

RECOMBINANT TRANSFORMING GROWTH FACTOR BETA IN YEAST

FIELD OF THE INVENTION

- 5 [1] The invention relates to the expression and secretion of transforming growth factor beta (TGF β) in methylotrophic yeast such as *Pichia pastoris* (*P. pastoris*), yeast cells whose genome comprises TGF β , and uses thereof, including use in growth media.

BACKGROUND OF THE INVENTION

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[2] Growth factors are naturally occurring cell signalling molecules that play a number of essential roles including regulating cell proliferation and development, wound healing and cellular differentiation.

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[3] Growth medium used in cell culture usually includes combinations of growth factors. It is therefore important to be able to produce growth factors suitable for use in growth medium efficiently and in a cost-effective manner. Currently, there is no such method of producing TGF β . Methods used to produce TGF β are labour intensive, and it is therefore an expensive material. There is a need for an efficient method of production of purified TGF β .

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[4] In the growing field of cultivated meat, cell culture is a fundamental aspect of the process. One of the limiting steps in the production of cultivated meat is the high cost of growth media. Climate and other environmental concerns are continuing to drive the demand for cultivated meat, and therefore also the need for improved growth media to replace animal serum-based media.

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[5] Transforming growth factor beta (TGF β) are a family of cell regulatory proteins, and members of this family are involved in a wide variety of cellular processes. TGF β 1, TGF β 2 and TGF β 3 are members of this family. TGF β binds to specific TGF β receptors (TGF β Rs), such as TGF β R1 and TGF β R2. TGF β is a key signalling protein that can regulate a variety of functions, including cell proliferation, differentiation, apoptosis, and metabolism. TGF β is commonly used in growth media and there is an ongoing need for an improved method of producing TGF β polypeptides in an efficient manner.

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[6] The present invention meets this need by providing yeast cells whose genome comprises TGF β . To our knowledge, this is the first successful and efficient expression of TGF β in methylotrophic yeast, such as *P. pastoris*.

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[7] It is particularly beneficial to express TGFβ in methylotrophic yeast, including *P. pastoris*, because of the cells post translational modification machinery. TGFβ3, for example, contains five disulphide bridges and will correctly fold upon formation of these disulphide bridges. TGFβ3 has three N-linked glycosylation sites, advantageously *P. pastoris* offers a similar glycosylation pattern to the human pattern ensuring similar folding and activity of TGFβ3, while different enough that it minimised the risk of immune responses being induced.

[8] In addition, methylotrophic yeast, including *P. pastoris*, secrete recombinantly produced proteins more readily than other types of yeast, such as *Saccharomyces cerevisiae*, and therefore are much better suited as platforms for producing recombinant proteins.

SUMMARY OF THE INVENTION

[9] Provided herein is a methylotrophic yeast cell, optionally a *Pichia pastoris* cell, whose genome comprises a sequence encoding transforming growth factor beta.

[10] In some embodiments, the yeast cell genome comprises a sequence encoding TGFβ1, TGFβ2 or TGFβ3.

[11] In some embodiments, the yeast cell genome comprises a sequence encoding TGFβ3.

[12] In some embodiments, the yeast cell has been transformed using a plasmid, wherein the plasmid comprises the nucleic acid sequence of SEQ ID NO: 7, SEQ ID NO: 14 or SEQ ID NO: 15.

[13] In some embodiments, the yeast cell has been transformed using a plasmid, wherein the plasmid comprises the nucleic acid sequence of SEQ ID NO: 7.

[14] In some embodiments, the yeast cell has been transformed using a plasmid, wherein the plasmid comprises the nucleic acid sequence of SEQ ID NO: 14.

[15] In some embodiments, the yeast cell has been transformed using a plasmid, wherein the plasmid comprises the nucleic acid sequence of SEQ ID NO: 15.

[16] Also provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 3, obtained or obtainable from a yeast cell of the invention.

[17] Also provided herein is a method of producing a TGF β polypeptide, wherein the method comprises transforming a yeast cell to express TGF β .

5 [18] Also provided herein is a method of growing an animal cell, wherein the method comprises cultivating the animal cell in a growth media containing a TGF β polypeptide of the invention.

[19] Also provided herein is use of a polypeptide produced according to the invention in an animal cell growth media.

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BRIEF DESCRIPTION OF THE FIGURES

[20] Figs. 1A and 1B show an example of a plasmid (pGT86) used to transform *P. pastoris*, in both circularised form (A) and linearised form (B).

15 [21] Fig. 2 shows dotblot expression results.

[22] Fig. 3 shows western blot expression analysis results.

20 [23] Figs. 4A-4D show examples of other plasmids (pBM64 and pBM65) used to transform *P. pastoris*, in both circularised form (A, C) and linearised form (B, D).

[24] Fig. 5 shows dotplot expression results for plasmid pBM64.

25 [25] Fig. 6 shows dotplot expression results for plasmid pBM65.

[26] Fig. 7 shows dotplot expression results for plasmid pBM65 following liquid PVTA.

[27] Fig. 8 shows western blot results for plasmid pBM65 following liquid PVTA.

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DETAILED DESCRIPTION OF THE INVENTION

[28] The present invention provides novel yeast cells, whose genomes comprise TGF β and are capable of expressing TGF β . The present invention also provides methods of obtaining said yeast cells by transformation and polypeptides produced by these yeast cells.

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[29] Novel yeast cells of the invention include methylotrophic yeast cells such as *P. pastoris* cells. Methylotrophic yeasts can use methanol as a sole source of carbon and energy. All methylotrophic yeasts possess a common methanol-utilizing pathway. Methanol is first oxidized

by AOX in peroxisomes to form formaldehyde and hydrogen peroxide (H₂O₂), both of which are toxic to cells and are broken down by the enzymes located in the peroxisome. Methylophilic yeast cells achieve surprisingly high levels of protein expression in the present invention compared to non-methylophilic yeast cells, such as *Saccharomyces* cells.

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[30] In an embodiment, the invention provides TGFβ coding sequences which are particularly effective in the yeast cell of the invention. In particular, such coding sequences produce surprisingly high levels of expression of TGFβ.

10 [31] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by a person skilled in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, nucleic acid chemistry and hybridisation are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and
15 peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art. The nomenclature used herein, and the laboratory procedures of synthetic biology described below, are those well-known and commonly employed in the art.

[32] CELLS OF THE INVENTION

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[33] In some embodiments, the invention provides a methylophilic yeast cell whose genome comprises transforming growth factor beta (TGFβ).

25 [34] The yeast cell of the invention is a recombinant yeast cell and contains at least one nucleic acid sequence that is not naturally present in the cell.

[35] In some embodiments, the yeast cell of the invention is a *Pichia*, *Candida*, *Hansenula*, *Ogataea*, *Torulopsis* or *Komagataella* cell.

30 [36] In some embodiments, the yeast cell of the invention is a *Pichia pastoris*, *Candida boidinii*, *Ogataea methanolica*, *Ogataea polymorpha*, *Ogataea thermomethanolica* or *Meyerozyma guilliermondii* cell.

[37] In some embodiments, the yeast cell of the invention is a *Pichia pastoris* cell.

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[38] *Pichia pastoris* is also known as *Komagataella phaffii*, *K. phaffii*, *Komagataella phaffii* (*Pichia pastoris*), *Pichia pastoris* (*Komagataella phaffii*), *Komagataella kurtzmanii* (*K. kurtzmanii*)

and other such variations. These terms are intended to refer to the same species and are used interchangeably herein.

[39] *Pichia pastoris* is an example of a methylotrophic yeast.

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[40] The terms 'TGFβ1', 'TGFb1', 'TGFbeta1', 'TGFβ-1', 'TGFb-1', 'TGFbeta-1', 'transforming growth factor beta 1' and 'transforming growth factor beta-1', and similar terms all refer to the same growth factor, and can be used interchangeably.

10 [41] The terms 'TGFβ2', 'TGFb2', 'TGFbeta2', 'TGFβ-2', 'TGFb-2', 'TGFbeta-2', 'transforming growth factor beta 2' and 'transforming growth factor beta-2', and similar terms all refer to the same growth factor, and can be used interchangeably.

15 [42] The terms 'TGFβ3', 'TGFb3', 'TGFbeta3', 'TGFβ-3', 'TGFb-3', 'TGFbeta-3', 'transforming growth factor beta 3' and 'transforming growth factor beta-3', and similar terms all refer to the same growth factor, and can be used interchangeably.

[43] Human TGFβ1 comprises the amino acid sequence of SEQ ID NO: 1, and is shown below

20 MPPSGRLRLPLLLPLLWLLVLTTPGRPAAGLSTCKTIDMELVKKRKRIRGQILSKLRLASPPSQG
EVPPGPLPEAVLALYNSTRDRVAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKFKQSTH
SIYMFNTSELREAVPEPVLLSRAELRLLRLKLKVEQHVELYQKYSNNSWRYLSNRLLAPSDSP
EWLSFDVTGVVRQWLSRGGEIEGFRLSAHCSCDSRDNTLQVDINGFTTGRRGDLATIHGMNR
PFLLLMATPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQLYIDFRKDLGWKWIHEPKGYH
25 ANFCLGPCPYIWSLDTQYSKVLALYNQHNPASAAAPCCVPQALEPLPIVYVGRKPKVEQLSN
MIVRSCKCS

[44] Human TGFβ2 comprises the amino acid sequence of SEQ ID NO: 2, and is shown below

30 MHYCVLSAFLILHLVTVALSLSTCSTLDMQDFMRKRIRGQILSKLKLTSPPEDYPEPEEVPPE
VISIYNSTRDLLQEASRRAAACERERSDEEYAKEVYKIDMPPFFPSETVCPVVTTPSGSVGS
LCSRQSQVLCGYLDAIPPTFYRPFYRIVRFDVSAMEKNASNLVKAEFRVFRLQNPKEQRI
ELYQILKSKDLTSPTRQYIDSKVVKTRAEGEWLSFDVTDVHEWLHHKDRNLGFKISLHPCCT
FVPSNNYIIPNKSEELARFAGIDGTSTYTSQDQKTIKSTRKKNKGKTPHLLMLLPSYRLESQQ
35 TNRRKKRALDAAYCFRNVQDNCCRLPLYIDFRKDLGWKWIHEPKGYNANFCAGACPYLWSSD
TQHSRVLSLYNTINPEASASPCCVSQDLEPLTILYYIGKTPKIEQLSNMIVKSKCS

[45] Human TGFβ3 comprises the amino acid sequence of SEQ ID NO: 3, and is shown below:

ALDTNYCFRNLEENCCVRPLYIDFRQDLGWKWWHEPKGYANFCSGPCPYLRSADTTHSTVL
GLYNTLNPEASASPCCVQDLEPLTILYYVGRTPKVEQLSNMVVKSCCKCS

5 [46] TGF β 3 has a number of functions, such as cell proliferation, differentiation, wound healing, apoptosis, and metabolism.

[47] TGF β 3 binds to transforming growth factor beta receptors (TGF β Rs), stimulating the receptors. This bioactivity is required for effective function of TGF β 3 proteins.

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[48] In some embodiments, the yeast cell of the invention comprises the nucleic acid sequence of SEQ ID NO: 4, SEQ ID NO: 12 or SEQ ID NO: 13.

[49] In some embodiments, the yeast cell of the invention comprises a sequence with at least
15 80% similarity to SEQ ID NO: 4, SEQ ID NO: 12 or SEQ ID NO: 13 that encodes TGF β 3. In some
embodiments, the yeast cell of the invention comprises a sequence with at least 85% similarity to
SEQ ID NO: 4, SEQ ID NO: 12 or SEQ ID NO: 13 that encodes TGF β 3. In some embodiments,
the yeast cell of the invention comprises a sequence with at least 90% similarity to SEQ ID NO:
4, SEQ ID NO: 12 or SEQ ID NO: 13 that encodes TGF β 3. In some embodiments, the yeast cell
20 of the invention comprises a sequence with at least 95% similarity to SEQ ID NO: 4, SEQ ID NO:
12 or SEQ ID NO: 13 that encodes TGF β 3. In some embodiments, the yeast cell of the invention
comprises a sequence with at least 99% similarity to SEQ ID NO: 4, SEQ ID NO: 12 or SEQ ID
NO: 13 that encodes TGF β 3.

25 [50] In some embodiments, the yeast cell of the invention comprises the nucleic acid sequence
of SEQ ID NO: 4.

[51] In some embodiments, the yeast cell of the invention comprises a sequence with at least
80% similarity to SEQ ID NO: 4 that encodes TGF β 3. In some embodiments, the yeast cell of the
30 invention comprises a sequence with at least 85% similarity to SEQ ID NO: 4 that encodes
TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least
90% similarity to SEQ ID NO: 4 that encodes TGF β 3. In some embodiments, the yeast cell of the
invention comprises a sequence with at least 95% similarity to SEQ ID NO: 4 that encodes
TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least
35 99% similarity to SEQ ID NO: 4 that encodes TGF β 3.

[52] In some embodiments, the yeast cell of the invention comprises the nucleic acid sequence
of SEQ ID NO: 12.

[53] In some embodiments, the yeast cell of the invention comprises a sequence with at least 80% similarity to SEQ ID NO: 12 that encodes TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least 85% similarity to SEQ ID NO: 12 that encodes TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least 90% similarity to SEQ ID NO: 12 that encodes TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least 95% similarity to SEQ ID NO: 12 that encodes TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least 99% similarity to SEQ ID NO: 12 that encodes TGF β 3.

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[54] In some embodiments, the yeast cell of the invention comprises the nucleic acid sequence of SEQ ID NO: 13.

[55] In some embodiments, the yeast cell of the invention comprises a sequence with at least 80% similarity to SEQ ID NO: 13 that encodes TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least 85% similarity to SEQ ID NO: 13 that encodes TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least 90% similarity to SEQ ID NO: 13 that encodes TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least 95% similarity to SEQ ID NO: 13 that encodes TGF β 3. In some embodiments, the yeast cell of the invention comprises a sequence with at least 99% similarity to SEQ ID NO: 13 that encodes TGF β 3.

[56] Percent similarity (or 'percentage similarity') between two sequences can be calculated by multiplying the number of matches in the pair by 100 and dividing by the length of the aligned region, including gaps. Identity scoring only counts perfect matches. Gaps at the end of sequences are not included, and internal gaps are included in the length. BLAST (NCBI) can be used to produce an alignment.

[57] In some embodiments, SEQ ID NO: 4 encodes TGF β 3 that binds at least one transforming growth factor beta receptor (TGF β R).

[58] In some embodiments, SEQ ID NO: 4 encodes TGF β 3 that binds TGF β R1, TGF β R2 and/or TGF β R3.

[59] In some embodiments, SEQ ID NO: 12 encodes TGF β 3 that binds at least one transforming growth factor beta receptor (TGF β R).

[60] In some embodiments, SEQ ID NO: 12 encodes TGF β 3 that binds TGF β R1, TGF β R2 and/or TGF β R3.

5 [61] In some embodiments, SEQ ID NO: 13 encodes TGF β 3 that binds at least one transforming growth factor beta receptor (TGF β R).

[62] In some embodiments, SEQ ID NO: 13 encodes TGF β 3 that binds TGF β R1, TGF β R2 and/or TGF β R3.

10 [63] In some embodiments, the yeast cell of the invention expresses a polypeptide comprising the amino acid sequence of SEQ ID NO: 1, or a fragment, analog or derivative thereof that binds at least one transforming growth factor beta receptor (TGF β R).

15 [64] In some embodiments, the yeast cell of the invention expresses a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, or a fragment, analog or derivative thereof that binds at least one transforming growth factor beta receptor (TGF β R).

20 [65] In some embodiments, the yeast cell of the invention expresses a polypeptide comprising the amino acid sequence of SEQ ID NO: 3, or a fragment, analog or derivative thereof that binds at least one transforming growth factor beta receptor (TGF β R).

[66] In some embodiments, the yeast cell of the invention expresses a polypeptide comprising a sequence at least 80% similarity to SEQ ID NO: 1, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGF β R). In some embodiments, the polypeptide of the invention comprises a sequence with at least 85% similarity to SEQ ID NO: 1. In some
25 embodiments, the polypeptide of the invention comprises a sequence with at least 90% similarity to SEQ ID NO: 1, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGF β R). In some embodiments, the polypeptide of the invention comprises a sequence with at least 95% similarity to SEQ ID NO: 1, wherein the polypeptide binds at least one
30 transforming growth factor beta receptor (TGF β R).

[67] In some embodiments, the yeast cell of the invention expresses a polypeptide comprising a sequence at least 80% similarity to SEQ ID NO: 2, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGF β R). In some embodiments, the polypeptide of the
35 invention comprises a sequence with at least 85% similarity to SEQ ID NO: 2. In some embodiments, the polypeptide of the invention comprises a sequence with at least 90% similarity to SEQ ID NO: 2, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGF β R). In some embodiments, the polypeptide of the invention comprises a sequence

with at least 95% similarity to SEQ ID NO: 2, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGF β R).

5 [68] In some embodiments, the yeast cell of the invention expresses a polypeptide comprising a sequence at least 80% similarity to SEQ ID NO: 3, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGF β R). In some embodiments, the polypeptide of the invention comprises a sequence with at least 85% similarity to SEQ ID NO: 3. In some
10 embodiments, the polypeptide of the invention comprises a sequence with at least 90% similarity to SEQ ID NO: 3, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGF β R). In some embodiments, the polypeptide of the invention comprises a sequence with at least 95% similarity to SEQ ID NO: 3, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGF β R).

[69] In some embodiments, the yeast cell of the invention expresses a polypeptide that binds
15 TGF β R1, TGF β R2 and/or TGF β R3.

[70] In some embodiments, the TGF β 3 comprised by a yeast cell of the invention is human TGF β 3.

20 [71] In some embodiments, the TGF β 3 is bovine TGF β 3. In some embodiments, the TGF β 3 is porcine TGF β 3. In some embodiments, the TGF β 3 is avian TGF β 3.

[72] In some embodiments, the TGF β 3 comprises the nucleic acid sequence of SEQ ID NO: 4, SEQ ID NO: 12 or SEQ ID NO: 13.
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[73] In some embodiments, the TGF β 3 comprises the nucleic acid sequence of SEQ ID NO: 4.
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[74] In some embodiments, the TGF β 3 comprises the nucleic acid sequence of SEQ ID NO: 12.
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[75] In some embodiments, the TGF β 3 comprises the nucleic acid sequence of SEQ ID NO: 13.

35 [76] In some embodiments, the sequence encoding TGF β 3 comprises the nucleic acid sequence of SEQ ID NO: 4, SEQ ID NO: 12 or SEQ ID NO: 13.

[77] In some embodiments, the sequence encoding TGF β 3 comprises the nucleic acid sequence of SEQ ID NO: 4.

- [78] In some embodiments, the sequence encoding TGF β 3 comprises the nucleic acid sequence of SEQ ID NO: 12.
- 5 [79] In some embodiments, the sequence encoding TGF β 3 comprises the nucleic acid sequence of SEQ ID NO: 13.
- [80] In some embodiments, the yeast cell has been transformed with a plasmid comprising sequence encoding TGF β 1, TGF β 2 or TGF β 3.
- 10 [81] In some embodiments, the plasmid comprises a promoter.
- [82] In some embodiments, the plasmid comprises a coding sequence.
- 15 [83] In some embodiments, the plasmid comprises a terminator.
- [84] In some embodiments, the plasmid comprises a promoter, a coding sequence and a terminator.
- 20 [85] In some embodiments, the plasmid comprises a signal peptide.
- [86] In some embodiments, the plasmid comprises a promoter, a signal peptide, a coding sequence and a terminator.
- 25 [87] In some embodiments, the promoter is an alcohol oxidase I (AOX1) promoter. In some embodiments, the promoter comprises the nucleic acid sequence of SEQ ID NO: 5.
- [88] In some embodiments, the signal peptide is an alpha mating-factor signal peptide. In some embodiments, the signal peptide comprises the nucleic acid sequence of SEQ ID NO: 6.
- 30 [89] In some embodiments, the coding sequence comprises TGF β 3, optionally human TGF β 3. In some embodiments, the coding sequence comprises the nucleic acid sequence of SEQ ID NO: 4, SEQ ID NO: 12 or SEQ ID NO: 13.
- 35 [90] In some embodiments, the coding sequence comprises TGF β 3, optionally human TGF β 3. In some embodiments, the coding sequence comprises the nucleic acid sequence of SEQ ID NO: 4.

- [91] In some embodiments, the coding sequence comprises TGF β 3, optionally human TGF β 3. In some embodiments, the coding sequence comprises the nucleic acid sequence of SEQ ID NO: 12.
- 5 [92] In some embodiments, the coding sequence comprises TGF β 3, optionally human TGF β 3. In some embodiments, the coding sequence comprises the nucleic acid sequence of SEQ ID NO: 13.
- [93] In some embodiments, the terminator is an alcohol oxidase I (AOX1) terminator. In some
10 embodiments, the terminator comprises the nucleic acid sequence of SEQ ID NO: 7,
- [94] In some embodiments, the plasmid comprises the nucleic acid sequences of SEQ ID Nos: 4-7.
- 15 [95] In some embodiments, the plasmid comprises the nucleic acid sequences of SEQ ID NOs: 12 and 5-7.
- [96] In some embodiments, the plasmid comprises the nucleic acid sequences of SEQ ID NOs: 13 and 5-7.
- 20 [97] In some embodiments, the plasmid comprises at least one tag.
- [98] In some embodiments, the tag comprises a His-tag fused to a HiBiT tag. In some
25 embodiments, the tag comprises the nucleic acid sequence of SEQ ID NO: 8.
- [99] In some embodiments, the tag comprises a His-tag fused to a TEV cleavage site. In some
embodiments, the tag comprises the nucleic acid sequence of SEQ ID NO: 18.
- [100] In some embodiments, the plasmid comprises the nucleic acid sequences of SEQ ID NOs:
30 4-8.
- [101] In some embodiments, the plasmid comprises the nucleic acid sequences of SEQ ID NOs: 12, 5-7 and 18.
- 35 [102] In some embodiments, the plasmid comprises the nucleic acid sequences of SEQ ID NOs: 13, 5-7 and 18.

- [103] In some embodiments, the plasmid comprises the nucleic acid sequence of SEQ ID NO: 9, SEQ ID NO: 16 or SEQ ID NO: 17.
- 5 [104] In some embodiments, the plasmid comprises the nucleic acid sequence of SEQ ID NO: 9.
- [105] In some embodiments, the plasmid comprises the nucleic acid sequence of SEQ ID NO: 16.
- 10 [106] In some embodiments, the plasmid comprises the nucleic acid sequence of SEQ ID NO: 17.
- [107] In some embodiments, the plasmid is the plasmid shown in Fig. 1A.
- 15 [108] In some embodiments, the plasmid is the plasmid shown in Fig. 1B.
- [109] In some embodiments, the plasmid is the plasmid shown in Fig. 4A.
- [110] In some embodiments, the plasmid is the plasmid shown in Fig. 4B.
- 20 [111] In some embodiments, the plasmid is the plasmid shown in Fig. 4C.
- [112] In some embodiments, the plasmid is the plasmid shown in Fig. 4D.
- 25 [113] The plasmid described herein may be circular.
- [114] The plasmid described herein may be linearised.
- [115] **POLYPEPTIDES OBTAINED OR OBTAINABLE FROM A YEAST CELL OF THE**
- 30 **INVENTION**
- [116] In some embodiments, the invention provides a polypeptide obtained or obtainable from a yeast cell disclosed herein.
- 35 [117] In some embodiments, the polypeptide of the invention comprises the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

[118] In some embodiments, the polypeptide of the invention comprises a sequence at least 80% similarity to SEQ ID NO: 1, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 85% similarity to SEQ ID NO: 1, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 90% similarity to SEQ ID NO: 1, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 95% similarity to SEQ ID NO: 1, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR).

[119] In some embodiments, the polypeptide of the invention comprises a sequence at least 80% similarity to SEQ ID NO: 2, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 85% similarity to SEQ ID NO: 2, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 90% similarity to SEQ ID NO: 2, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 95% similarity to SEQ ID NO: 2, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR).

[120] In some embodiments, the polypeptide of the invention comprises a sequence at least 80% similarity to SEQ ID NO: 3, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 85% similarity to SEQ ID NO: 3, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 90% similarity to SEQ ID NO: 3, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR). In some embodiments, the polypeptide of the invention comprises a sequence with at least 95% similarity to SEQ ID NO: 3, wherein the polypeptide binds at least one transforming growth factor beta receptor (TGFβR).

[121] Percent similarity (or 'percentage similarity') between two sequences can be calculated by multiplying the number of matches in the pair by 100 and dividing by the length of the aligned region, including gaps. Identity scoring only counts perfect matches and does not consider the degree of similarity of amino acids to one another. Gaps at the end of sequences are not included, and internal gaps are included in the length.

[122] The present invention further relates to fragments, analogs and derivatives of a polypeptide obtained or obtainable from a yeast cell disclosed therein, where the "fragment," "derivative" and "analog" retains essentially the same biological function or activity as a polypeptide as set forth in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

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[123] METHODS OF THE INVENTION

[124] In some embodiments, a method of producing a TGF β polypeptide is provided, wherein the method comprises cultivating the yeast cell of the invention under conditions which allow for expression of said polypeptide and, optionally, recovering the expressed polypeptide.

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[125] In some embodiments, the method of producing of a TGF β polypeptide further comprises i) optimising a nucleic acid encoding a TGF β polypeptide for expression in yeast, ii) screening more than ten clones for expression of TGF β and/or iii) detecting expression with a tag.

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[126] In some embodiments, the method of producing of a TGF β polypeptide further comprises i) optimising a nucleic acid encoding a TGF β polypeptide for expression in yeast, ii) screening more than ten clones for expression of TGF β and/or iii) detecting expression with a HiBiT peptide tag.

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[127] In some embodiments, the method of producing of a TGF β polypeptide further comprises i) optimising a nucleic acid encoding a TGF β polypeptide for expression in yeast, ii) screening more than ten clones for expression of TGF β and/or iii) detecting expression with a His-tag.

[128] In some embodiments, the invention provides a method of producing a TGF β 3 polypeptide comprising transforming a methylotrophic yeast with a linearised plasmid comprising the nucleic acid sequences of SEQ ID NOs: 4-7 by electroporation and screening more than 10 clones for expression.

[129] In some embodiments, the invention provides a method of producing a TGF β 3 polypeptide comprising transforming a methylotrophic yeast with a linearised plasmid comprising the nucleic acid sequences of SEQ ID NOs: 12 and 5-7 by electroporation and screening more than 10 clones for expression.

[130] In some embodiments, the invention provides a method of producing a TGF β 3 polypeptide comprising transforming a methylotrophic yeast with a linearised plasmid comprising the nucleic acid sequences of SEQ ID NOs: 13 and 5-7 by electroporation and screening more than 10 clones for expression.

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5 [131] In some embodiments, the invention provides a method of producing a TGFβ3 polypeptide comprising transforming *Pichia pastoris* with a linearised plasmid comprising the nucleic acid sequences of SEQ ID NOs: 4-7 by electroporation and screening more than 10 clones for expression.

10 [132] In some embodiments, the invention provides a method of producing a TGFβ3 polypeptide comprising transforming *Pichia pastoris* with a linearised plasmid comprising the nucleic acid sequences of SEQ ID NOs: 12 and 5-7 by electroporation and screening more than 10 clones for expression.

15 [133] In some embodiments, the invention provides a method of producing a TGFβ3 polypeptide comprising transforming *Pichia pastoris* with a linearised plasmid comprising the nucleic acid sequences of SEQ ID NOs: 13 and 5-7 by electroporation and screening more than 10 clones for expression.

[134] In some embodiments, over 20 clones or over 30 clones are screened for expression. In a preferred embodiment, over 40 clones are screened.

20 [135] In some embodiments, the invention comprises screening clones for expression by detection of a tag.

[136] In some embodiments, the invention comprises screening clones for expression by detection of a HiBiT tag.

25 [137] In some embodiments, the invention comprises screening clones for expression by detection of a His-tag.

30 [138] In some embodiments, the method of the invention comprises growing yeast cells of the invention in baffled shake flasks.

[139] In some embodiments, the method of the invention comprises culturing yeast cells of the invention in a stirred bioreactor.

35 [140] In some embodiments, the yeast cell of the invention is cultured in fermentation media in a stirred bioreactor.

[141] In some embodiments, the yeast cell of the invention is cultured in fermentation media in a stirred bioreactor for a defined time, a defined pH, and/or a defined temperature.

5 [142] In some embodiments, the defined time is between 6 - 144 hours, preferably between 24 - 96 hours.

[143] In some embodiments, the defined pH is between 2.0 - 10.0, preferably between 4.0 - 8.0.

10 [144] In some embodiments, the defined temperature is between 4 - 37 °C, preferably between 8 - 30 °C.

[145] In some embodiments, TGF β is expressed and secreted out of the yeast cell and into the fermentation media. In some embodiments, the TGF β is not stored intracellularly in the yeast cell. Thus, the present invention also relates to secreted expression of TGF β using yeast cells.

15 [146] In some embodiments, following the cultivation period, the TGF β -containing supernatant is separated from the yeast cells by centrifugation.

20 [147] In some embodiments, following the cultivation period, the TGF β -containing supernatant is separated from the yeast cells by centrifugation and subjected to filtration-based purification.

[148] In some embodiments, the filtration-based purification removes unwanted molecules.

25 [149] In some embodiments, the filtered TGF β -containing sample will be freeze-dried, optionally for long term stable storage.

[150] It will be understood that the invention can relate to expression of functional variants of TGF β 1, TGF β 2 and TGF β 3, and proteins in the same biological pathway, which achieve the same, or similar, downstream effect to wild-type TGF β .

30 [151] For example, the invention can also relate to expression of any activator of a TGF β family receptor. The invention also can also relate expression of an activator of the TGF β 3 receptor. The invention also relates to the formation of TGF-beta-3-TGFR complex (EMBL-EBI ComplexAc: CPX-2544). The invention also relates to an activator of the TGF β signalling pathway. The
35 invention also relates to an activator of the Transforming growth factor beta receptor signalling pathway (EMBL-EBI GO:0007179).

[152] **USES OF POLYPEPTIDES PRODUCED ACCORDING TO THE INVENTION**

[153] In some embodiments, the invention provides use of the polypeptide described herein in a growth media. In some embodiments, the growth media is an animal growth media. The growth media may be serum-free.

5 [154] A growth media is a medium used for the viability, growth and/or storage of cells. In some embodiments, the growth media of the invention is used for culture of fibroblasts, myoblasts, adipocytes, mesenchymal stem cells or induced pluripotent stem cells (iPSCs).

10 [155] A growth media of the invention can additionally comprise one or more additional growth factors, serum or serum replacement, one or more hormones, one or more antibiotics, one or more trace elements and/or one or more antioxidants.

15 [156] In some embodiments, the invention provides a method of growing a cell, wherein the method comprises cultivating the cell in a growth media containing the polypeptide described herein. In some embodiments, the cell is an animal cell. In some embodiments the growth media is an animal growth media.

20 [157] It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognise, or be able to ascertain using no more than routine study, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims. All publications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention
25 pertains.

[158] All publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

30 [159] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives
35 and "and/or."

[160] Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the feature in the below.

[161] As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or
5 "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps

[162] The term "or combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, MB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination,
15 unless otherwise apparent from the context.

[163] Any part of this disclosure may be read in combination with any other part of the disclosure, unless otherwise apparent from the context.

[164] All of the cells, polypeptides, nucleic acids and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the invention has been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the cells, polypeptides, nucleic acids and/or methods and in the steps or in the sequence of steps of the method described herein without
25 departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

[165] The invention can be further described in the following numbered clauses:
30

1. A yeast cell whose genome comprises a sequence encoding transforming growth factor beta 3 (TGF β 3).
2. The yeast cell of claim 1, wherein the TGF β 3 is human TGF β 3.
3. The yeast cell of claim 1 or claim 2, wherein the TGF β 3 comprises the nucleic acid
35 sequence of SEQ ID NO: 4.

4. The yeast cell of any preceding claim, wherein the yeast cell is a *Pichia pastoris* cell.
5. The yeast cell of any preceding claim, wherein the yeast cell has been transformed using a plasmid.
- 5 6. The yeast cell of claim 5, wherein the plasmid comprises a promoter, the coding sequence, and a terminator.
7. The yeast cell of claim 6, wherein the plasmid additionally comprises a signal peptide.
- 10 8. The yeast cell of claim 6 or claim 7, wherein the promoter is an alcohol oxidase I (AOX1) promoter.
9. The yeast cell of any of claim 6-8, wherein the promoter comprises the nucleic acid sequence of SEQ ID NO: 5.
- 15 10. The yeast cell of any of claims 7-9, wherein the signal peptide is an alpha mating-factor signal peptide.
11. The yeast cell of any of claims 7-10, wherein the signal peptide comprises the nucleic acid sequence of SEQ ID NO: 6.
12. The yeast cell of any of claims 6-11, wherein the terminator is an alcohol oxidase I (AOX1) terminator.
- 25 13. The yeast cell of any of claims 6-12, wherein the terminator comprises the nucleic acid sequence of SEQ ID NO: 7.
14. The yeast cell of any of claims 6-13, wherein the plasmid comprises the nucleic acid sequences of SEQ ID NOs: 4-7.
- 30 15. The yeast cell of any of claims 6-14, wherein the plasmid comprises the nucleic acid sequence of SEQ ID NO: 9.
16. A yeast cell capable of expressing TGFβ3.
- 35 17. The yeast cell of claim 16, wherein the yeast cell is a *Pichia pastoris* cell.

18. A polypeptide comprising the amino acid sequence of SEQ ID NO: 3, obtained or obtainable from the yeast cell of any preceding claim.
19. A method of producing a TGFβ3 polypeptide, wherein the method comprises transforming a yeast cell with a plasmid encoding TGFβ3.
20. A method of producing a TGFβ3 polypeptide, wherein the method comprises cultivating the yeast cell of any of claims 1-17 under conditions which allow for expression of said polypeptide and, optionally, recovering the expressed polypeptide.
21. The method of claim 20, further comprising i) optimising a nucleic acid encoding a TGFβ3 polypeptide for expression in yeast, optionally *Pichia pastoris*, ii) screening more than ten clones for expression of TGFβ3, optionally at least twenty clones, and/or iii) detecting expression with a HiBiT peptide tag.
22. A method of growing an animal cell, wherein the method comprises cultivating the animal cell in a growth media containing the polypeptide of claim 18.
23. Use of the polypeptide of claim 18 in an animal cell growth media.
- [166] The present invention is described in more detail in the following non limiting exemplification.

EXAMPLES

The following examples will be useful in demonstrating the present invention.

Example 1: Plasmid assembly

[167] *Escherichia coli* strain DH5α was used for all DNA assembly and all other cloning described. *E. coli* DH5α cells were routinely grown in LB medium (tryptone 10 g l⁻¹, yeast extract 5 g l⁻¹ and NaCl 5 g l⁻¹) at 37 °C, with shaking at 225 rpm, or on LB agar plates (containing 15 g l⁻¹ bacteriological agar) at 37 °C. LB was supplemented with kanamycin (50 µg ml⁻¹) or chloramphenicol (25 µg ml⁻¹) as appropriate. Chemically competent *E. coli* DH5α cells were routinely used for transformation.

[168] Previously assembled plasmids used in this study are as described in Obst *et al.* "A Modular Toolkit for Generating *Pichia pastoris* Secretion Libraries", ACS Synth. Biol., 2017 and

in Lee *et al.* "A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly", ACS Synth. Biol., 2015.

5 [169] Plasmid pGT86 was assembled from five individual parts: Paox1 (pPTK001), α -MF (pPTK005), TGF β 3 CDS (pGT2), 6xHis-tag-HiBiT (pMMp014) and Taox1(pMMp010), into the backbone pMMc002. The Golden Gate reaction was performed as described in Taylor *et al.* "Start-Stop Assembly: a functionally scarless DNA assembly system optimized for metabolic engineering", Nucleic Acids Res., 2019. Chemically competent *E. coli* cells were transformed with 10 μ l of the pGT86 Golden Gate assembly reaction. The plasmids of transformant colonies were
10 purified and sequence verified.

[170] The TGF β 3 CDS (pGT2), 6xHis-tag-HiBiT (pMMp014) and Taox1(pMMp010) were stored in the Level 0 storage plasmid pYTK001.

15 [171] The coding sequence (SEQ ID NO: 4) of the human Transforming growth factor beta isoform 3 (TGF β 3) was codon optimised for *Pichia pastoris* using TWIST Biosciences software (rather than optimised for the host from which it was derived, as would be normal practice) and synthesized by TWIST Biosciences. The synthetic DNA was directly mixed with pYTK001 in a Golden Gate reaction using the conditions described by Lee *et al.* "A Highly Characterized Yeast
20 Toolkit for Modular, Multipart Assembly" ACS Synth Biol., 2015. *E. coli* was transformed with the resultant reaction mixture. The plasmid (pGT2) of transformant colonies were purified and sequence verified.

25 [172] The tags were generated by annealing complementary pairs of oligonucleotides, oBM37 and oBM38 (sequences shown below), as described in Taylor *et al.* The resultant double stranded DNA was mixed with pYTK001 in a Golden Gate reaction using the conditions described by Lee *et al.* *E. coli* was transformed with the resultant reaction mixture. The plasmid (pMMp014) of transformant colonies were purified and sequence verified.

30 [173] The DNA sequence for the terminator Taox1 was synthesized using TWIST Biosciences. The synthetic DNA was directly mixed with pYTK001 in a Golden Gate reaction using the conditions described by Lee *et al.* *E. coli* was transformed with the resultant reaction mixture. The plasmid (pMMp010) of transformant colonies were purified and sequence verified.

35 [174] oBM37 (SEQ ID NO: 10)

GCATCGTCTCATCGGTCTCAATCCCATCATCATCATCATGTGAGCGGCTGGCGGCTGT
TCAAGAAGATTAGCTGGCTGAGACCTGAGACGGCAT

[175] oBM38 (SEQ ID NO: 11)

ATGCCGTCTCAGGTCTCAGCCATTAGCTAATCTTCTTGAACAGCCGCCAGCCGCTCACATG
5 ATGATGATGATGATGGGATTGAGACCGATGAGACGATGC

[176] pMMc002 was assembled using pYTK002, pYTK047, pYTK072, pYTK080 and pYTK084 via Golden Gate assembly using the reaction conditions described by Lee *et al.* *E. coli* was transformed with the resultant reaction mixture. The plasmid (pMMc002) of transformant colonies
10 were purified and sequence verified.

[177] The resultant plasmid is shown in Figs. 1A and 1B.

Example 2: *Pichia pastoris* transformation

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[178] *Pichia pastoris* strain CBS7435 was used for transforming growth factor beta isoform 3 (TGFβ3) expression. *P. pastoris* cells were routinely grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C, with shaking at 225 rpm, or on YPD agar plates (containing 15 g l⁻¹ bacteriological agar) at 30 °C. YPD was supplemented with Zeocin (100 µg ml⁻¹) as
20 appropriate.

[179] 1000 µg of pGT86 was linearised using PmeI (NEB), as per manufacturers instructions. Linearised pGT86 was cleaned-up by gel purification using the QIAquick Gel Extraction kit (Qiagen). *P. pastoris* cells were transformed with 100 µg of linearised pGT86 using the condensed
25 protocol for electroporation as described in Lin-Cereghino, J *et al.* "Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*" Biotechniques, 2005.

Example 3: TGFβ3 expression

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[180] *P. pastoris* protein expression was carried out in buffered dextrose/methanol complex medium (BMDY/ BMMY; 1% yeast extract, 2% peptone, 100mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 4x10⁻⁵% biotin, 2% dextrose or 0.5% methanol).

35 [181] For high throughput screening of protein expression in *P. pastoris* deep 96-well plates sealed with sterile breathable sealing film (Breathe Easier, Sigma Aldrich) containing 300 µl of medium were used. For larger scale expression, 500 ml baffled shake flasks containing 100 ml of medium were used. Isolated transformant clones of *P. pastoris* were used to inoculate YPD

supplemented with zeocin and grown at 30 °C at 225 rpm for 24 hours. Cultures were diluted 1:100 into BMDY supplemented with zeocin and grown at 30 °C at 225 rpm for 24 hours. Following incubation, cells were pelleted at 4000 rpm for 5 minutes, supernatant was removed, and fresh BMMY media added to induce protein expression. Cultures were incubated at 30 °C, 225 rpm for 5 48 hours, at 24 hours cultures were supplemented with methanol (100%) to a final concentration 0.5% v/v. Following incubation, cultures were pelleted at 4000 rpm for 5 min, and the supernatants were collected for protein expression assays.

[182] Trichloroacetic acid (100%) was added to supernatant to a final concentration of 10%. 10 Supernatant was incubated at -20 °C for 15 minutes, before protein precipitates were pelleted at 10,000 g, 4 °C for 15 minutes. The pellet was washed twice with acetone (100%) and then air dried. The washed pellet was resuspended in 1/10 of the supernatant volume in Tris-HCl (50mM, pH 8).

15 [183] 100 µl of supernatant was loaded into individual wells of the Dot Blotter apparatus (Wolflabs). A vacuum was applied to transfer the proteins onto a nitrocellulose membrane (Amersham). TGFβ3 was detected using a 1:1000 dilution of the Mouse anti-HiBiT primary antibody (CS2006A01) followed by 1:2500 dilution of the horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody (W4021). Dots were observed using the ECL Western 20 Blotting Substrate (Promega W1001).

[184] 20 µl of sample (supernatant or concentrated supernatant) were denatured by boiling for 10 minutes in reducing SDS sample buffer (4x Laemmli protein sample buffer supplemented with 50mM DTT). The prestained protein ladder (10-250 kDa, PageRuler, Thermo Scientific) was used 25 to estimate molecular weight. Proteins were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% Surepage precast gels (GenScript). Proteins were fixed to a nitrocellulose membrane (Amersham) using the wet/tank blotting systems (Bio Rad; 100V, 1 hour, 4°C). TGFβ3 was detected using a 1:1000 dilution of the Mouse anti-HiBiT primary antibody (Promega CS2006A01) followed by 1:2500 dilution of the horseradish peroxidase (HRP) 30 conjugated anti-mouse secondary antibody (Promega W4021). Dots were observed using the ECL Western Blotting Substrate (Promega W1001).

[185] In this experimental protocol, contrary to usual practice (6-10 verified clones as recommended by the Invitrogen Pichia Expression Kit manual), we screened greater than 50 35 transformant clones. This was significant because only 6.25% of clones screened expressed TGFβ3.

[186] The results are shown in Figs. 2 and 3.

[187] As seen in Figs. 2 and 3, for both colonies assayed (c3 and c25), expression of TGFβ3 is seen, showing that successful integration and expression in *Pichia pastoris* has been achieved.

5 [188] 3.4% of clones express TGFβ3.

[189] Concentration was assessed for TGFβ3 colony c3, because it gave the strongest band on western blot (Fig. 3). TGFβ3 concentration was established using a calibration curve to enable quantitative dotblot. A calibration curve was generated by plotting the dot intensity against known
10 protein concentration. Dot intensity was measured using ImageJ. The intensity of TGFβ3 c3 dot was measured (using ImageJ) and compared using $y=mx+c$ to the calibration curve to establish concentration.

[190] The concentration of TGFβ3 was 8.49 mg/L.
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Example 4: Plasmid assembly 2

[191] *Escherichia coli* strain DH5α was used for all DNA assembly and all other cloning described. *E. coli* DH5α cells were routinely grown in LB medium (tryptone 10 g l⁻¹, yeast extract
20 5 g l⁻¹ and NaCl 5 g l⁻¹) at 37 °C, with shaking at 225 rpm, or on LB agar plates (containing 15 g l⁻¹ bacteriological agar) at 37 °C. LB was supplemented with kanamycin (50 µg ml⁻¹) or chloramphenicol (25 µg ml⁻¹) as appropriate. Chemically competent *E. coli* DH5α cells were routinely used for transformation.

25 [192] Previously assembled plasmids used in this study are as described in Obst *et al.* "A Modular Toolkit for Generating *Pichia pastoris* Secretion Libraries", ACS Synth. Biol., 2017 and in Lee *et al.* "A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly", ACS Synth. Biol., 2015.

30 [193] Plasmid pBM64 (SEQ ID NO: 16) was assembled from five individual parts: Paox1 (pPTK001), α-MF (pPTK005), TGFβ3 CDS2 (pGT102), TEV_His_Tag_Linkers and Taox1 (pMMp010), into the backbone pMMc002. The Golden Gate reaction was performed as described in Taylor *et al.* "Start-Stop Assembly: a functionally scarless DNA assembly system optimized for metabolic engineering", Nucleic Acids Res., 2019. Chemically competent *E. coli* cells were
35 transformed with 10 µl of the pBM64 Golden Gate assembly reaction. The plasmids of transformant colonies were purified and sequence verified.

[194] Plasmid pBM65 (SEQ ID NO: 17) was assembled from five individual parts: Paox1 (pPTK001), α -MF (pPTK005), TGF β 3 CDS3 (pGT103), TEV_His_Tag_Linker and Taox1 (pMMp010), into the backbone pMMc002. The Golden Gate reaction was performed as described in Taylor *et al.* "Start-Stop Assembly: a functionally scarless DNA assembly system optimized for metabolic engineering", Nucleic Acids Res., 2019. Chemically competent *E. coli* cells were transformed with 10 μ l of the pBM65 Golden Gate assembly reaction. The plasmids of transformant colonies were purified and sequence verified.

[195] The TGF β 3 CDS2 (pGT102) was stored in the Level 0 storage plasmid pYTK001.

[196] The TGF β 3 CDS3 (pGT103) was stored in the Level 0 storage plasmid pYTK001.

[197] The coding sequences (CDS2 and CDS3) that encode the human Transforming growth factor beta isoform 3 (TGF β 3) were codon optimised for expression in *Pichia pastoris* (rather than optimised for the host from which it was derived, as would be normal practice). TGF β 3 CDS2 (SEQ ID NO: 12) was codon optimised using GeneART software (Thermo Fisher Scientific). TGF β 3 CDS3 (SEQ ID NO: 13) was codon optimised using the OPTIMIZER one AA – one codon methodology (<http://genomes.urv.es/OPTIMIZER/>). DNA synthesis was carried out by TWIST Biosciences. The synthetic DNA was directly mixed with pYTK001 in a Golden Gate reaction using the conditions described by Lee *et al.* "A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly" ACS Synth Biol., 2015. *E. coli* was transformed with the resultant reaction mixtures. The plasmids of transformant colonies were purified and sequence verified.

[198] The tag was generated by annealing complementary pairs of oligonucleotides, as described in Taylor *et al.* 2019. The TEV_His_Tag_Linker (SEQ ID NO: 18) resulted from annealing oGT44 and oGT45 (sequences shown below). The resultant double stranded DNA tags were used directly in Golden Gate reactions.

[199] oGT44 (SEQ ID NO: 14)

ATCCGAAAACCTTTATTTTCAAGGTCATCATCACCACCATCATTA

[200] oGT45 (SEQ ID NO: 15)

GCCATTAATGATGGTGGTGATGATGACCTTGAAAATAAAGGTTTTC

[201] Taox1 was synthesized as described above in Example 1 and pMMc002 was assembled as described in Example 1.

[202] The resultant plasmids (pBM64 and pBM65) are shown in Figs. 4A-4D.

Example 5: *Pichia pastoris* transformation 2

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[203] *Pichia pastoris* strain CBS7435 was used for transforming growth factor beta isoform 3 (TGFβ3) expression. *P. pastoris* cells were routinely grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C, with shaking at 225 rpm, or on YPD agar plates (containing 15 g l⁻¹ bacteriological agar) at 30 °C. YPD was supplemented with Zeocin (100 µg ml⁻¹) as appropriate.

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[204] 1000 µg of each plasmid (pBM64 and pBM65) were linearised using PmeI (NEB), as per manufacturers instructions. Linearised plasmid was cleaned-up by gel purification using the QIAquick Gel Extraction kit (Qiagen). *P. pastoris* cells were transformed with 100 µg of linearised plasmid using the condensed protocol for electroporation as described in Lin-Cereghino, J *et al.* "Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*" Biotechniques, 2005.

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Example 6: TGFβ3 expression 2

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[205] *P. pastoris* protein expression, using each plasmid, was carried out in buffered dextrose/methanol complex medium (BMDY/ BMMY; 1% yeast extract, 2% peptone, 100mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 4x10⁻⁵% biotin, 2% dextrose or 0.5% methanol).

25

[206] For high throughput screening of protein expression in *P. pastoris* deep 96-well plates sealed with sterile breathable sealing film (Breathe Easier, Sigma Aldrich) containing 300 µl of medium were used. For larger scale expression, 500 ml baffled shake flasks containing 100 ml of medium were used. Isolated transformant clones of *P. pastoris* were used to inoculate YPD supplemented with zeocin and grown at 30 °C at 225 rpm for 24 hours. Cultures were diluted 1:100 into BMDY supplemented with zeocin and grown at 30 °C at 225 rpm for 24 hours. Following incubation, cells were pelleted at 4000 rpm for 5 minutes, supernatant was removed, and fresh BMMY media added to induce protein expression. Cultures were incubated at 30 °C, 225 rpm for 48 hours, at 24 hours cultures were supplemented with methanol (100%) to a final concentration 0.5% v/v. Following incubation, cultures were pelleted at 4000 rpm for 5 min, and the supernatants were collected for protein expression assays.

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[207] Trichloroacetic acid (100%) was added to supernatant to a final concentration of 10%. Supernatant was incubated at -20 °C for 15 minutes, before protein precipitates were pelleted at 10,000 g, 4 °C for 15 minutes. The pellet was washed twice with acetone (100%) and then air dried. The washed pellet was resuspended in 1/10 of the supernatant volume in Tris-HCl (50mM, pH 8).

[208] 100 µl of supernatant was loaded into individual wells of the Dot Blotter apparatus (Wolflabs). A vacuum was applied to transfer the proteins onto a nitrocellulose membrane (Amersham). TGFβ3 was detected using a 1:1000 dilution of the Mouse anti-HiBiT primary antibody (CS2006A01) followed by 1:2500 dilution of the horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody (W4021). Dots were observed using the ECL Western Blotting Substrate (Promega W1001).

[209] 20 µl of sample (supernatant or concentrated supernatant) were denatured by boiling for 10 minutes in reducing SDS sample buffer (4x Laemmli protein sample buffer supplemented with 50mM DTT). The prestained protein ladder (10-250 kDa, PageRuler, Thermo Scientific) was used to estimate molecular weight. Proteins were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% Surepage precast gels (GenScript). Proteins were fixed to a nitrocellulose membrane (Amersham) using the wet/tank blotting systems (Bio Rad; 100V, 1 hour, 4°C). TGFβ3 was detected using a 1:1000 dilution of the Mouse anti-HiBiT primary antibody (Promega CS2006A01) followed by 1:2500 dilution of the horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody (Promega W4021). Dots were observed using the ECL Western Blotting Substrate (Promega W1001).

[210] In this experimental protocol, contrary to usual practice (6-10 verified clones as recommended by the Invitrogen Pichia Expression Kit manual), we screened greater than 50 transformant clones. This was significant because only 6.25% of clones screened expressed TGFβ3.

[211] The results are shown in Figs. 5 and 6.

[212] As seen in Figs. 5 and 6 expression of TGFβ3 is seen, showing that successful integration and expression in *Pichia pastoris* has been achieved.

[213] For pBM64 (Fig. 5), 25.9% of clones express TGFβ3. For pBM65 (Fig. 6), 37.8% of clones express TGFβ3.

Example 7: Liquid PTVA

[214] To further increase TGFβ3 protein concentration, liquid post-transformational vector amplification (PTVA) was used. Liquid PTVA applies increasing selective pressure on *Pichia* strains, typically via antibiotic concentration, to generate more copies of the inserted foreign DNA that contains the gene of interest as well as an antibiotic marker. The clones that survive at the higher concentrations typically have multiple copies of the antibiotic marker, to accommodate increased antibiotic concentration, and therefore multiple copies of the gene of interest, which leads to increase protein concentration.

[215] *Pichia* strain BM65c56 (BM102) (Fig. 6) was chosen as the parental clone for Liquid PTVA, which was performed as described in Aw, R and Polizzi, K "Liquid PTVA: a faster and cheaper alternative for generating multi-copy clones in *Pichia pastoris*".

[216] The results are shown in Fig. 7.

[217] Expression of clone c5 (Fig. 7) was scaled up to 100ml (as described above in Example 6) as it gave a very strong dot intensity (Fig. 7), the protein was visualised using western blot (Fig. 8) and quantified using the His Tag ELISA Detection Kit (GenScript), in accordance with manufacturer's instructions.

[218] The concentration of TGFβ3 was 17 mg/L.

SEQUENCES

SEQ ID NO:	Description	Sequence	Length
1	Human TGFβ1 (aa)	MPPSGLRLLPLLLPLLWLLVLTTPGRPAAGLSTCKTIDMEL VKRKRIEAIKQILSKLRLASPPSQGEVPPGPLPEAVLAL YNSTRDRVAGESAEPEPEPEADYYAKEVTRVLMVETHN EIYDKFKQSTHSIYMFNTSELREAVPEPVLLSRAELRLL RLKLKVEQHVELYQKYSNNSWRYLSNRLLAPSDSPEWL SFDVTGVVRQWLSRGGEIEGFRLSAHCSCDSRDNTLQV DINGFTTGRRGLATIHGMNRPFLLMATPLERAQHLQS SRHRRALDTNYCFSSTEKNCCVRQLYIDFRKDLGWKWI	390

		HEPKGYHANFCLGPCPYIWSLDTQYSKVLALYNQHNPG ASAAPCCVPQALEPLPIVYVGRKPKVEQLSNMIVRSCK CS	
2	Human TGFβ2 (aa)	MHYCVLSAFLILHLVTVALSLSTCSTLDMDQFMRKRIEAI RGQILSKLKLTSPPEDYPEPEEVPPEVISIYNSTRDLLQE KASRRAAACERERSDEEYYAKEVYKIDMPPFFPSETVCP VVTTPSGSVGSLCSRQSQVLCGYLDAIPPTFYRPFYFRIV RFDVSAMEKNASNLVKAEFRVFRQLQNPKEVPEQRIELY QILKSKDLTSPTQRYIDSKVVKTRAEGEWLSFDVTDVH EVLHHKDRNLGFKISLHPCCTFVPSNNYIIPNKSEEELEA RFAGIDGTSTYTSQDQKTIKSTRKKNSGKTPHLLMLLPS YRLESQQTNRKKRALDAAYCFRNVQDNCCLRPLYIDF KRD LGWKWIHEPKGYNANFCAGACPYLWSSDTQHRSRV LSLYNTINPEASASPCCVSQDLEPLTILYYIGKTPKIEQLS NMIVKSCKCS	442
3	Human TGFβ3 (aa)	ALDTNYCFRNLEENCCVRPLYIDFRQDLGWKWWHEPKG YYANFCSGPCPYLRSADTTHSTVLGLYNTLNPEASASPC CVPQDLEPLTILYYVGRTPKVEQLSNMVVKSCCKCS	112
4	Human TGFβ3 (nt)	gcactcgatacgaattactgttttagaaatctgaagagaactgtgcgtg cgtcccctgtacattgattccgtcaagatctgggatggaaatgggttcattg aaccaaagggttactatgccaaactttgttctggccttgccttacctgcgg tcggctgatactactcattccaccgttctggggctgtacaacactctgaatc ctgaagctccgcctcccatgttgttccctcaagacctggagccactg accatcctgtactacgttgacgcacccccaaagtggaacaactctcca acatggtgtcaagagctgcaaattgtca	336
5	AOX1 promoter (nt)	gatctaacaatccaaagacgaaaggtgaatgaaaccttttgccatccga catccacaggtcattctcacacataagtgccaaacgcaacaggaggg gatacactagcagcagaccgttgcaaacgcaggacctccactcctctc tcctcaacacccacttttgccatcgaaaaaccagcccagttattgggcttg attggagctcgctcattccaattccttctattaggctactaacaccatgacttt attagcctgtctatcctggccccctggcgagggtcatgtttgtttattccga atgcaacaagctccgcattacacccgaacatcactccagatgagggtt tctgagtggtgggtcaaatagtttcatgttcccaaatggcccaaaactga cagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatcc aagatgaactaagtttggtcgttgaaatgtaacggccagttggtcaaa aagaaacttcaaaaagtcggcataccgtttgtctgtttggtattgattgac	939

		gaatgctcaaaaataatctcattaatgcttagcgagctctctatcgcttct gaaccccggtgcacctgtgccgaaacgcaaattggggaacacccgct tttgatgattatgcattgtctccacattgtatgctccaagattctggtggg aatactgctgatagcctaacgttcatgatcaaaatttaactgttctaacccc tacttgacagcaatatataaacagaaggaagctgccctgtcttaaaccttt tttttatcatcattattagcttactttcataattgcgactggtccaattgacaa gcttttgatttaacgactttaacgacaactgagaagatcaaaaaacaa ctaattattcgaaacg	
6	Alpha mating- factor (nt)	atgagatttcctcaattttactgctgtttattcgagcatcctccgcattag ctgctccagtcacactacaacagaagatgaaacggcacaattccg gctgaagctgtcatcggttactcagatttagaaggggatttcgatgttgctg tttgccattttcaacagcacaataacgggtattgtttataataactacta ttgccagcattgctgctaagaagaaggggtatctctcgagaaaagag aggctgaagct	267
7	AOX1 terminator (nt)	tcaagaggatgtcagaatgccattgcctgagagatgcaggcttcatttt gatactttttatttgaacctatatagtataggattttttgtcattttgttcttc gtacgagcttgctcctgatcagcctatctcgagctgatgaatatcttggtg taggggtttgggaaaatcattcgagtttgatgttttcttggtatttccactcc tcttcagagtacagaagattaagtgaga	247
8	His-tag fused to HiBit tag (nt)	catcatcatcatcatcatgtgagcggctggcggctgttcaagaagattag c	51
9	pGT86 plasmid sequence (nt)	cgtgcggccgcccctgaattcgcatctagatggtagagccacaaacag ccgggtacaagcaacgatctccaggaccatctgaatcatgcgcggatga cacgaactcacgacggcgatcacagacattaacccacagtacagaca ctgcgacaacgtggcaattcgctgcaataccgtctcactgaactggccg ataattgcagacgaacggatctaacaatccaaagacgaaaggttgatg aaaccttttgccatccgacatccacaggtccattctcacacataagtgcc aaacgcaacaggaggggatacactagcagcagaccgttgcaaacgc aggacctccactcctcttctcctcaacacccacttttgccatcgaaaaacc agcccagttattgggcttgattggagctcgctcattccaattccttctattag gctactaacaccatgactttattagcctgtctatcctggccccctggcga gggtcatgtttgtttatttccgaatgcaacaagctccgcattacacccgaac atcactccagatgagggcttctgagtggtgggtcaaatagtttcatgttcc ccaaatggcccaaaactgacagtttaaacgctgtcttggaaacctaatatg	5161

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11	oBM38 oligonucleotide (nt)	atgccgtctcaggtctcagccattagctaattcttgaacagccgccagc cgctcacatgatgatgatgatgggattgagaccgatgagacgatgc	100
12	Human TGFβ3 (nt) – CDS2	gcttggacaccaactactgcttcagaaacctggaagagaactgttgcgt cagaccactgtacatcgacttcagacaagacctgggtggaagtgggttc acgaacctaaagggtactacgccaacttctgttctggtccatgtccatactt gagatccgctgacactactcactctaccgttttgggtctgtacaacactttg aaccagaggcttctgcttcccatgttgtgtccacaagacttgagcca ttgaccatctgtactacgttggtagaaccccaaaggctcagacagttgtcc aacatggttgttaagtctgcaagtgtct	336
13	Human TGFβ3 (nt) – CDS3	gcttggatactaactactgttttagaaacttggaagaaaactgttgttag accattgtacattgatttagacaagattgggttgaagtgggtcatgaa ccaaagggttactacgctaactttgttctggtccatgtccatacttgagatc	336

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15	oGT45 oligonucleotide (nt)	gccattaatgatgggtgatgatgaccttgaaaataaagggtttc	46
16	pBM64 plasmid sequence (nt)	cgtgcggccgcccctgaattcgcatctagatggtagagccacaaacag ccggtacaagcaacgatctccaggaccatctgaatcatgcgaggatga cacgaactcacgacggcgatcacagacattaacccacagtacagaca ctgcgacaacgtggcaattcgtcgcaataccgtctcactgaactggccg ataattgcagacgaacggatctaacaatccaaagacgaaagggtgaatg aaaccttttgccatccgacatccacagggtccattctcacacataagtgcc aaacgcaacaggaggggatacactagcagcagaccgttgcaaacgc aggacctccactcctctctcctcaacacccacttttgccatcgaaaaacc agcccagttattgggcttgattggagctcgctcattccaattccttctattag gctactaacacatgactttattagcctgtctatcctggccccctggcga gggtcatgtttgtttatttccgaatgaacaagctccgcattaccccgaac atcactccagatgagggcttctgagtgtgggggtcaaatagtttcatgttcc ccaaatggcccaaaactgacagtttaaacgctgtcttggaaacctaatatg acaaaagcgtgatctcatccaagatgaactaagtttggttcgttgaaatg ctaacggccagttggtcaaaaagaaacttcaaaaagtcggcataccggt tgtctgtttggtattgattgacgaatgctcaaaaataatctcattaatgctta gcgcagctctctatcgcttctgaaccccggtgcacctgtgccgaaacgc aatggggaaacacccgcttttgatgattatgcattgtctccacattgtat gctccaagattctggtgggaataactgctgatagcctaacggtcatgatca aaatttaactgttctaaccctacttgacagcaatatataaacagaagga agctgccctgtcttaaaccttttttatcatcattattagcttactttcataattg cgactggttcaattgacaagcttttgatttaacgacttttaacgacaactt gagaagatcaaaaaacaactaattatcgaaacgtatgagatttcttca atcttactgctgtttattcgagcatcctccgattagctgtccagtcacac actacaacagaagatgaaacggcacaattccggctgaagctgtcatc	5149

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18	TEV_His_Ta g_Linkers (nt)	gaaaacctttattttcaaggatcatcatcaccaccatcattaa	42

CLAIMS

1. A methylotrophic yeast cell whose genome comprises a sequence encoding transforming growth factor beta (TGF β).
- 5 2. The yeast cell of claim 1, wherein the yeast cell is a *Pichia pastoris* cell.
3. The yeast cell of claim 1 or claim 2, wherein the TGF β is TGF β 1, TGF β 2 or TGF β 3.
- 10 4. The yeast cell of any preceding claim, wherein the TGF β is TGF β 3, optionally human TGF β 3.
5. The yeast cell of any preceding claim, wherein the sequence comprises the nucleic acid sequence of SEQ ID NO: 13, SEQ ID NO: 12 or SEQ ID NO: 4.
- 15 6. The yeast cell of any preceding claim, wherein the sequence comprises the nucleic acid sequence of SEQ ID NO: 13.
7. The yeast cell of any preceding claim, wherein the sequence comprises the nucleic acid sequence of SEQ ID NO: 12.
- 20 8. The yeast cell of any preceding claim, wherein the sequence comprises the nucleic acid sequence of SEQ ID NO: 4.
- 25 9. The yeast cell of any preceding claim, wherein the yeast cell has been transformed using a plasmid.
10. The yeast cell of claim 9, wherein the plasmid comprises a promoter, the coding sequence, and a terminator.
- 30 11. The yeast cell of claim 10, wherein the plasmid additionally comprises a signal peptide.
12. The yeast cell of claim 10 or claim 11, wherein the promoter is an alcohol oxidase I (AOX1) promoter.
- 35 13. The yeast cell of any one of claim 10-12, wherein the promoter comprises the nucleic acid sequence of SEQ ID NO: 5.

14. The yeast cell of any one of claims 11-13, wherein the signal peptide is an alpha mating-factor signal peptide.
- 5 15. The yeast cell of any one of claims 11-14, wherein the signal peptide comprises the nucleic acid sequence of SEQ ID NO: 6.
16. The yeast cell of any one of claims 10-15, wherein the terminator is an alcohol oxidase I (AOX1) terminator.
- 10 17. The yeast cell of any one of claims 10-16, wherein the terminator comprises the nucleic acid sequence of SEQ ID NO: 7.
18. The yeast cell of any one of claims 10-17, wherein the plasmid comprises the nucleic acid sequences of SEQ ID NOs: 4-7.
- 15 19. The yeast cell of any one of claims 10-18, wherein the plasmid comprises the nucleic acid sequence of SEQ ID NO: 9.
- 20 20. The yeast cell of any one of claims 10-17, wherein the plasmid comprises the nucleic acid sequences of SEQ ID NOs: 12 and 5-7.
21. The yeast cell of any one of claims 10-17 and 20, wherein the plasmid comprises the nucleic acid sequence of SEQ ID NO: 16.
- 25 22. The yeast cell of any one of claims 10-17, wherein the plasmid comprises the nucleic acid sequences of SEQ ID NOs: 13 and 5-7.
23. The yeast cell of any one of claims 10-17 and 22, wherein the plasmid comprises the nucleic acid sequence of SEQ ID NO: 17.
- 30 24. A methylotrophic yeast cell capable of expressing TGF β .
25. The yeast cell of claim 24, wherein the yeast cell is capable of expressing TGF β 1, TGF β 2 or TGF β 3.
- 35 26. The yeast cell of claim 24 or claim 25, wherein the yeast cell is a *Pichia pastoris* cell.

27. A polypeptide comprising the amino acid sequence of SEQ ID NO: 3, obtained or obtainable from the yeast cell of any preceding claim.
28. A method of producing a TGF β polypeptide, wherein the method comprises transforming a methylotrophic yeast cell with a plasmid encoding TGF β .
29. A method of producing a TGF β polypeptide, wherein the method comprises cultivating the yeast cell of any of claims 1-26 under conditions which allow for expression of said polypeptide and, optionally, recovering the expressed polypeptide.
30. The method of claim 29, further comprising i) optimising a nucleic acid encoding a TGF β polypeptide for expression in yeast, optionally methylotrophic yeast, further optionally *Pichia pastoris*, ii) screening more than ten clones for expression of TGF β , optionally at least twenty clones, and/or iii) detecting expression with a HiBiT peptide tag or a his-tag.
31. The method of any one of claims 28 to 30, wherein the TGF β polypeptide is a TGF β 3 polypeptide.
32. A method of growing an animal cell, wherein the method comprises cultivating the animal cell in a growth media containing the polypeptide of claim 27.
33. Use of the polypeptide of claim 27 in an animal cell growth media.