

and B) or by culturing in the vitamin-deprived culture medium containing limiting (B5  $10^{-3}$ X / H  $10^{-4}$ X) vitamin concentrations (condition C). Note that the crossed circle indicates that cells that had not been transfected with the SLC5a6 expression vector did not survive selection in the vitamin-deprived culture medium. After selection, cells were cultured in a non-selective culture medium until analysis by FACS or by immunoglobulin secretion assays of the resulting polyclonal cell pools (Fig. 19-20), or during the generation and analysis of monoclonal populations (Fig. 20-21).

**Fig. 19** shows the enrichment of cells expressing the GFP reporter protein transfected according to the protocol shown in Fig. 18. Analysis by cytofluorometry for GFP fluorescence (**A**) showed high levels of polyclonal populations following vitamin-deprivation based selection (circled C). The enrichment of GFP-positive fluorescent cells (**B**) and the geometric mean of the GFP fluorescence of the cells (**C**) are represented for the polyclonal cell pools.

**Fig. 20** shows the enrichment of cells expressing a therapeutic immunoglobulin (rather than GFP) at high levels in polyclonal populations following vitamin-deprivation based selection, according to the protocols shown in Fig. 18. The production levels of cells selected by vitamin deprivation (labeled C) are higher at the polyclonal cell pool level (panel **A**), and for 10 randomly selected cells clones obtained by limiting dilutions (**panel B**) (see also the legend of Fig. 18).

**Fig. 21** shows the high level of IgG secretion by cell surface staining for one of the IgG-producing clones (Clone C\_a) obtained by vitamin selection (**A**), the stability of production for two such clones (Clones C\_a and C\_b) (**B**), as well as the high viable cell density and production levels of the two clones in fed-batch culture conditions (**C and D**), in comparison to a previously obtained high producer reference clone grown in parallel (BS03).

**Fig. 22** illustrates the selection (via an antibiotic or by culture in vitamin depleted medium (“metabolic”)) of polyclonal populations expressing various therapeutic proteins, one easy-to-express antibody (**A and B**) and one difficult-to-express protein (interferon beta, panel **C**). This shows the versatility of the selection system for the selection of cells producing therapeutic proteins of interest at improved levels relative to conventional antibiotic selection.

## SUMMARY

The disclosure is directed at a eukaryotic expression system comprising:

- at least one first polynucleotide encoding at least one vitamin metabolic protein under the control of at least one first regulatory sequence, and

- under the control of at least one second regulatory sequence, at least one restriction enzyme cleavage site and/or at least one second polynucleotide encoding at least one product of interest.

5 In one aspect, there is provided a eukaryotic expression system comprising:

- at least one first polynucleotide encoding at least one vitamin metabolic protein under the control of at least one first regulatory sequence, and
- under the control of at least one second regulatory sequence, at least one restriction enzyme cleavage site and/or at least one second polynucleotide encoding at least one product of interest,

10 - wherein the at least one vitamin metabolic protein is a vitamin transport protein, and wherein said vitamin transport protein is thiamine transporter-1 (THTR-1), thiamine transporter-2 (THTR-2), thiamine pyrophosphokinase (TPK), thiamine pyrophosphate Carrier (TPC) and/or sodium dependent multi vitamin transporter (SMVT).

15 The at least one vitamin metabolic protein may be a vitamin transport protein. The at least one second polynucleotide may be inserted into said at least one restriction enzyme cleavage site. The vitamin transport protein may transport a soluble vitamin such as vitamin B1, B5 and/or H. The vitamin transport protein may be THTR-1 (thiamine transporter-1), THTR-2 (thiamine transporter-1), TPC (thiamine pyrophosphate Carrier), TPK (thiamine pyrophosphokinase) and/or, in particular SMVT (sodium dependent multi vitamin transporter). An expression vector may comprise the expression system. In particular, a singular vector may comprise said at least one first and said at least one second polynucleotide.

20 The first and/or second regulatory sequence may be promoters, enhancers, locus control regions (LCRs), matrix attachment regions (MARs), scaffold attachment regions (SARs), insulator elements and/or nuclear matrix-associating DNAs.

25 The disclosure is also directed at a kit comprising in one container, the eukaryotic expression system disclosed herein (in particular on one or more vectors) and, in a second container, instructions of how to use said system. In another aspect, there is provided a kit comprising in one container, said eukaryotic expression system disclosed herein and, in a second container, instructions of how to use said system. The kit may further comprise a cell culture medium, preferably having a limiting and/or saturating concentration of at least one vitamin, such as of vitamin B1, B5 and/ or H.

30 The disclosure is also directed at a recombinant eukaryotic cell comprising the expression system described herein; and/or

having an up or down mutation in a vitamin metabolic protein, and a polynucleotide (second polynucleotide) encoding a product of interest, or a regulatory sequence regulating the expression of a polynucleotide encoding the vitamin metabolic protein, wherein the vitamin metabolic protein is

optionally intrinsic to the cell. The cell may be a Chinese Hamster Ovary (CHO) cell. The at least one first polynucleotides may be mutated/ contain an up or down mutation. The vitamin metabolic protein may interfere with vitamin metabolism and/or bind the vitamin within a cell. The vitamin metabolic protein may be pantothenate 1, 2 and/or 3 and/or a thiamin pyrophosphate kinase, such as  
5 TPK1 (thiamin pyrophosphate kinase 1). In another aspect, there is provided a recombinant eukaryotic cell comprising the expression system described herein; and/or having an up or down mutation of a vitamin metabolic protein, and comprising a second polynucleotide encoding a product of interest, wherein the vitamin metabolic protein is optionally intrinsic to the cell, wherein the at least one vitamin metabolic protein is a vitamin transport protein, and wherein said vitamin transport protein is  
10 THTR-1, THTR-2, TPK, TPC and/or SMVT.

The vitamin metabolic protein may be a selectable marker for said recombinant eukaryotic cell and said recombinant eukaryotic cell may produce and, preferably secrete said product of interest.

15 The disclosure is also directed at a eukaryotic cell culture medium comprising the recombinant eukaryotic cells disclosed herein, preferably polyclonal, preferably expressing (i) a vitamin transport protein as the

selectable marker and (ii) a protein of interest. The medium may be a limiting medium for B5, or a saturated medium for B5 but a limiting medium for H. In another aspect, there is provided a eukaryotic cell culture medium comprising the recombinant eukaryotic cells described herein expressing (i) a vitamin transport protein as a selectable marker and (ii) said protein of interest, wherein the at least one vitamin metabolic protein is a vitamin transport protein, and wherein said vitamin transport protein is THTR-1, THTR-2, TPK, TPC and/or SMVT.

The disclosure is also directed at a method for culturing and, optionally selecting recombinant eukaryotic cells comprising:

providing the expression system as disclosed herein,  
providing eukaryotic cells, wherein viability, growth and/or division of said eukaryotic cells is dependent on vitamin uptake,  
introducing said expression system into said eukaryotic cells to produce said recombinant eukaryotic cells expressing said vitamin metabolic protein and said protein of interest,  
culturing said eukaryotic cells in a cell culture medium, e.g., a limiting medium for B5, or a saturated medium for B5 but a limiting medium or not limiting medium for H, or a saturated medium for H but a limiting medium or not limiting medium for B5, and  
optionally, selecting via said vitamin metabolic protein, which is preferably expressed on the surface of said recombinant eukaryotic cells, said recombinant eukaryotic cells that stably express said product of interest.

In another aspect, there is provided a method for culturing and, optionally selecting recombinant eukaryotic cells comprising:

providing the expression system described herein,  
providing eukaryotic cells, wherein viability, growth and/or division of said eukaryotic cells is dependent on vitamin uptake,  
introducing said expression system into said eukaryotic cells to produce said recombinant eukaryotic cells expressing said vitamin metabolic protein and said protein of interest,  
culturing said eukaryotic cells in a cell culture medium, and  
optionally, selecting via said vitamin metabolic protein, which is preferably expressed on the surface of said recombinant eukaryotic cells, said recombinant eukaryotic cells that stably express said product of interest, wherein the at least one vitamin metabolic protein is a vitamin transport protein, and wherein said vitamin transport protein is THTR-1, THTR-2, TPK, TPC and/or SMVT.

A selection medium as disclosed herein might be a limiting medium for B5, or a saturated medium for B5 but a limiting or non-limiting medium for H.

The present disclosure is also directed at the use of a vitamin metabolic protein and its DNA coding sequence as a selection marker for selection of recombinant eukaryotic cells stably expressing a product of interest, wherein viability, growth and/or division of said cell may be dependent on the uptake of a vitamin. In another aspect, there is provided use of the eukaryotic expression system described herein for expressing the vitamin metabolic protein as a selection marker for selection of a recombinant eukaryotic cell stably expressing a product of interest, wherein viability, growth and/or division of said cell is dependent on the uptake of a vitamin

- 10 The present disclosure is also directed at a culture medium comprising at least one vitamin:
- in a concentration of less than 10nM, and/or
  - in a concentration of 20μM or more, wherein said at least one vitamin is an essential vitamin.

15 The at least one vitamin may be vitamin B1, B5 and/ or H. The culture medium may comprise one or more recombinant eukaryotic cells expressing, preferably secreting, a protein of interest. The protein of interest may be a therapeutic protein. Growth and/or division of the cells may be arrested, and the protein of interest may be produced at a maximum arrested level (MAL in [g/l]) that exceeds a maximum level (ML in [g/l]) of protein expressed by the cells when grown in a medium, preferably a standard medium, in which growth is not arrested, wherein the MAL is

20 more than 1,5 x the ML, more than 2 x the ML or even more than 2,5x or 3x the ML.

In another aspect, there is provided a culture medium comprising at least one vitamin:

- in a concentration of less than 10nM, and/or
  - in a concentration of 20μM or more, wherein said at least one vitamin is an essential vitamin,
- 25 wherein said culture medium selects for cells containing at least one copy of the eukaryotic expression system described herein.

The disclosure is also directed at a method of producing a protein of interest, comprising:

(a) transforming eukaryotic cells with an expression system disclosed herein to produce recombinant eukaryotic cells;

(b) culturing said recombinant eukaryotic cells in a culture medium in which viability and/or growth or division of the recombinant eukaryotic cells is dependent upon activity of one or more vitamin metabolic protein;

(c) selecting for recombinant eukaryotic cells expressing said one or more vitamin metabolic protein, wherein said vitamin metabolic protein is a selectable marker to obtain selected recombinant eukaryotic cells, preferably when said recombinant eukaryotic cells are part of a monoclonal cell population (originating from a single cell); and

(d) purifying the protein of interest from said selected recombinant eukaryotic cells or from a culture medium thereof comprising said selected recombinant eukaryotic cells.

In another aspect, there is provided a method of producing a protein of interest, comprising:

(a) transforming eukaryotic cells with an expression system disclosed herein to produce recombinant eukaryotic cells;

(b) culturing said recombinant eukaryotic cells in a culture medium in which viability, growth and/or division of the recombinant eukaryotic cells is dependent upon activity of one or more vitamin metabolic protein,

(c) selecting for recombinant eukaryotic cells expressing said one or more vitamin metabolic protein, wherein said vitamin metabolic protein is a selectable marker to obtain selected recombinant eukaryotic cells; and

(d) purifying the protein of interest from said selected recombinant eukaryotic cells or from a culture medium thereof comprising said selected recombinant eukaryotic cells.

The vitamin metabolic protein may be a vitamin transport protein preferably transporting vitamin B5, B1 and/or H and said culture medium may be limiting and/or saturating for one of more of said vitamins. The vitamin transport protein may be SMVT and the culture medium may be a limiting medium for B5, or a saturated medium for B5 but a limiting medium for H.

The disclosure is also directed at cells, methods, systems and expression vectors disclosed herein, wherein said SMVT protein is encoded by a Slc5a6 gene or a derivative thereof, and/or wherein said eukaryotic cells are part of a monoclonal cell population.

The present disclosure is also more generally directed at assessing whether the strict vitamin requirements of eukaryotic cells could be used as selection tool for transformed cells, in particular transformed cells that stably express high levels of a gene of interest, when co-expressed with a vitamin uptake gene. The present disclosure is also more generally directed at assessing

whether vitamin-depleted or enriched culture media may be used to further improve protein production by such cells.

5 In one specific embodiment, the present disclosure is directed at decreasing the availability of vitamin B5 at the late phase of recombinant protein production to slow cell division, and thereby to increase the level of therapeutic proteins produced in a bioreactor.

10 In one other specific embodiment, the disclosure is also directed at cloning and expressing the multivitamin transporter Slc5a6 (SMVT), involved in the uptake of both vitamin B5 and H into the cell,

in particular CHO-M cells. The disclosure is also directed at cells overexpressing this vitamin transporter to result in faster growth and higher viability in B5-limiting media when compared to non-transformed cells. The disclosure is also directed at co-expressing SLC5A6 as a selection marker to obtain cell lines having higher levels of recombinant protein production. The disclosure is furthermore directed at overexpressing SLC5A6 in cells to produce better cell viability even in a non-depleted culture media, preferably contributing to even more favorable expression levels of therapeutic proteins.

## DISCUSSION OF VARIOUS AND PREFERRED EMBODIMENTS

### Definitions

A eukaryotic expression system according to the present invention comprises elements that allow for expression of a gene of interest in a eukaryotic cells such as a CHO cell, preferably a CHO K1 cell, preferably a CHO-M cell. Generally, the eukaryotic expression system comprises at least one expression vector. However, the eukaryotic expression system might also be part of the genome of a eukaryotic cell. The system/ expression vector comprises regulatory sequences such as promoters, enhancers, locus control regions (LCRs), matrix attachment regions (MARs), scaffold attachment regions (SARs), insulator elements and/or nuclear matrix-associating DNAs that lead to efficient transcription of a transgene integrated into the expression system. These regulatory sequences as any other sequences referred to herein are often heterologous (i.e., foreign to the host cell being utilized, e.g., derived from a different species as the host cell being utilized) or, while being homologous (i.e., endogenous to the host cell being utilized) are present at different genomic location(s) than any counterpart intrinsic to the cells (hereinafter referred to as "heterolocal"). An expression vector may also contain an origin of replication.

The first polynucleotide encoding at least one vitamin metabolic protein and the second polynucleotide encoding at least one product of interest according to the present invention are added to a eukaryotic cell to create a recombinant eukaryotic cell. Genes or proteins intrinsic to the eukaryotic cell are not added to the cell, but exist in the cell independent of any transformation. However, as the person skilled in the art will realize, the first and second polynucleotide might be copies of an intrinsic gene, such as heterolocal copies of the gene. In many instances it is preferred that some or all of the coding DNA sequences (CDSs) of a wild type gene make up the polynucleotides of the present invention, including the first polynucleotide encoding at least one vitamin metabolic protein.

As used herein, "plasmid" and "vector" are used interchangeably, as a plasmid is the most commonly used vector form. However, the invention is intended to include such other forms of expression vectors, including, but not limited to, viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), or transposable vectors, which serve equivalent functions. Herein,

**What we claim is:**

1) A eukaryotic expression system comprising:

- at least one first polynucleotide encoding at least one vitamin metabolic protein under the control of at least one first regulatory sequence, and
- under the control of at least one second regulatory sequence, at least one restriction enzyme cleavage site and/or at least one second polynucleotide encoding at least one product of interest,
- wherein the at least one vitamin metabolic protein is a vitamin transport protein, and wherein said vitamin transport protein is thiamine transporter-1 (THTR-1), thiamine transporter-2 (THTR-2), thiamine pyrophosphokinase (TPK), thiamine pyrophosphate Carrier (TPC) and/or sodium dependent multi vitamin transporter (SMVT).

2) The eukaryotic expression system of claim 1, wherein the at least one second polynucleotide is inserted into said at least one restriction enzyme cleavage site.

3) The eukaryotic expression system of claim 1 or 2, wherein said vitamin transport protein transports a soluble vitamin such as vitamin B1, B5 and/or H.

4) The eukaryotic expression system of any one of claims 1 to 3, wherein said vitamin transport protein is SMVT.

5) The eukaryotic expression system of any one of the preceding claims, wherein the expression system is at least one expression vector.

6) The eukaryotic expression system of claim 5, wherein a singular vector comprises said at least one first and said at least one second polynucleotide.

7) The eukaryotic expression system of any one of the preceding claims, wherein said first and/or second regulatory sequence are promoters, enhancers, locus control regions (LCRs), matrix attachment regions (MARs), scaffold attachment regions (SARs), insulator elements and/or nuclear matrix-associating DNAs.

8) A kit comprising in one container, said eukaryotic expression system of any one of the preceding claims and, in a second container, instructions of how to use said system.

9) The kit of claim 8 further comprising a cell culture medium, preferably having a limiting and/or saturating concentration of at least one vitamin.

10) The kit of claim 9, wherein the cell culture medium has a limiting and/or saturating concentration of vitamin B1, B5 and/ or H.

11) A recombinant eukaryotic cell comprising the expression system of any one of claims 1 to 7; and/or

having an up or down mutation of a vitamin metabolic protein, and comprising a second polynucleotide encoding a product of interest, wherein the vitamin metabolic protein is optionally intrinsic to the cell, wherein the at least one vitamin metabolic protein is a vitamin transport protein, and wherein said vitamin transport protein is THTR-1, THTR-2, TPK, TPC and/or SMVT.

12) The recombinant eukaryotic cell of claim 11, wherein the cell is a Chinese Hamster Ovary (CHO) cell.

13) The recombinant eukaryotic cell of claim 11 or 12, wherein the at least one first polynucleotide or a sequence regulating the expression the at least one first polynucleotide has an up or down mutation.

14) The recombinant eukaryotic cell of claim 11 or 12, wherein the vitamin metabolic protein interferes with vitamin metabolism and/or binds a vitamin within a cell.

15) The recombinant eukaryotic cell of claim 14, wherein the vitamin metabolic protein is TPK1 (thiamin pyrophosphate kinase 1).

16) The recombinant eukaryotic cell of any one of claims 11 to 15 comprising said expression system, wherein the vitamin metabolic protein is a selectable marker for said recombinant eukaryotic cell and said recombinant eukaryotic cell produces and, preferably secretes said product of interest.

17) A eukaryotic cell culture medium comprising the recombinant eukaryotic cells according to any one of claims 11 to 16 expressing (i) a vitamin transport protein as a selectable marker and (ii) said protein of interest, wherein the at least one vitamin metabolic protein is a vitamin transport protein, and wherein said vitamin transport protein is THTR-1, THTR-2, TPK, TPC and/or SMVT.

18) The cell culture medium of claim 17, wherein said medium is a limiting medium for B5, or a saturated medium for B5 but a limiting medium or not limiting medium for H or a saturated medium for H but a limiting medium or not limiting medium for B5, preferably a limiting medium for B5, or a saturated medium for B5 but a limiting medium for H.

19) A method for culturing and, optionally selecting recombinant eukaryotic cells comprising:  
providing the expression system of any one of claims 1 to 7, preferably claims 5 and 6,  
providing eukaryotic cells, wherein viability, growth and/or division of said eukaryotic cells is dependent on vitamin uptake,  
introducing said expression system into said eukaryotic cells to produce said recombinant eukaryotic cells expressing said vitamin metabolic protein and said protein of interest,  
culturing said eukaryotic cells in a cell culture medium, and  
optionally, selecting via said vitamin metabolic protein, which is preferably expressed on the surface of said recombinant eukaryotic cells, said recombinant eukaryotic cells that stably express said product of interest, wherein the at least one vitamin metabolic protein is a vitamin transport protein, and wherein said vitamin transport protein is THTR-1, THTR-2, TPK, TPC and/or SMVT.

20) The method of claim 19, wherein said medium is a limiting medium for B5, or a saturated medium for B5 but a limiting medium for H.

21) Use of the eukaryotic expression system of claim 1 for expressing the vitamin metabolic protein as a selection marker for selection of a recombinant eukaryotic cell stably expressing a product of interest, wherein viability, growth and/or division of said cell is dependent on the uptake of a vitamin.

22) A culture medium comprising at least one vitamin:

- in a concentration of less than 10nM, and/or

- in a concentration of 20μM or more, wherein said at least one vitamin is an essential vitamin, wherein said culture medium selects for cells containing at least one copy of the eukaryotic expression system of claim 1.

23) The culture medium of claim 22, wherein said at least one vitamin is vitamin B1, B5 and/ or H.

24) The culture medium of claim 22 or 23, wherein said culture medium comprises one or more recombinant eukaryotic cells expressing, preferably secreting, a protein of interest.

25) The culture medium of claim 24, wherein said protein of interest is a therapeutic protein.

26) The culture medium of claim 24 or 25 or a method of providing said culture medium, wherein the growth and/or division of said cells is arrested, and said protein of interest is produced at a maximum arrested level (MAL in [g/l]) that exceeds a maximum level (ML in [g/l]) of protein expressed by said cells when grown in a medium, preferably a standard medium, in which growth is not arrested,

wherein the MAL is more than 1,5 x the ML, more than 2 x the ML or even more than 2,5x or 3x the ML.

27) A method of producing a protein of interest, comprising:

(a) transforming eukaryotic cells with an expression system of any one of claims 1 to 7 to produce recombinant eukaryotic cells;

(b) culturing said recombinant eukaryotic cells in a culture medium in which viability, growth and/or division of the recombinant eukaryotic cells is dependent upon activity of one or more vitamin metabolic protein,

(c) selecting for recombinant eukaryotic cells expressing said one or more vitamin metabolic protein, wherein said vitamin metabolic protein is a selectable marker to obtain selected recombinant eukaryotic cells; and

(d) purifying the protein of interest from said selected recombinant eukaryotic cells or from a culture medium thereof comprising said selected recombinant eukaryotic cells.

28) The method of claim 27, wherein the vitamin metabolic protein is a vitamin transport protein preferably transporting vitamins B5, B1 and/or H and said culture medium is limiting and/or saturating for one of more of said vitamins.

29) The method of claim 28, wherein the vitamin transport protein is SMVT and said culture medium is a limiting medium for B5, or a saturated medium for B5 but a limiting medium for H.