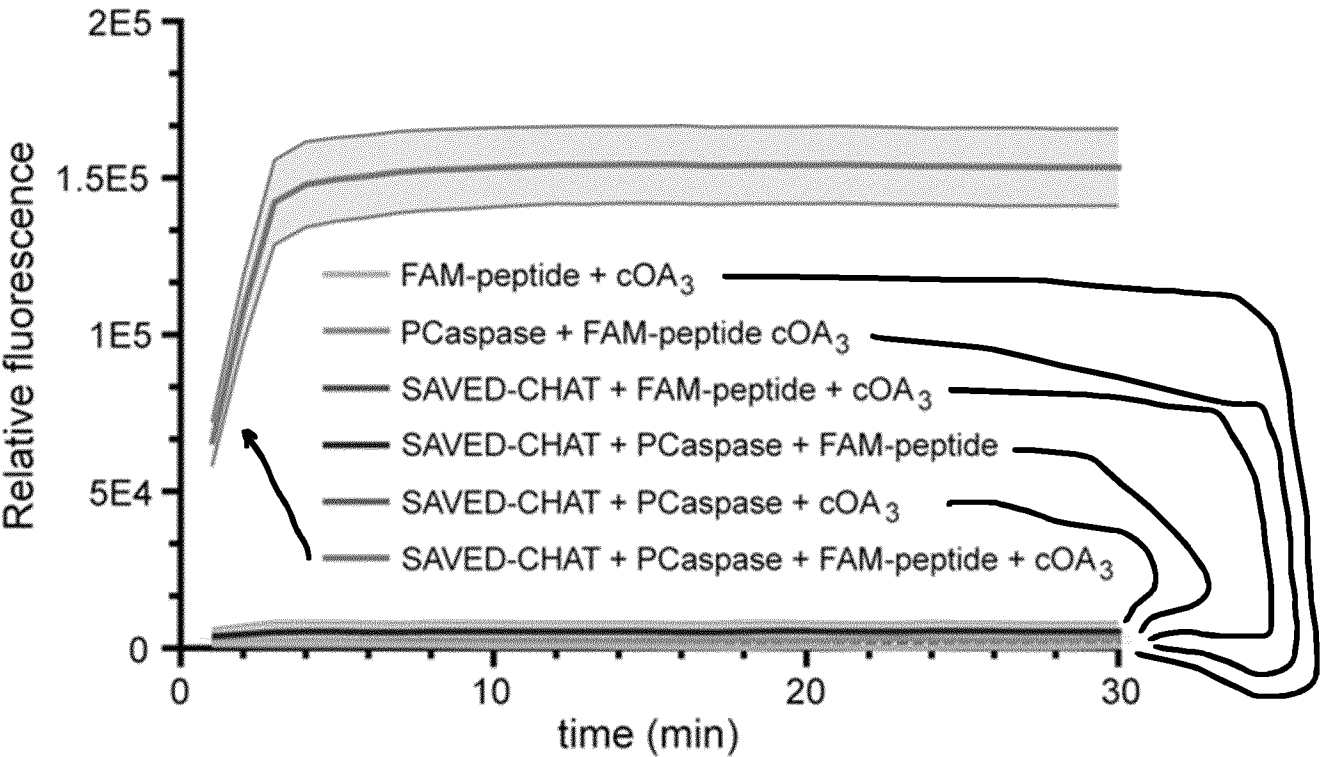


FIGURE 13

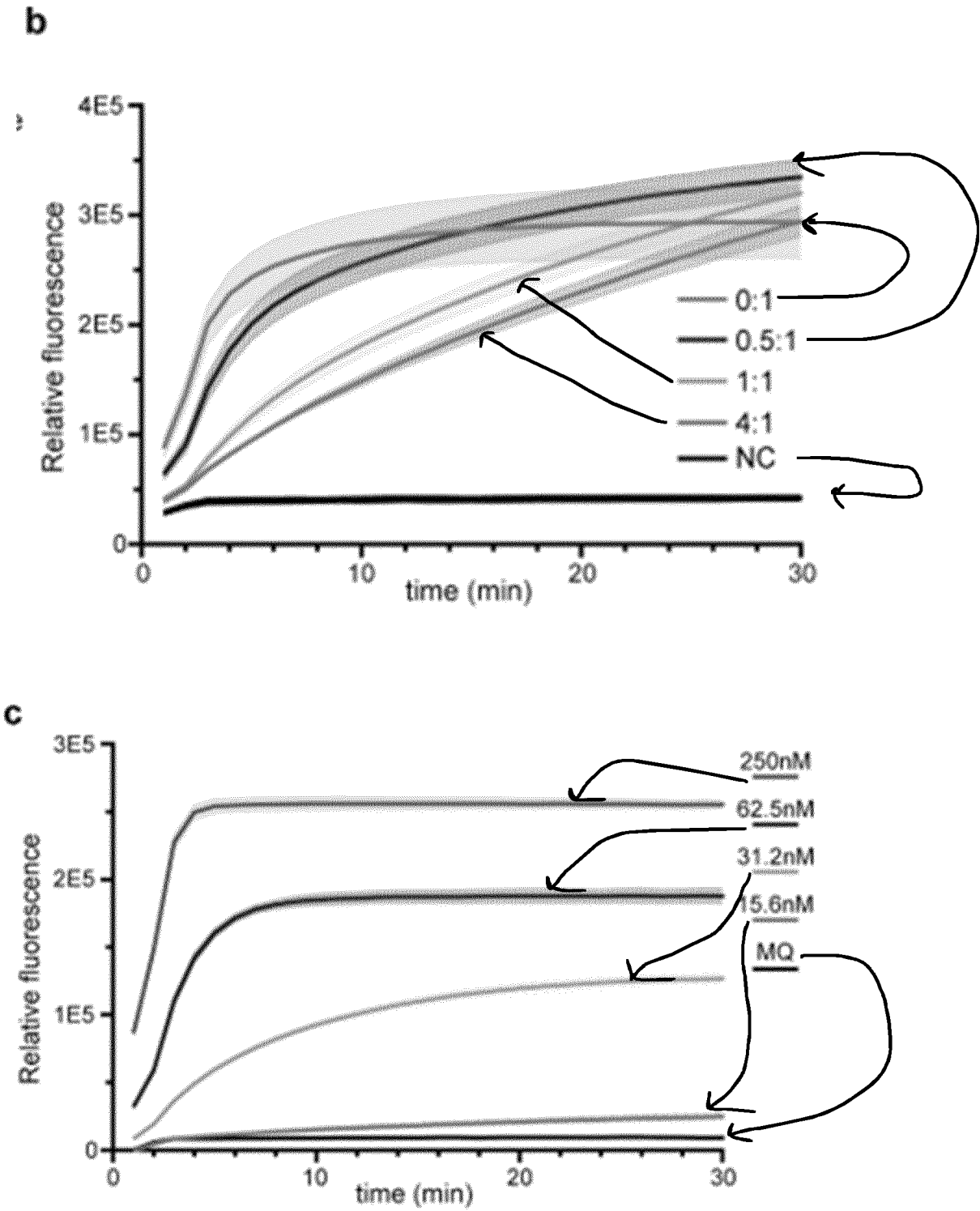


FIGURE 13 ctd.

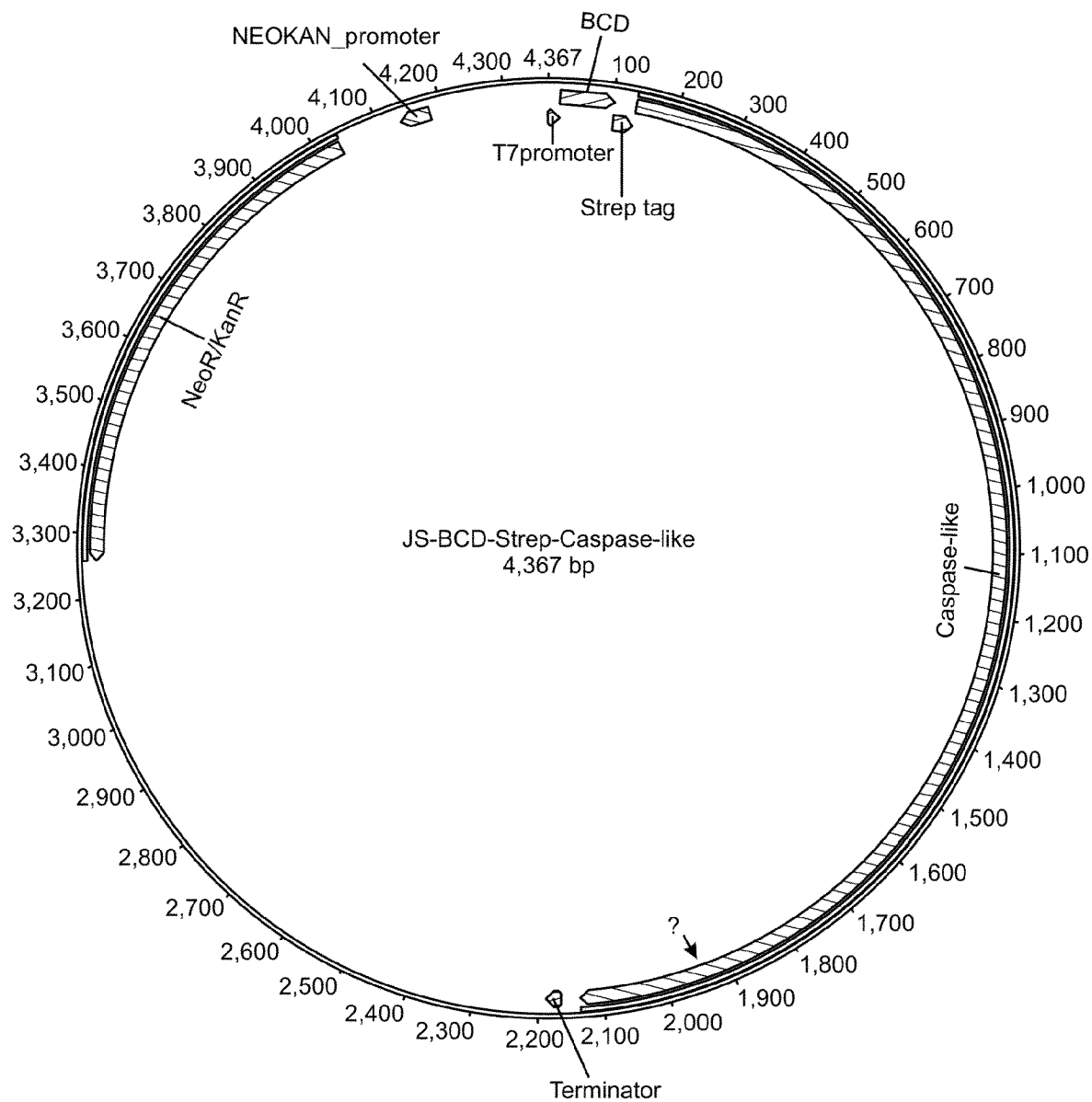
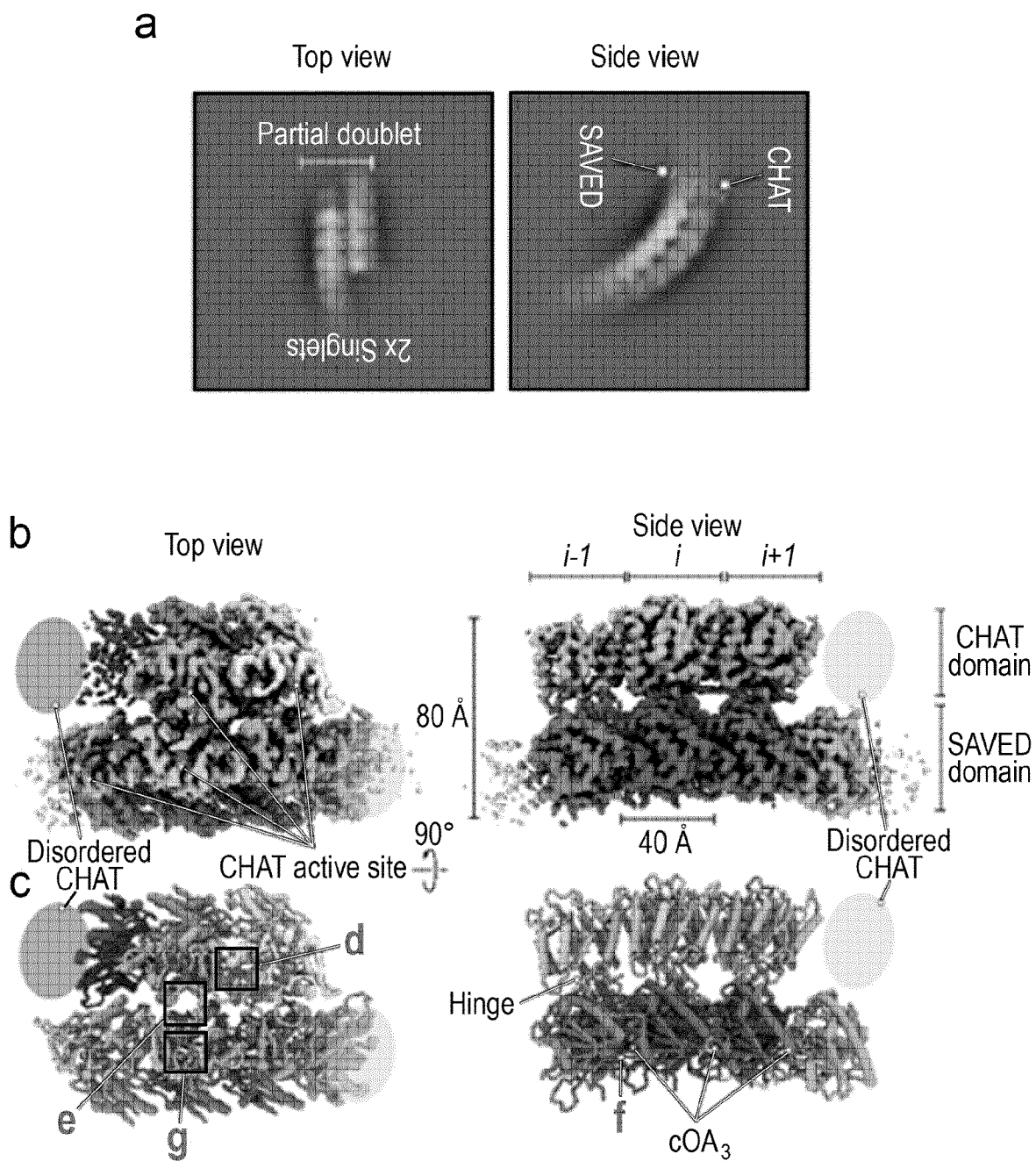


FIG. 14



CHAT-CHAT singlet interface

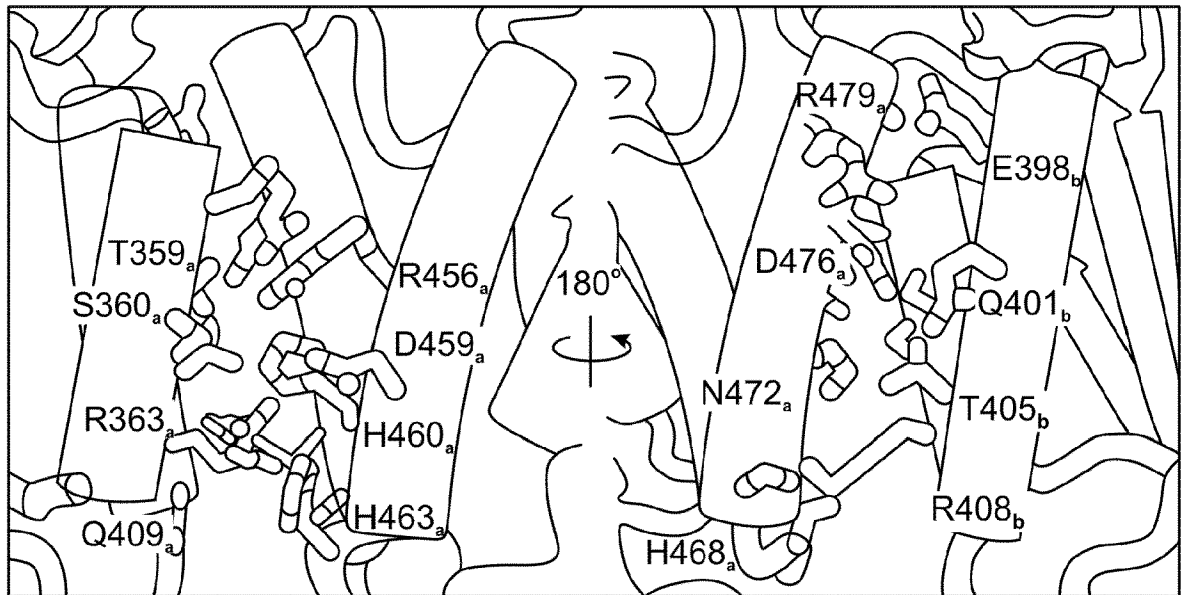


FIG. 15D

CHAT-CHAT doublet interface

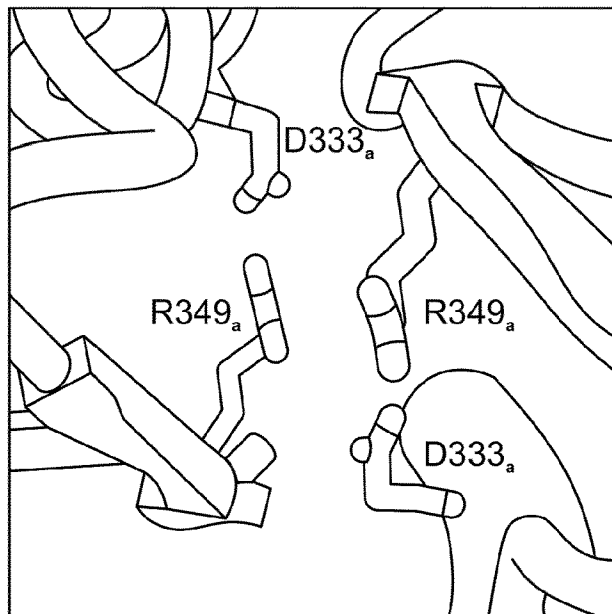


FIG. 15E

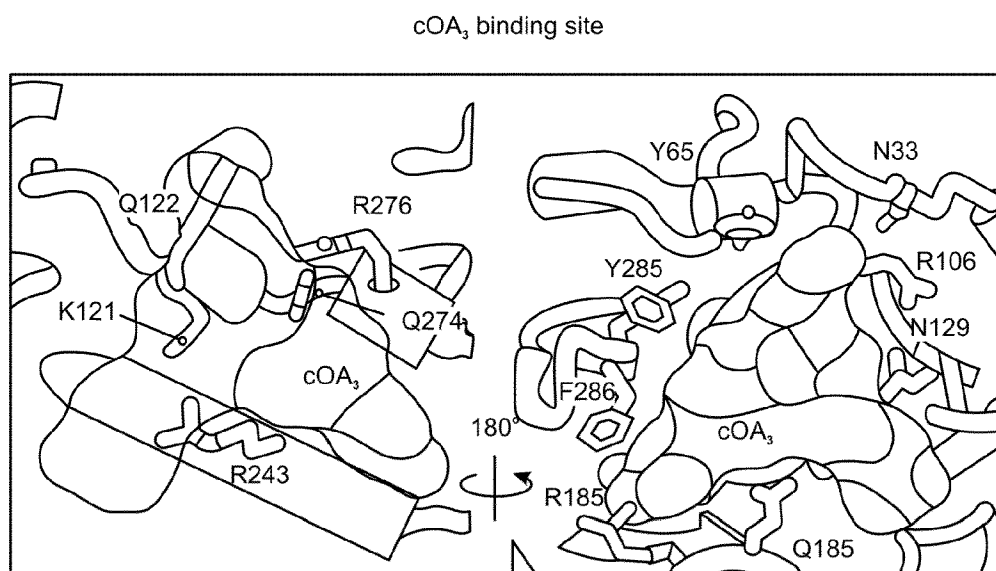


FIG. 15F

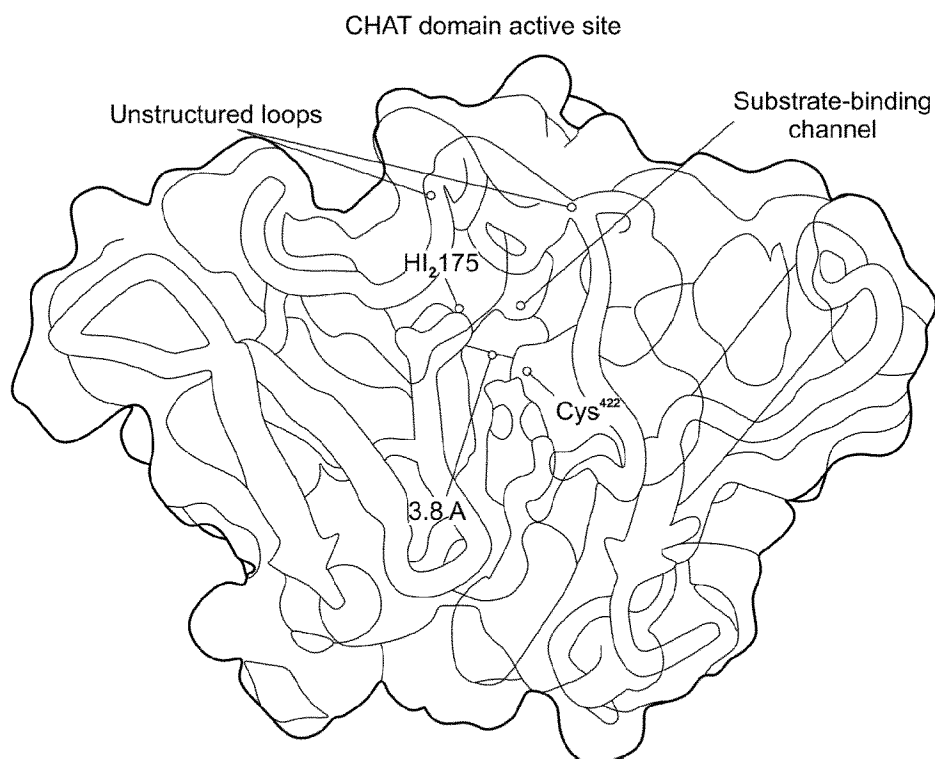


FIG. 15G

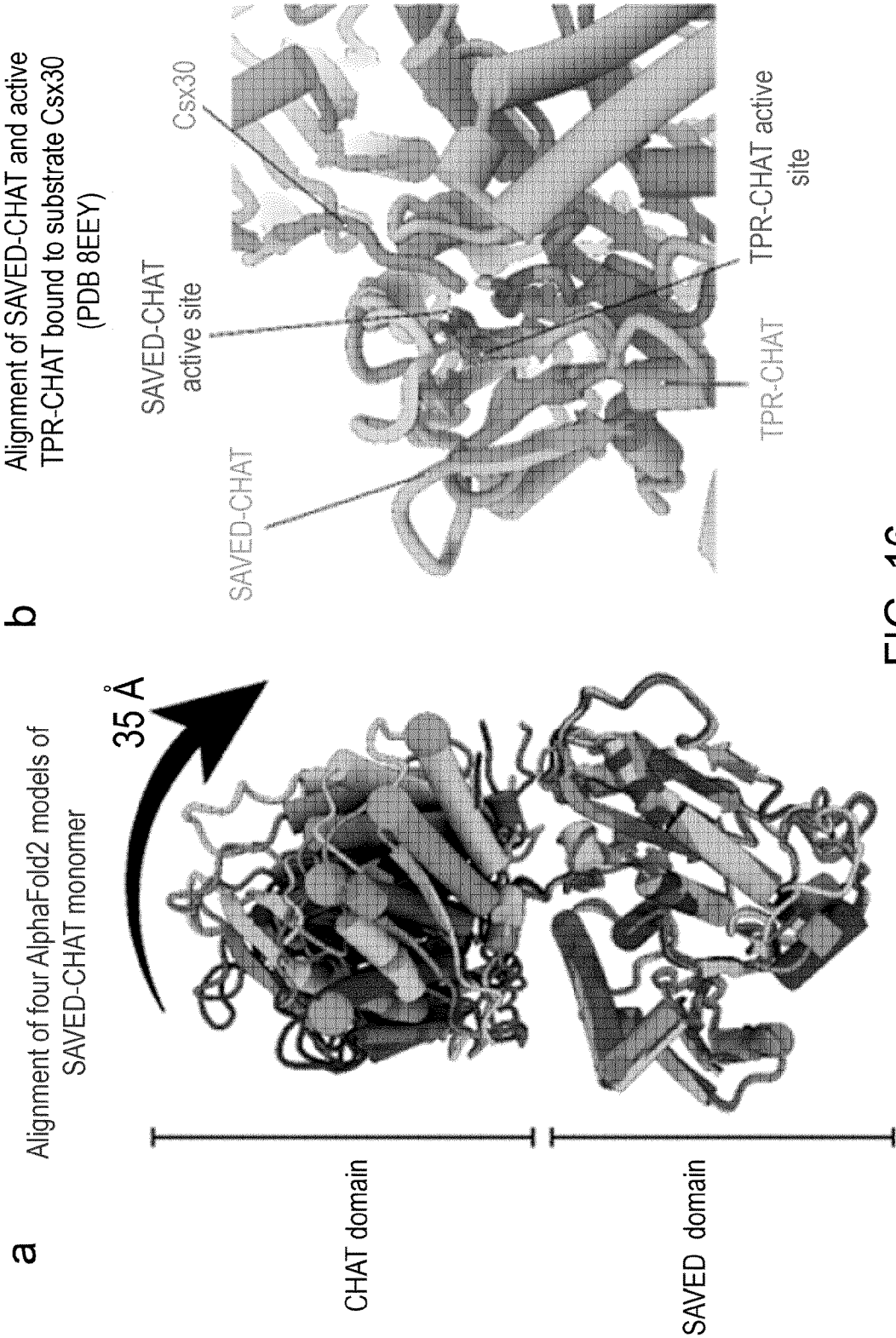


FIG. 16

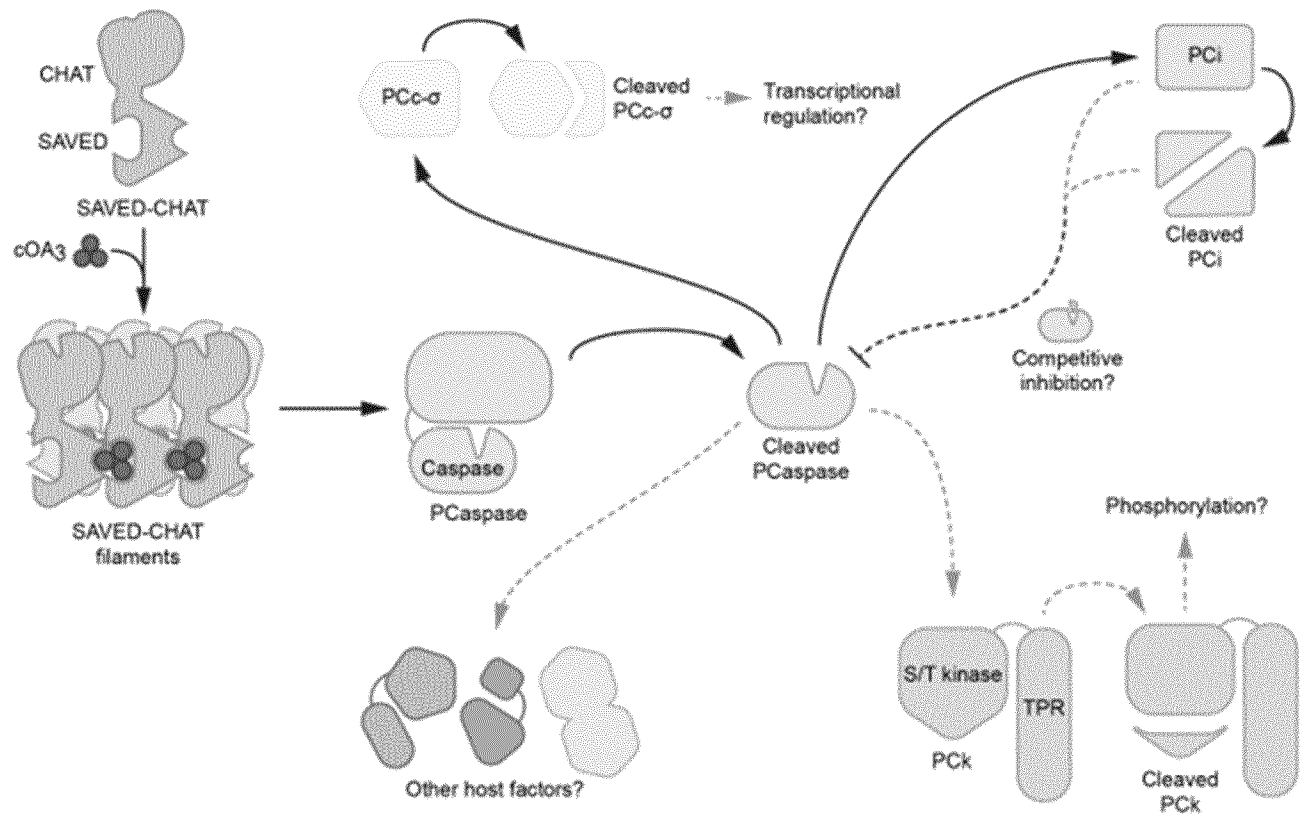


FIGURE 17

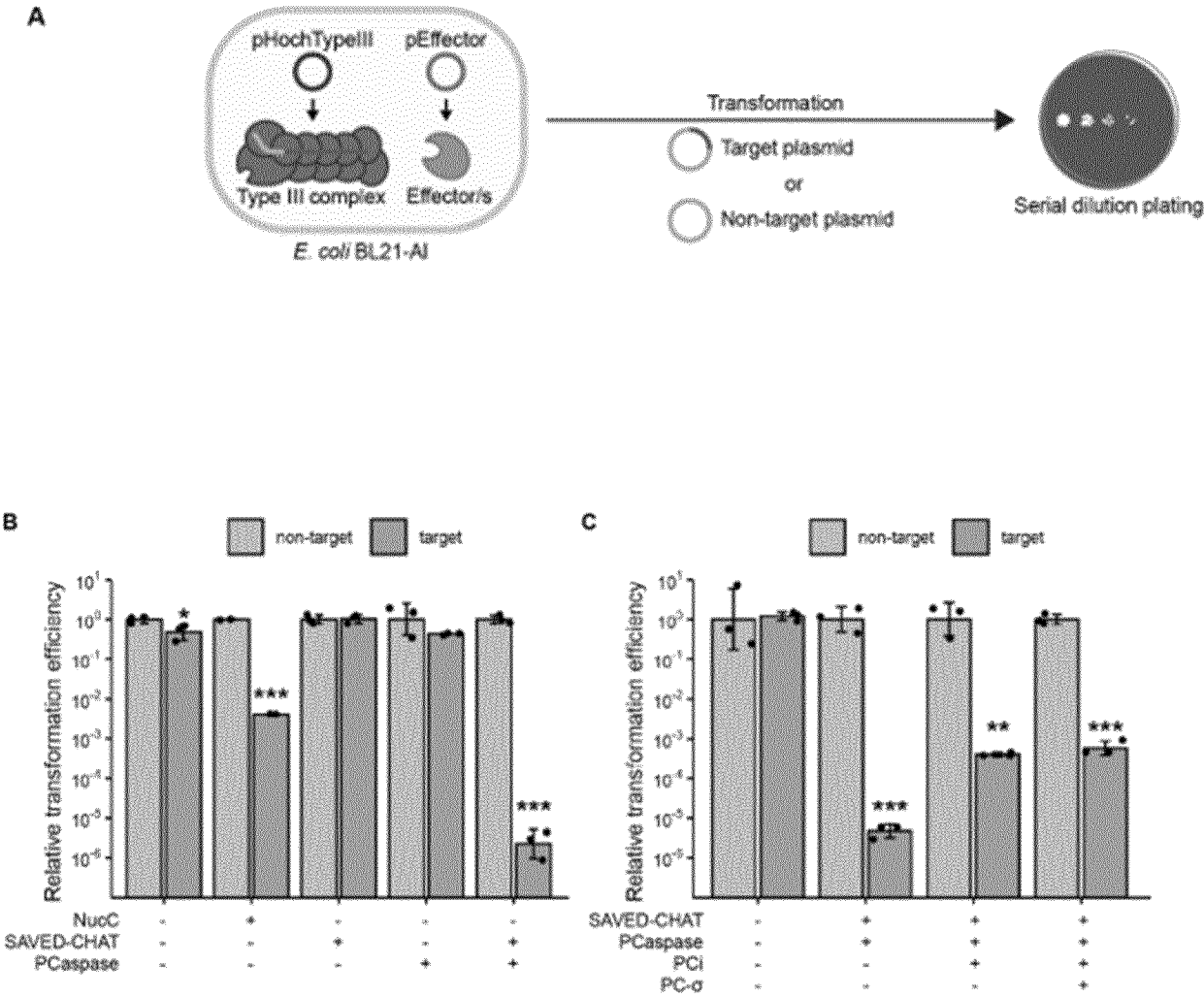


FIGURE 18

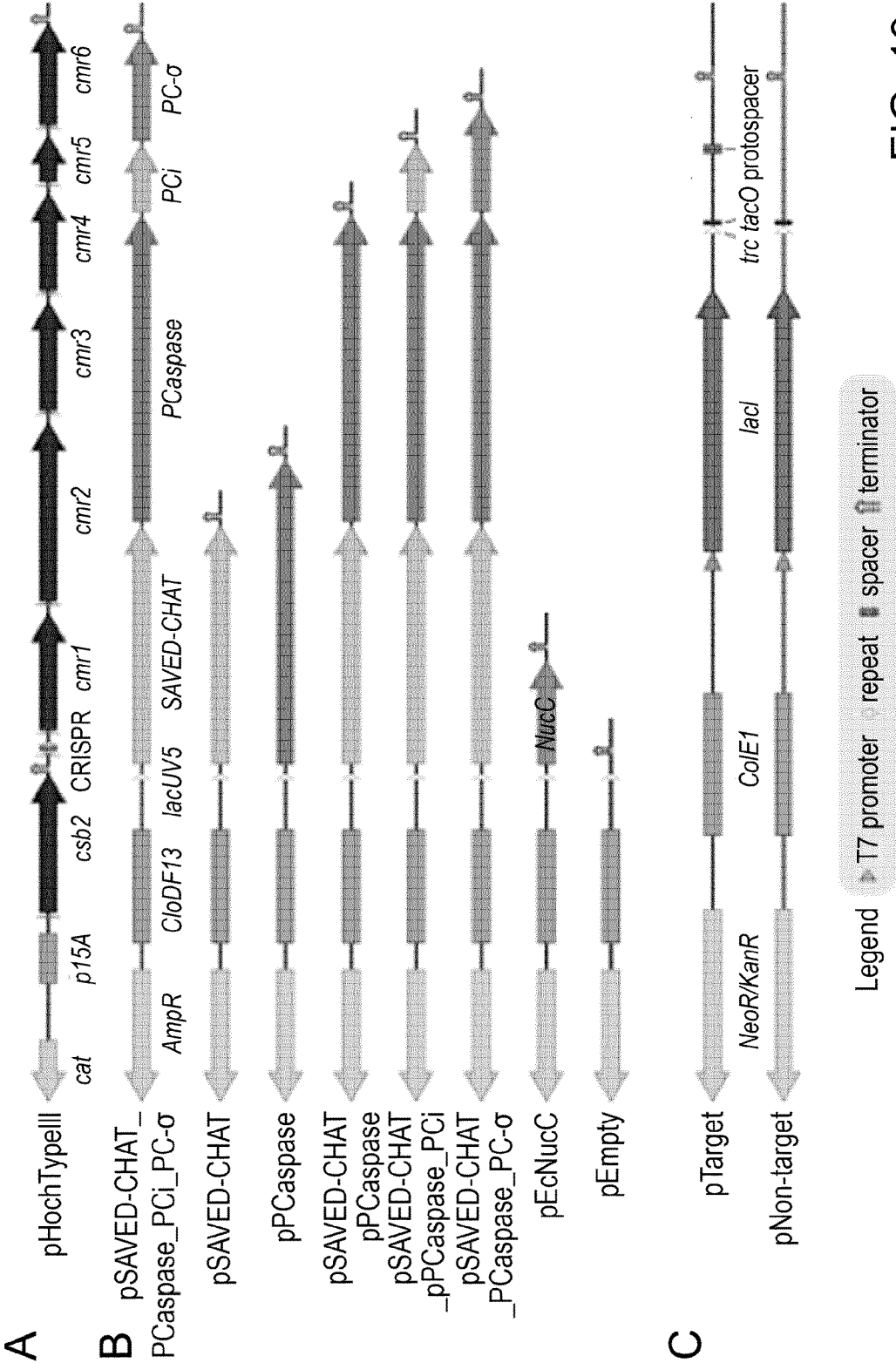


FIG. 19

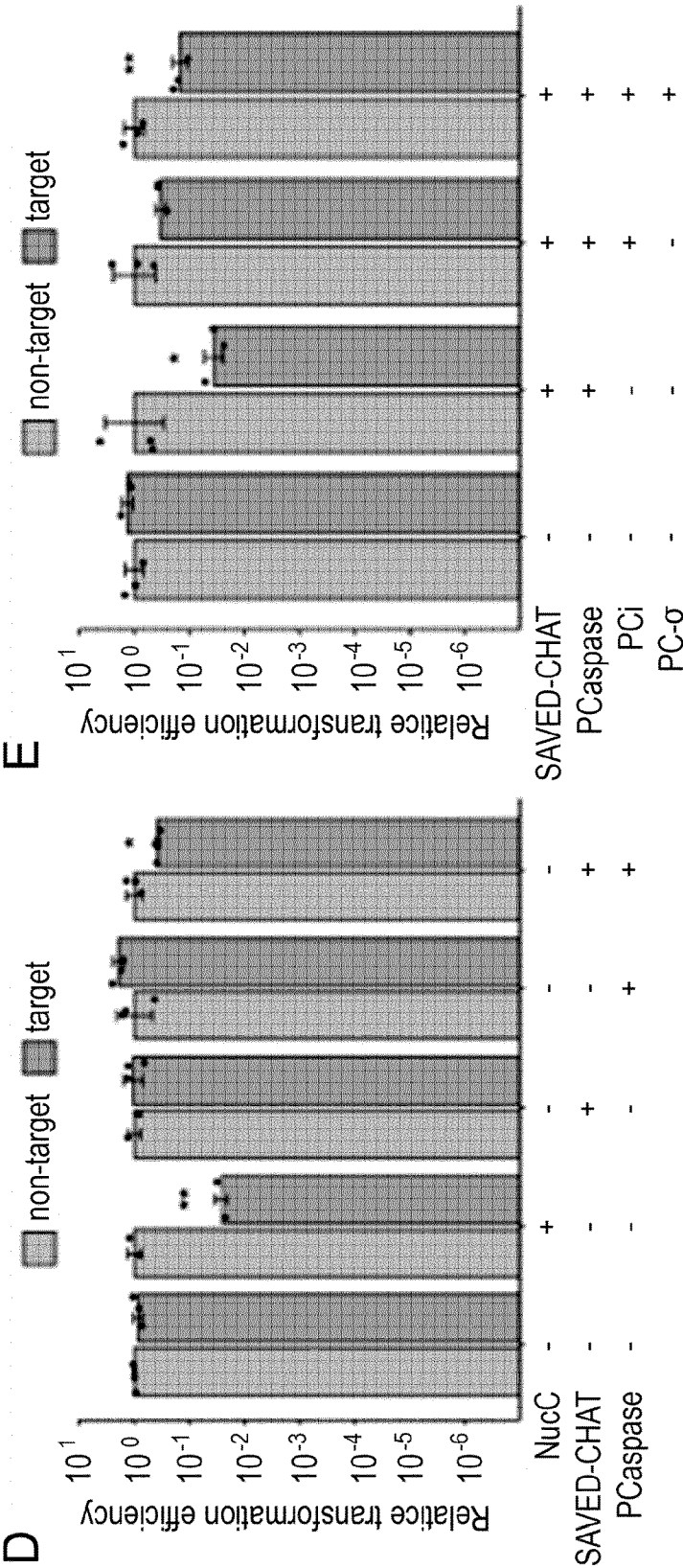


FIG. 19
(continued)

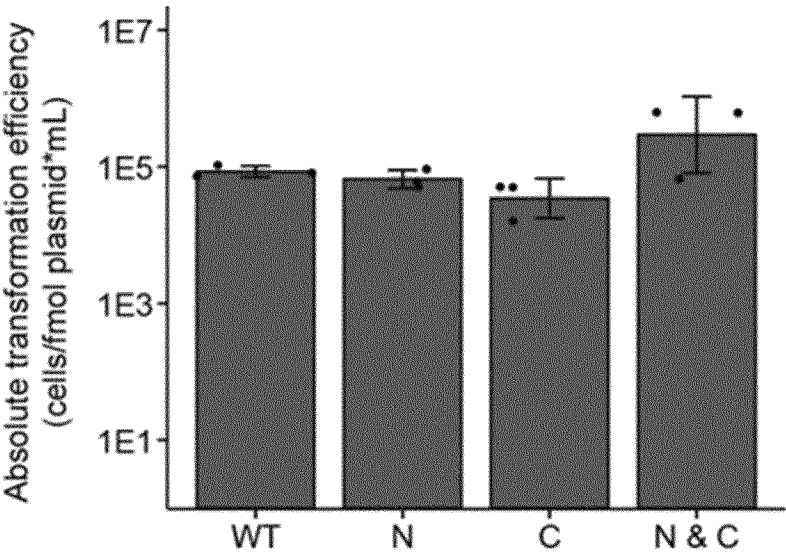


FIGURE 20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2024/066358

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2024/066358

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:
1 - 36, 49 - 65

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/066358

A. CLASSIFICATION OF SUBJECT MATTER INV. C12Q1/682 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2020/256553 A1 (UNIV WAGENINGEN [NL]) 24 December 2020 (2020-12-24) cited in the application claims 1, 9 -----	1 - 36, 49 - 59
X	Rouillon Christophe ET AL: "SAVED by a toxin: Structure and function of the CRISPR Lon protease", bioRxiv, 6 December 2021 (2021-12-06), XP093180560, DOI: 10.1101/2021.12.06.471393 Retrieved from the Internet: URL:https://www.biorxiv.org/content/10.110 1/2021.12.06.471393v1.full.pdf figures 2, 4 -----	49
☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 5 July 2024		Date of mailing of the international search report 26/09/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Aslund, Fredrik

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/066358

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2020256553 A1	24-12-2020	CN 114207145 A	18-03-2022
		EP 3987058 A1	27-04-2022
		JP 2022538010 A	31-08-2022
		KR 20220038668 A	29-03-2022
		US 2022325328 A1	13-10-2022
		WO 2020256553 A1	24-12-2020

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-36, 49-65

System, method and kit comprising: (a) a type III CRISPR-associated effector protein complex which when comprised with a targeting RNA substantially complementary to a desired target polynucleotide sequence recognises that target sequence in a nucleic acid molecule and cleaves the nucleic acid molecule and produces a cyclic oligoadenylate (cAx) messenger; (b) a cyclic oligoadenylate (cAx)-dependent protease; and (c) a protein or polypeptide susceptible to cleavage by the protease of (b) and from which a detectable signal is generated whether directly or indirectly by cleavage of said protein or polypeptide by the protease of (b).

2. claims: 37-48

A polynucleotide that encodes of specified Seq IDs that relates to a type III CRISPR complex comprising proteins, SAVED-CHAT proteins, CHAT-SAVED proteins, PCaspase or a a PCK.
