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(54) Title: SYSTEMS AND METHODS FOR ENHANCING PROTEIN PRODUCTION

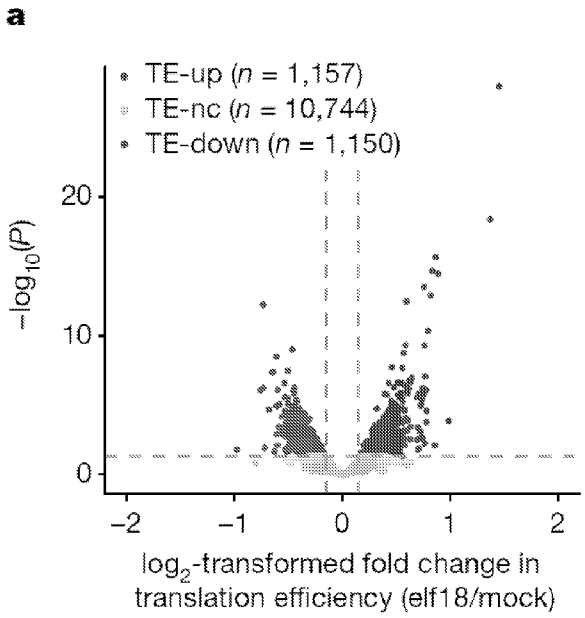

FIG. 1

(57) Abstract: The present disclosure describes, in part, systems and methods for modulating or enhancing protein production.

[Continued on next page]

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SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report:
02 August 2024 (02.08.2024)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 23/83989

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. C12N 15/113 (2024.01)
ADD. C12N 15/82, C12Q 1/6897 (2024.01)

CPC - INV. C12N 15/1138
ADD. C12N 15/8216, C12N 15/8279

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)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	GU et al., Bi-directional ribosome scanning controls the stringency of start codon selection. Nature Communications, 15 November 2021, volume 12, Article number: 6604. Abstract; pg 3, col 2, para 2; Fig. 2 legend	27-29 --------- 1-3
Y	US 2019/0352664 A1 (DUKE UNIVERSITY) 21 November 2019 (21.11.2019) Claim 1; Claim 7; para [0016]; [0021]; [0027]; [0028]; [0170]	1-3

☐ 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
"D" document cited by the applicant in the international application
"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	Date of mailing of the international search report
10 March 2024 (10.03.2024)	JUN 10 2024
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Kari Rodriquez Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 23/83989

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 fi led.

   b. ☐ furnished subsequent to the international fi ling date for the purposes of international search (Rule 13ter.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/US 23/83989

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.: 4-19, 21, 30-34
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:
----Please see continuation in first extra 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 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.:
1-3, 27-29

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/US 23/83989

Continuation of Box No. III. Observations where unity of invention is lacking.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-3, 27-29, directed to a messenger RNA (mRNA).

Group II, claim 20, directed to a DNA molecule encoding a RNA.

Group III, claims 22-26, 35-37, directed to a method for generating a cell in which a polypeptide can be inducibly expressed, and increasing translation of an ORF in a cell.

The inventions listed as Groups I-III do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features:

Group I has the special technical feature of a messenger RNA (mRNA), that is not required by Groups II-III.

Group II has the special technical feature of a DNA molecule encoding a RNA, that is not required by Groups I and III.

Group III has the special technical feature of a method for generating a cell in which a polypeptide can be inducibly expressed, and increasing translation of an ORF in a cell, that is not required by Groups I-II.

Common technical features:

Groups I-III share the common technical feature of:
a messenger RNA (mRNA) comprising:
(a) an upstream open reading frame (uORF) comprising:
(i) an upstream start codon; and
(ii) a sequence that forms a stem loop structure operably linked to the upstream start codon, wherein the stem loop structure is about 1 to about 35 nucleotides downstream of the upstream start codon, and wherein the stem loop structure comprises a stem having about 12 to about 20 base pairs or a sequence that forms a secondary structure operably linked to the upstream start codon, wherein the secondary structure begins about 1 to about 35 nucleotides downstream of the upstream start codon and has a folding energy of about —19.9 kcal mol-1 to about —34.1 kcal mol-1 when calculated for nucleotides +4 to +104 relative to the upstream start codon; and
(b) a heterologous open reading frame (ORF) encoding a polypeptide, wherein the uORF is 5' of the heterologous ORF and is operably linked to the heterologous ORF, and wherein the uORF regulates translation of the heterologous ORF.

However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is made obvious by US 2019/0352664 A1 to Howard Hughes Medical Institute et al., (hereinafter 'HHMI') in view of the article entitled "mRNA structural elements immediately upstream of the start codon dictate dependence upon eIF4A helicase activity" by Waldron et al., (Genome Biology, 2019, Vol. 20:300) (hereinafter 'Waldron').

HHMI teaches a messenger RNA (mRNA) comprising:
(a) an upstream open reading frame (uORF) comprising:
(i) an upstream start codon; and
(b) a heterologous open reading frame (ORF) encoding a polypeptide, wherein the uORF is 5' of the heterologous ORF and is operably linked to the heterologous ORF, and wherein the uORF regulates translation of the heterologous ORF (Claim 1 - 'A DNA construct comprising a heterologous promoter operably connected to a DNA polynucleotide encoding a RNA transcript comprising a 5' regulatory sequence located 5' to an insert site, wherein the 5' regulatory sequence comprises an R-motif sequence.'; Claim 7 - 'The DNA construct of any one of the preceding claims, wherein the 5' regulatory sequence further comprises a uORF polynucleotide encoding any one of the uORF polypeptides of SEQ ID NOs: 1-38, or a variant thereof.'; para [0021] - 'FIGS. 12A-12L show R-motif-mediated translational control in response elf18 induction, related to FIGS. 3A-3G. FIG. 12A, Effects of R-motif containing 5' leader sequences on basal translational activities after normalization to mRNA (mean±s.e.m., n=3). FIG. 12B, Effects of R-motif deletions (.delta.R) on mRNA abundance (mean±s.d., 2 experiments with 3 technical replicates).'; para [0027] - 'FIGS. 18A-18D show conservation of uORF2TBF1 nucleotide and peptide sequences in plant species. FIG. 18A, Schematic of TBF1 mRNA structure. The 5' leader sequence contains two uORFs, uORF1 and uORF2. CDS, coding sequence. FIGS. 18B-18D'; para [0016] - 'FIG. 7E, Nucleotide resolution of the coverage around start and stop codons using the 15th nucleotide of 30-nt reads of RF.').

Continuation of Box No. III. Observations where unity of invention is lacking.

HHMI does not expressly teach (ii) a sequence that forms a stem loop structure operably linked to the upstream start codon, wherein the stem loop structure is about 1 to about 35 nucleotides downstream of the upstream start codon, and wherein the stem loop structure comprises a stem having about 12 to about 20 base pairs or a sequence that forms a secondary structure operably linked to the upstream start codon, wherein the secondary structure begins about 1 to about 35 nucleotides downstream of the upstream start codon and has a folding energy of about —19.9 kcal mol-1 to about —34.1 kcal mol-1 when calculated for nucleotides +4 to +104 relative to the upstream start codon.

Waldron teaches a sequence that forms a secondary structure operably linked to the upstream start codon, wherein the secondary structure begins about 1 to about 35 nucleotides downstream of the upstream start codon and has a folding energy of about —19.9 kcal mol-1 to about —34.1 kcal mol-1 when calculated for nucleotides +4 to +104 relative to the upstream start codon and can be unwound by eIF4A (Abstract - 'We find that eIF4A inhibition does not lead to global increases in 5'UTR structure, but rather it leads to 5'UTR remodeling, with localized gains and losses of structure. The degree of these localized structural changes is associated with 5'UTR length, meaning that eIF4A-dependent mRNAs have greater localized gains of structure due to their increased 5'UTR length. ... By measuring changes in RNA structure following eIF4A inhibition, we show that eIF4A remodels local 5'UTR structures. The location of these structural elements ultimately determines the dependency on eIF4A, with increased structure just upstream of the CDS being the major limiting factor in translation, which is overcome by eIF4A activity.'; Fig. 1 legend - 'g Mean binned minimum free energy (MFE) of all 50-nt windows, with a step of 10nt, after folding with restraints derived from DMS reactivities under control or hippuristanol conditions, within the 5'UTRs of all transcripts included in panel c. Shaded area represents 95% confidence intervals of the mean'; pg 8, col 1, last para - 'The cytosines within a (GGC)4 motif that has folded into a G-quadruplex would be within the loop position of the quadruplex (Fig. 3f). We therefore reasoned that the reactivity of these cytosines to DMS should be higher when these sequences are folded into a G-quadruplex than when folded into canonical Watson-Crick based structures, due to increased accessibility, as is seen with the SHAPE reagent NAI [23, 46]'; pg 8, col 2, last para - 'This clearly shows that following hipp treatment, 4A-dep mRNAs gain in structure the most just upstream of the CDS and that this is the region in which we see the biggest difference in .delta. reactivity between 4A-dep and 4A-indep mRNAs'; pg 10, col 2, last para - 'Furthermore, this difference is most statistically significant with windows of 15 nt (Fig. 5b), indicating perhaps the optimal length of secondary structure which eIF4A can efficiently unwind within the 5'UTRs of cellular mRNAs. Interestingly, this is in rough agreement with the hairpin size with which eIF4A has been shown to efficiently unwind in vitro [47], and also the translocation step size of eIF4A in single molecule experiments [48].'; pg 15, col 2, para 1 - 'Both the sliding window (Fig. 5b) and dStruct analysis (Additional file 1: Figure S8B) support the in vitro data that eIF4A can only efficiently unwind hairpins up to roughly 15–20 nt [47, 48].'). It would have been obvious to one of ordinary skill in the art that by including stem loop structure forming sequence of Waldron in the mRNA of HHMI, translation of said mRNA could be further modulated according to HHMI and Waldron.

As the technical features were known in the art, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-III inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.

Continuation of Item 4 above: claims 4-19, 21, 30-34 are held unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).