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1 **Constitutively active Rheb mutants [T23M] and [E40K] drive increased production and secretion of**
2 **recombinant protein in Chinese hamster ovary cells**

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14

15 **Abstract**

16 Monoclonal antibodies (mAbs) are high value agents used for disease therapy ('biologic drugs') or as
17 diagnostic tools which are widely used in the health care sector. They are generally manufactured in
18 mammalian cells, in particular Chinese hamster ovary (CHO) cells cultured in defined media, and are
19 harvested from the medium. Rheb is a small GTPase which, when bound to GTP, activates mechanistic
20 target of rapamycin complex 1 (mTORC1), a protein kinase that drives anabolic processes including
21 protein synthesis and ribosome biogenesis. Here we show that certain constitutively-active mutants
22 of Rheb drive faster protein synthesis in CHO cells and increase the expression of proteins involved in
23 the processing of secreted proteins in the endoplasmic reticulum, which expands in response to
24 expression of Rheb mutants. Active Rheb mutants, in particular Rheb[T23M], drive increased cell
25 number under serum-free conditions similar to those used in the biotechnology industry. Rheb[T23M]
26 also enhances the expression of the reporter protein luciferase and, especially strongly, the secreted
27 Gaussia luciferase. Moreover, Rheb[T23M] markedly (2-3 fold) enhances the amount of this luciferase
28 and of a model immunoglobulin secreted into the medium. Our data clearly demonstrate that
29 expressing Rheb[T23M] in CHO cells provides a simple approach to promoting their growth in defined
30 medium and the production of secreted proteins of high commercial value.

31 **Keywords**

32 Recombinant proteins; biologic drug; mTORC1; defined medium; Rheb

33 **Introduction**

34 Mammalian cell culture, most notably Chinese Hamster Ovary (CHO) cells, are commonly used to
35 produce recombinant proteins such as monoclonal antibodies (mAbs) by the biopharmaceutical
36 industry for use in both therapy and diagnosis (Goulet & Atkins, 2020). Such proteins offer enormous
37 advances in disease therapy (or diagnosis) and are products of high commercial value. Over the last
38 several decades there have been significant advances in the efficiency of CHO cells to produce these
39 recombinant proteins (Budge et al., 2020; Kunert & Reinhart, 2016; Sharker & Rahman, 2020;
40 Srirangan et al., 2020). However, the rate of protein production remains a significant bottleneck,

1 particularly for many of the novel format based biotherapeutics now in development, and therefore
2 there remains substantial interest in further advancing the productivity of CHO cells (Budge et al.,
3 2020; Sharker & Rahman, 2020).

4 The rate of protein synthesis (mRNA translation) is a key limiting determinant of recombinant protein
5 production (Godfrey et al., 2017; Khoo & Al-Rubeai, 2009; O'Callaghan et al., 2010; Roobol et al., 2020;
6 Smales et al., 2004). The mechanistic target of rapamycin complex 1 (mTORC1) is a key regulator of
7 protein synthesis (X. Wang & Proud, 2006). mTORC1 is a protein kinase that is activated by a variety
8 of upstream signals, including amino acids (Kim et al., 2008; Sancak et al., 2008) and growth factors
9 (Inoki et al., 2002; B. D. Manning et al., 2002), and acts as a master regulator of diverse anabolic
10 processes including protein synthesis (Proud, 2019) and ribosomal biogenesis (Iadevaia et al., 2014;
11 Saxton & Sabatini, 2017), both of which are critical for efficient protein production. This is achieved
12 through phosphorylation of a number of downstream effectors such as ribosomal protein S6 kinase 1
13 (S6K1) (Chung et al., 1992), eukaryotic elongation factor 2 kinase (eEF2K) (X. Wang et al., 2014), eIF4E-
14 binding protein 1 (4E-BP1) (Beretta et al., 1996), and Maf1 (Michels et al., 2010), a regulator of
15 ribosomal RNA transcription. mTORC1 signalling is thus potentially a major positive regulator of
16 efficient, high-level production of recombinant proteins in mammalian cells.

17 mTORC1 is activated by the small GTPase Rheb (Ras homologue enriched in brain) when it is in its GTP-
18 bound form; its conversion to the inactive GDP-bound state is promoted by the tuberous sclerosis
19 complex which includes the proteins TSC1 and TSC2, the latter acting as a GTPase-activator protein
20 (GAP) for Rheb, thereby converting Rheb to its inactive GDP-bound state (Garami et al., 2003; Inoki et
21 al., 2003; Tee et al., 2003). In turn, the ability of TSC1/2 to impair Rheb function is inhibited by
22 signalling events activated by hormones, mitogenic stimuli and growth factors (Huang & Manning,
23 2008; Inoki et al., 2002; B.D. Manning & Cantley, 2003; Zhang et al., 2003). We have recently
24 discovered that several mutants of Rheb (which occur in certain human cancers) are resistant to the
25 GAP activity of TSC2 and are thus 'constitutively active', promoting high levels of mTORC1 activity in
26 human cells (Xie et al., 2020).

27 The folding, assembly and maturation of most secreted proteins occurs, with the assistance of
28 chaperones, within the endoplasmic reticulum (ER). Homeostatic control of the ER is mediated by the
29 unfolded protein response (UPR) (Preissler & Ron, 2019). In response to protein disequilibrium in the
30 ER, two contrasting consequences are achieved which in combination mediate homeostasis of the ER.
31 Firstly, such stress leads to the global inhibition of protein synthesis. This is due to the phosphorylation
32 of the α -subunit of eIF2, catalysed by the ER-resident kinase, PERK, and in turn P-eIF2 inhibits the
33 activity of eIF2's guanine nucleotide-exchange factor, eIF2B. This decreases the 'load' of new proteins
34 to be folded within the ER (Wek, 2018). However, secondly, by virtue of their upstream open reading
35 frames (uORFs), certain mRNAs undergo preferentially translation in response to P-eIF2 α mediated
36 inhibition of general protein synthesis (Harding et al., 2000). One such protein is 'activating
37 transcription factor 4' (ATF4). ATF4 drives the expression of genes responsible for ensuring protein
38 homeostasis, which are collectively called ER quality control (ERQC) genes (Preissler & Ron, 2019).
39 ERQC genes mainly comprise one encoding chaperones and other proteins involved in folding new
40 polypeptides. Thus the upregulation of ATF4 attenuates ER stress by increasing the protein folding
41 capacity of the cell (Shaffer et al., 2004; Sriburi et al., 2004; M. Wang & Kaufman, 2016).

42 Here we show that two Rheb mutants, T23M and E40K, drive constitutive mTORC1 signalling in human
43 embryonic kidney (HEK293) and CHO cells boosting levels of proteins involved in ER homeostasis
44 which enhances the production of recombinant protein including, importantly, its secretion from the

1 cells. Manipulation of mTORC1 signalling by these Rheb mutants therefore has the clear potential to
2 enhance the production of proteins of high commercial value.

3 **Materials and Methods**

4 *Cell Culture, Vectors and Transfection*

5 HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal
6 bovine serum and 1% penicillin/streptomycin at 37°C with 5% (v/v) CO₂. Firefly Luciferase (FLuc) and
7 *Gaussia* Luciferase (GLuc) expressing Chinese Hamster Ovary (CHO) cells were cultured in Ham's F12
8 medium containing 10% foetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% (v/v)
9 CO₂. Expi-CHO cells were acquired from ThermoFisher Scientific (Thebarton SA, Australia; catalogue
10 number A29127) and cultured in Expi-CHO™ Expression Medium. All cells were regularly tested for
11 mycoplasma contamination. pRK7-FLAG-Rheb vectors were used as previously described (Xie et al.,
12 2020). peGFP-N1 neomycin selection vector was a kind gift from Dr. Timothy Sargeant. To create stable
13 transfection vectors, DNA sequence encoding FLAG-Rheb was amplified from pRK7-FLAG-Rheb vectors
14 via PCR with the addition of 5' *EcoRI* and 3' *BamHI* restriction sites. Amplicons were cloned into peGFP-
15 N1 neomycin selection vector via the *EcoRI* and *BamHI* restriction sites. Transfection into HEK293,
16 FLuc-CHO and GLuc-CHO was performed using Lipofectamine 3000 (ThermoFisher Scientific, SA,
17 Australia) according to the manufacturer's instructions. Transfections into EXPI-CHO™ was performed
18 via the Expifectamine™ transfection system as per the manufacturer's instructions. To create cells
19 stably expressing Rheb mutants, vectors were transfected into EXPI-CHO™ cells which were cultured
20 in the presence of 1 mg/mL G418 for 6 weeks to select stable transfectants. Rheb expression was
21 assessed via SDS-PAGE and Western Blot.

22 *Immunofluorescence*

23 HEK293 cells were seeded into 2 cm x 1 cm chamber slides at a density of 50,000 viable cells/slide 24
24 h prior to transfection. Cells were grown until they had reached 80% confluence. Medium was then
25 removed, and cells washed 3x in PBS before being fixed in 1 mL of 4% (v/v) paraformaldehyde in PBS
26 for 15 min. Cells were then washed 3x with PBS before being permeabilized with 1 mL of freshly made
27 0.05% (v/v) Triton X-100 in PBS for 5 min. Cells were washed 3x with PBS and blocked for 1 h in 10%
28 (v/v) normal donkey serum. Cells were washed 3x with PBS before 400 µL of PBS containing 2% (v/v)
29 normal donkey serum and 1/200 dilutions of indicated primary antibodies was added. Cells were left
30 in primary antibody at 4°C overnight. Primary antibodies were removed, and cells washed 3x in PBS
31 before secondary antibody solution of 400 µL PBS containing 2% (v/v) normal donkey serum and 1/200
32 dilutions of donkey anti-rabbit AF488 and donkey anti-mouse AF594 antibodies were added to each
33 sample. Cells were incubated under foil at room temperature for 1 h before secondary antibodies
34 were removed and cells washed 3x with PBS. Cells were mounted with 400 µL of VectaShield Hardset
35 containing DAPI and cover slips were carefully applied. Cells were then incubated overnight in a 5%
36 (v/v) CO₂ incubator before being sealed with nail polish. Images were collected using a Leica TCS
37 SP8X/MP (Leica Microsystems, NSW, Australia) confocal microscope and processed with ImageJ
38 software package. Cell volume was calculated relative to the β-actin counterstain. Antibodies used in
39 this study are described in Supplementary Table 1.

40 *SUnSET Assay*

41 To perform surface sensing of translation (SUnSET) assays, cells were grown to 70-80% confluency for
42 adherent cells or 4-6x10⁶ cells/mL for suspension cells. in fully supplemented medium. Medium was
43 then replaced for Earle's Balanced Salts Solution (EBSS). For one well, medium was replaced with EBSS
44 containing 1 µM cycloheximide (CHX) as a positive control. After 30 min, puromycin was added to the

1 cells to a final concentration of 1 μ M and allowed to incorporate for 30 min. Cell lysates were then
2 harvested for SDS-PAGE immunoblot analysis as described below, with detection of incorporated
3 puromycin via an anti-puromycin antibody.

4 *SDS-PAGE and Immunoblot Analysis*

5 SDS-PAGE/Western blot analysis was carried out as previously described (Xie et al., 2020). Briefly, cells
6 were lysed in ice-cold lysis buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl pH 7.5,
7 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM
8 dithiothreitol (DTT) and 1 mM β -glycerophosphate supplemented with protease inhibitor cocktail.
9 Protein concentrations were determined via Bradford assay and normalized. Equal aliquots of protein
10 (20 μ g) were denatured in Laemmli loading buffer, heated at 95°C for 3 min and separated by SDS-
11 PAGE using gels containing 7-13% acrylamide and 0.1%-0.36% bis-acrylamide as required. Proteins
12 were transferred to nitrocellulose membranes, which were blocked and incubated with primary
13 antibody as indicated (Supplementary Table S1). The relevant fluorescently-conjugated secondary
14 antibody was applied and signals were imaged using a LiCor Odyssey[®] CLx imager (Millennium Science,
15 VIC, Australia).

16 *RT-qPCR*

17 Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed by first
18 extracting RNA via phenol-chloroform extraction using TriReagent (Sigma-Aldrich, NSW, Australia) as
19 per the manufacturer's instructions (Xie et al., 2020). Contaminating DNA removal and reverse
20 transcription were then performed on equal amounts of RNA using QuantiNova[™] Reverse
21 Transcription Kit. cDNA diluted 1:10 in nuclease free water was used as a template for qPCR reactions
22 with SYBR Green PCR Master Mix (Sigma-Aldrich). For a list of primers used for qPCR reactions, see
23 Supplementary Table S2.

24 *Bromodeoxyuridine (BrdU) Assay*

25 Was performed as previously described (Xie et al., 2020). BrdU (bromodeoxyuridine) Cell Proliferation
26 Assay Kit (Cell Signalling Technology, Danvers, MA #6813) was performed as per the manufacturer's
27 instructions. Cells were plated at 10,000 cells per well in a 96-well plate 2 h prior to addition of 10 μ l
28 of 10x BrdU solution. Two h after adding BrdU, cells were fixed/denatured for 30 min. BrdU detection
29 antibody was diluted 1:100 and added to cells for 1 h. Cells were washed 3x in wash buffer before
30 addition of HRP-linked secondary antibody for 30 min. Cells were washed 3x before addition of
31 3,3',5,5'-tetramethylbenzidine. After 30 min, STOP solution was added and absorbance determined at
32 450 nm. BrdU data are presented as absorbance and not plotted against a standard curve and thus
33 cell number is not calculated.

34 *Firefly and Gaussia Luciferase Assays*

35 Firefly luciferase assay was performed using Steady-Glo[®] Luciferase Assay System (Promega, NSW
36 Sydney) as per the manufacturer's instructions. To prepare cells, FLuc-CHO cells were seeded in 6-well
37 plates and transfected with vector encoding Rheb mutants. Cells were grown for 24 h and then seeded
38 into 96-well plates. Cells were allowed to grow for 24 h before Firefly Luciferase assay was performed.
39 100 μ L of assay reagent was added to each well and 5 min allowed for cell lysis. Luminescence was
40 then measured using GloMax[®] Discover microplate reader (Promega Australia, NSW, Australia)

41 *Gaussia* luciferase (GLuc) assays were performed using the BioLux[®] *Gaussia* Luciferase Assay Kit (New
42 England Biolabs, Ipswich, MA) as per the manufacturer's instructions. Cells were prepared as per the
43 Firefly luciferase assay. GLuc Assay solution was prepared by mixing 1:1000 BioLux[®] GLuc Substrate

1 and BioLux® GLuc Assay buffer. 20 µL of cell growth medium was transferred to a black 96-well plate
2 and 50 µL GLuc Assay solution added. Luminescence was read using a GloMax® Discover microplate
3 reader with a 5 second integration.

4 *Cell Growth Curves*

5 Cell growth curves were produced by seeding cells at a density of 1000 cells per well in 6-well plates.
6 Cells were allowed to grow for 24 h before medium was replaced as indicated. Cells were counted on
7 a haemocytometer every 24 h for 7 days. For GLuc-CHO cells, cells were counted every 4 h from 24 h
8 post-seeding to 60 h post seeding.

9 *IgG Production and Secretion Assay*

10 ExpiCHO-S™ cells stably expressing Rheb[WT], [T23M] or [E40K] were seeded at a density of 4×10^6
11 cells/mL in 125 mL shaker flasks and allowed to grow for 16 h to a density of 10×10^6 cells/mL. Cells
12 were then diluted to 6×10^6 cells/mL and transfected with pcDNA3.2 vector encoding heavy and light
13 chains of rabbit IgG1 at 2:1 light:heavy chain ratio via ExpiFectamine™ CHO Transfection Kit according
14 to the manufacturer's instructions. ExpiFectamine™ CHO/plasmid complexes were prepared by
15 diluting 12 µg plasmid in 1 mL OptiPRO™ medium and 80 µL of ExpiFectamine™ CHO Reagent in 920
16 µL OptiPRO™ medium. Diluted ExpiFectamine™ reagent was added to diluted DNA and incubated at
17 room temperature for 5 min before ExpiFectamine™ CHO/DNA complex was added to cells. Cells were
18 incubated at 37°C and 8% CO₂ on an orbital shaker at 125 rpm at 19 mm shaking diameter. After 24 h,
19 150 µL of ExpiCHO™ Enhancer and 6 mL of ExpiCHO™ Feed were added to cells. Twenty µL of medium
20 were removed each day in order to determine rate of secretion. 10-days post transfection, cells were
21 pelleted via centrifugation at 1000 rpm for 10 min and supernatant collected.

22 IgG concentration in growth medium was determined via Easy-Titre™ Rabbit IgG Assay Kit as per the
23 manufacturer's instructions. Growth medium containing rabbit IgG1 were diluted 1:1000 in PBS. 20
24 µL of Anti-IgG Sensitized Beads were transferred to a 96-well plate and 20 µL of diluted cell medium
25 containing rabbit IgG added. Plates were mixed on a plate mixer for 5 min before 100 µL of blocking
26 buffer was added to each well. Absorbance was measured at 405 nm and IgG concentration calculated
27 from a standard curve of know IgG concentration generated from 1:2 serial dilutions starting at 500
28 ng/mL.

29 Specific productivity was calculated using the following equation:

$$30 \quad Qp \text{ (pg/cell/day)} = \frac{\text{titre}_2 - \text{titre}_1}{\text{density}_2 - \text{density}_1} \times DGR$$

31 where,

$$32 \quad \text{Daily Growth Rate (DGR)} = \frac{\ln(\text{density}_2) - \ln(\text{density}_1)}{\text{time}_2 - \text{time}_1} / 24$$

33

34 **Results**

35 ***Rheb-T23M and E40K drive mild endoplasmic reticulum stress through increased protein synthesis***

36 In a recent study (Xie et al., 2020), we showed that certain mutations in Rheb (which arise in human
37 cancers) are able to drive hyperactive mTORC1 signalling in mouse or human cells. As mTORC1 is well
38 known to promote multiple steps in mRNA translation (protein synthesis), we assessed whether these
39 Rheb mutants can drive increased protein synthesis. To do this we employed surface sensing of

1 translation western blot assay (SUnSET-WB (Schmidt et al., 2009)). In the SUnSET-WB technique, cells
2 are treated with a low concentration of puromycin that is incorporated into nascent polypeptides.
3 Incorporated puromycin can then be detected by western blot with puromycin-specific antibodies.
4 Thus SUnSET-WB is a radioactive-free assay to measure rates of protein synthesis. For our initial
5 experiments, we elected to utilise HEK293 cells as they are a common cell line used for cell signalling
6 studies, used for the production of biotherapeutic proteins, and cells in which we have previously
7 shown Rheb mutations drive constitutive mTORC1 signalling. HEK293 cells transiently transfected with
8 vectors encoding FLAG-Rheb[WT], [T23M], [Y35N] or [E40K] or an empty vector (EV) were transferred
9 to Dulbecco's phosphate buffered saline (D-PBS) for 30 min prior to the addition of 1 μ M puromycin
10 for an additional 30 min. One well of untransfected cells was pre-treated with 50 μ g/mL of
11 cycloheximide, a potent inhibitor of protein synthesis, for 30 minutes prior to the addition of
12 puromycin to provide a 'negative control' for any immunostaining that is not due to ongoing protein
13 synthesis. Cell lysates were then harvested for western blot analysis. Rheb[T23M] and [E40K] each
14 stimulated a large increase in puromycin incorporation compared to either Rheb[WT] or EV (Fig. 1a;
15 quantified in Fig. 1b). Interestingly, despite Rheb[Y35N] being known to drive hyperactive mTORC1
16 signalling, it did not increase puromycin incorporation, in line with the fact that Rheb mutants differ
17 in their downstream effects (Xie et al., 2020).

18 It has been shown that increases in overall protein synthesis can overload the protein folding capacity
19 of the endoplasmic reticulum (ER) resulting in ER stress and activation of the unfolded protein
20 response (Sriburi et al., 2004). This process has been observed in response to increased mTORC1
21 activity (Appenzeller-Herzog & Hall, 2012; Dong et al., 2015) and results in an expansion of the ER and
22 therefore increased protein folding capacity (Shaffer et al., 2004; Sriburi et al., 2004; M. Wang &
23 Kaufman, 2016). We hypothesised that Rheb-mutants may drive mild ER stress resulting in increased
24 ER volume and protein folding capacity. To test this, we first studied several proteins involved in the
25 ATF4 arm of the UPR or in protein folding. HEK293 cells expressing plasmids encoding Rheb[WT] or
26 mutants of Rheb showed increased expression of ATF4 compared to the empty vector (Fig. 1c).
27 Interestingly, there was no significantly greater change in ATF4 protein expression in cells expressing
28 Rheb mutants compared to WT. However, Rheb[T23M] and [E40K] did promote an increase, or tended
29 to cause an increase, in classical UPR markers or ER resident proteins including PERK, BiP/Grp78, IRE-
30 1 α , PDI and ERO1-1 α (Fig. 1c; quantified in Supplementary Figure 1). Calnexin did not change. There
31 was also an increase in the protein folding markers ERO1-L α (Fig. 1c; quantified in Supplementary Fig.
32 S1).

33 To assess whether these changes reflected increased levels of the corresponding mRNAs, we
34 performed RT-qPCR for the mRNAs encoding BiP (*HSPA5*; Fig. 1d), PDI (*PDI*; Fig. 1e), IRE1 α (*ERN1*; Fig.
35 1f), and ATF4 (*ATF4*; Fig. 1g) whose levels were increased by mutant Rheb expression. Increases in
36 mRNAs encoding both UPR and protein folding proteins correlated with protein increases with the
37 notable exception of ATF4 mRNA which was significantly higher in cells expressing Rheb mutants
38 compared to both the EV and Rheb[WT] (Fig. 1d-g). To determine if these observed changes are
39 associated with an increase in ER volume, we performed immunofluorescence on HEK293 cells
40 transiently expressing Rheb mutants or WT or an empty vector. To image the ER, we chose to probe
41 with an anti-calnexin antibody as calnexin is an ER surface protein and since there was no significant
42 change in the level of calnexin protein with the different Rheb mutants (Fig. 1b) (so that any alterations
43 seen in the extent of the ER assessed in this way would be independent of changes in its overall levels).
44 Both Rheb[T23M] and [E40K] promoted a significant increase in ER volume compared to Rheb[WT]
45 and EV. Rheb[Y35N] did not promote an increase in ER volume (Fig. 2a; quantified Fig.2b). These data
46 suggest the Rheb[T23M] and [E40K] mutants can each promote increased protein synthesis which, in

1 turn, drives a mild ER stress resulting in increased ER volume and concomitantly enhanced protein
2 folding capacity.

3 The data for HEK293 cells prompted us to extend our studies to CHO cells, the dominant type of cells
4 used in industry for producing recombinant proteins, particularly as an expanded ER has been
5 reported to enhance the ability of CHO cells to produce secretory recombinant proteins (Budge et al.,
6 2020).

7 ***Rheb mutants drive constitutive mTORC1 signalling in CHO cells***

8 Enabling cells to produce more protein is highly beneficial for the efficient production of therapeutic
9 proteins such as monoclonal antibodies (mAbs). mAbs are most commonly produced from Chinese
10 Hamster Ovary (CHO) cells (Kunert & Reinhart, 2016; Sharker & Rahman, 2020). Given that Rheb
11 mutants increase protein synthesis and enhance ER capacity in HEK293 cells, we hypothesised that
12 these mutants, when expressed in CHO cells, may also result in increased titres of secreted mAb. For
13 our initial experiments, we utilised our previously reported CHO cell lines (Mead et al., 2009) that
14 stably express either the secreted luciferase reporter *Gaussia* Luciferase (GLuc) or firefly Luciferase
15 (FLuc), which is not secreted. First of all, it was important to confirm that Rheb mutants can also drive
16 hyperactive mTORC1 signalling in CHO cells. Phosphorylation of all mTORC1 effectors was slightly
17 elevated in cells expressing Rheb[WT] compared to cells that received EV and was almost completely
18 abolished in both GLuc-CHO (Fig. 3a) and FLuc-CHO cells (Fig. 3b) that were co-transfected with FLAG-
19 TSC1/2 (and WT or mutant Rheb). This is an expected result given that over-expression of Rheb[WT]
20 but not TSC1/2 shifts the stoichiometric balance of Rheb and TSC in favour of Rheb whereas it is
21 expected that endogenous TSC is not enough to stimulate complete hydrolysis of all GTP-bound Rheb.

22 Cells expressing Rheb[T23M] or [E40K] showed higher levels of phosphorylation of S6K1 and 4E-BP1
23 in the absence of TSC1/2 overexpression compared to both EV and Rheb[WT] in GLuc-CHO (Fig. 3a)
24 cells and compared to EV in FLuc-CHO cells (Fig. 3b). However, in contrast to Rheb[WT], when co-
25 expressed with TSC1/2, P-S6K1 and P-4E-BP1 remained elevated in cells expressing Rheb[T23M] and
26 [E40K] and were elevated in Rheb[Y35N] suggesting these mutants drive hyperactivation of mTORC1
27 (i.e., which is insensitive to inhibition by TSC) in GLuc-CHO (Fig. 3a) and FLuc-CHO (Fig. 3b) cells. These
28 data are consistent with our findings for expression of these mutants of Rheb in human cells (Xie et
29 al., 2020).

30 We next sought to determine the effect of Rheb mutations on cell proliferation. None of the Rheb
31 mutants had any effect on cell proliferation when GLuc-CHO cells were grown in fully supplemented
32 medium or medium supplemented with 1% FBS (Fig. 3c, Supplementary Fig. S2a). However, when
33 GLuc-CHO cells were grown in medium supplemented with 0.5% FBS, Rheb[T23M] promoted both an
34 increased rate of proliferation and, presumably as a consequence, an increased total number of cells
35 (Supplementary Fig. S2b). This was even more pronounced when cells were grown in the absence of
36 FBS, with Rheb[T23M] promoting a marked increase in the rate of proliferation (Fig. 3d). To confirm
37 these results, BrdU incorporation assays were performed in order to quantify more directly the effect
38 of Rheb[T23M] on cell proliferation. Consistent with our findings for HEK293 cells (Xie et al., 2020),
39 Rheb[T23M], [Y35N] and [E40K] each drove faster proliferation of both GLuc-CHO and FLuc-CHO cells
40 compared to Rheb[WT] or EV (Fig. 3e/f).

41 ***Rheb[T23M] and [E40K] drive increased protein synthesis and secretion dependent on ER stress***

42 To test whether Rheb mutants drive an increase in recombinant protein production in CHO cells, we
43 transfected FLuc-CHO cells with vectors encoding Rheb mutants and performed firefly luciferase
44 assays every 48 h from days 1-7. In order to determine if the protein production capacity of *individual*

1 cells was increased (and to take into account possible differences in cell number due to the effect of
2 Rheb mutants on cell proliferation), the firefly luciferase assay data were normalised to the number
3 of cells present as counted on a haemocytometer. Cells expressing Rheb[T23M] showed a small yet
4 significant increase in Firefly luciferase (Fig. 4a/b) indicating an increase in protein production.
5 Interestingly, the other Rheb mutants tested did not significantly increase the accumulation of firefly
6 luciferase suggesting that while they promote constitutive mTORC1 activity, this does not result in the
7 general upregulation of protein production.

8 Next, we sought to determine the effect of Rheb mutants on the output of a secreted protein, which
9 is the key parameter for the production of recombinant proteins of commercial interest, which are
10 harvested from the culture medium. To do this, we transfected GLuc-CHO cells with vectors encoding
11 Rheb mutants and performed *Gaussia* luciferase assays on the growth media. As with the firefly
12 luciferase assays, results were normalised to cell number to give an indication of accumulated *Gaussia*
13 luciferase secretion on a per-cell basis. In contrast to firefly luciferase production, Rheb[T23M], [Y35N]
14 and [E40K] each promoted a significant increase in GLuc secretion as calculated in this way (Fig. 4c).
15 Cells expressing these mutants showed an approximately 3-fold increase in GLuc secretion after 7
16 days; no difference was seen in the amounts of intracellular GLuc (not shown), consistent with Rheb
17 mutants boosting secretion of the additional GLuc. As expected, treatment with the potent mTOR
18 inhibitor AZD8055 (Chresta et al., 2010) blocked the effect of mutant Rheb (Fig. 4c/d). These data
19 suggest that, as well as increasing protein synthesis, Rheb mutants may also increase cells' secretory
20 capacity, consistent with the findings for ER expansion in HEK293 cells. As increased ER volume is
21 known to increase secretory capacity (Budge et al., 2020; M. Wang & Kaufman, 2016), we also
22 performed the GLuc assay using cells treated with a combination of the ER stress response inhibitors
23 iPERK (Axten et al., 2012), a compound that inhibits the kinase activity of PERK, and ISRIB (Sidrauski et
24 al., 2013), a compound that interferes with the ability of phosphorylated eIF2 to inhibit the activity of
25 eIF2B (Sidrauski et al., 2015). Inhibition of the ER stress response dramatically decreased the secretion
26 of GLuc from cells expressing Rheb mutants (Fig. 4e) indicating Rheb-mediated ER expansion is crucial
27 for increased GLuc secretion. Consistent with this, western blot analysis of GLuc-CHO cells transiently
28 expressing Rheb mutants or transfected with an empty vector show that Rheb[T23M] and [E40K]
29 significantly drive increased expression of the ATF4 arm of the UPR (Fig. 4f).

30 ***Rheb[T23M] and [E40K] drive increased mAb secretion under conditions similar to those used in*** 31 ***industry***

32 While GLuc-CHO cells offer a good reporter system for interrogating the effect of Rheb mutants on
33 protein secretion, there are several limitations that make these cells unsuitable for use as an industrial
34 research tool. First, to be useful in an industrial context, CHO cells must be grown in suspension (in
35 chemically defined, serum-free media, lacking serum). Second, while GLuc is a secreted protein, it is
36 not a model biotherapeutic protein and, unlike antibodies, does not undergo glycosylation. To address
37 these issues, we acquired EXPI-CHO™ cells and modified them to stably express Rheb[WT], [T23M],
38 [Y35N] or [E40K] (Supplementary Fig. S3). Consistent with the data for GLuc-CHO cells, in EXPI-CHO
39 cells stably expressing Rheb mutants or Rheb[WT], [T23M] and [E40K] drove increased
40 phosphorylation of rpS6 at Ser240/244 as well as increased phosphorylation of 4E-BP1 at Thr37 and
41 Thr46 suggesting that Rheb[T23M] and [E40K] drive hyperactive mTORC1 signalling in these cells (Fig.
42 5a).

43 In addition, Rheb[T23M] and [E40K] each drove increased expression of the ATF4 arm of the UPR as
44 well as of ER stress markers such as BiP (Fig. 5b). Interestingly, we were unable to detect any
45 differences in protein synthesis via SUNSET assay (Fig. 5c). This is likely because these cells stably
46 express Rheb mutants and therefore increased protein synthesis likely results in increased total

1 amounts of protein; as the amounts of cell lysate loaded onto the immunoblot gels are adjusted for
2 total protein, such normalisation will effectively ‘eliminate’ any effect on the overall rate of protein
3 synthesis. Cells expressing Rheb[T23M] or [E40K] also showed significantly faster cell proliferation as
4 assessed by counting cells on a haemocytometer every 24 h from days 1-7 (Fig. 5d) as well as by BrdU
5 assay (for DNA synthesis; Fig. 5e).

6 To determine the effect of Rheb mutants on mAb production and secretion in the suspension-grown
7 cells, we transfected EXP1-CHO™ cells with a vector encoding the heavy and light chains of rabbit IgG1
8 and grew the cells in serum-free, chemically-defined medium for 10 days with the cells being fed on
9 day 2. This more closely mimics the type of conditions the biopharmaceutical industry would use when
10 using CHO cells and a transient expression approach to produce a recombinant IgG. We found that
11 after 10 days of culture, Rheb[T23M] and [E40K] promoted a 2-3 fold increase in IgG1 yield (Fig. 5f).
12 However, given that cells expressing Rheb[T23M] and [E40K] proliferate more quickly (Fig. 5d/e), it
13 was possible that this increase was the result of simply having more cells for longer. It was therefore
14 important to determine the specific productivity (Qp) of each cell line, Qp being a measure of how
15 much protein is being secreted from each cell per day (pg protein/cell/day). We found that the Qp of
16 cells expressing Rheb[T23M], [Y35N] or [E40K] at day 3 (the earliest time point where IgG can be
17 reliably detected) is approximately the same at about 25 pg protein/cell/day and slightly higher than
18 the approximately 20 pg protein/cell/day produced by cells expressing Rheb[WT]. However, the Qp of
19 cells expressing Rheb[WT] and [Y35N] decreased much more rapidly over the next 7 days than cells
20 expressing Rheb[T23M] or [E40K] (Fig.5g). To determine whether the increased and sustained
21 productivity of cells expressing Rheb[T23M] or [E40K] reflected increased synthesis of IgG or only
22 increased secretion, we performed western blot analysis on samples taken at days 0, 5 and 10 to
23 determine whether there was a change in intracellular IgG. At day 0 intracellular IgG could not be
24 detected, while at days 5 and 10 cells expressing Rheb[T23M] and [E40K] contained more intracellular
25 IgG than Rheb[WT] or [Y35N] cells (Fig. 5h). This suggests that Rheb[T23M] and [E40K] each promote
26 both the increased production and, very importantly, secretion of this recombinant antibody.

27 **Discussion**

28 It is clear that any increase in IgG titre in medium, provided the quality of the product is not
29 compromised, is beneficial to the biopharmaceutical industry in terms of enhancing the production of
30 the desired product and particularly its levels in the cell medium from which it is harvested. Here we
31 show that Rheb mutants that we previously identified (Xie et al., 2020) are capable of increasing IgG
32 titres in CHO cells grown in suspension in chemically-defined, serum-free medium replicating
33 industrial growth conditions.

34 Consistent with our previous study (Xie et al., 2020), we found that the exogenous expression of the
35 Rheb mutants Rheb[T23M], [Y35N] and [E40K] drive constitutive mTORC1 signalling to a greater
36 extent than overexpression of Rheb[WT] through insensitivity to TSC-GAP activity and thus rendering
37 mTORC1 insensitive to removal of upstream activators. We show that in HEK293 cells, Rheb[T23M]
38 and [E40K] drive an increased rate of protein synthesis consistent with previous studies (Xie et al.,
39 2020) as well as the known function of mTORC1 as a key regulator of protein synthesis (Proud, 2019)
40 and ribosome biogenesis (Iadevaia et al., 2014). mTORC1 also promotes mitochondrial function (de la
41 Cruz López et al., 2019). A very recent paper has also shown the mTORC1 signalling can stimulate ATF4
42 expression independently of the UPR and thereby upregulate a subset of ATF4 target genes (Torrence
43 et al., 2020). As a key activator of mTORC1 signalling, the expression of an active Rheb mutant such as
44 T23M can drive multiple anabolic pathways, so that this single manipulation can lead to enhanced cell
45 growth and proliferation and faster protein synthesis. This obviates the need to modify multiple genes
46 thereby offering a substantial advantage for cell engineering.

1 We, found that Rheb[WT] and, unexpectedly, despite promoting constitutive mTORC1 signalling under
2 specific conditions Rheb[Y35N], did not increase protein synthesis. This is consistent with our finding
3 that, while Rheb[T23M] and [E40K] increased the levels of ATF4, Rheb[Y35N] did not. Rheb[T23M] and
4 [E40K] also resulted in an increase in markers of the integrated stress response and expansion of the
5 endoplasmic reticulum (ER).

6 These data suggest that the increase in protein synthesis driven by constitutive activation of mTORC1
7 increases the protein folding load on the ER. As unfolded proteins accumulate in the ER, the integrated
8 stress response becomes activated; in the short term, this drives increased expression of proteins
9 required for protein folding and expansion of the ER. If this expansion is enough to accommodate the
10 increased protein folding requirements, cells can maintain increased protein synthesis without
11 progressing into apoptosis which can occur during prolonged activation of the UPR (Shaffer et al.,
12 2004; Sriburi et al., 2004; M. Wang & Kaufman, 2016). This mechanism allows cells to produce more
13 protein without adverse effects. The fact we only observe this phenomenon in cells expressing
14 Rheb[T23M] and [E40K] but not Rheb[WT] or Rheb[Y35N] is surprising and suggests that mTORC1
15 activation alone is not sufficient to support a long-term increase in protein synthesis. We previously
16 showed through proteomic analysis of NIH3T3 cells stably expressing Rheb[T23M] and [E40K] that
17 additional pathways are activated by these mutations. Cells expressing Rheb[T23M] showed increased
18 reliance on anaerobic glycolysis while cells expressing Rheb[E40K] showed increased autophagic flux
19 (Xie et al., 2020). It is possible that activation of these pathways does not occur in cells expressing
20 Rheb[Y35N] so that they cannot sustain the energy or nutrient requirements to sustain prolonged
21 increases in protein synthesis. This hypothesis also offers an explanation for the observation that the
22 effects of Rheb[T23M] and [E40K] are more evident under metabolic stress. The increased availability
23 of biological precursors due to increased glycolysis or autophagy may grant cells expressing
24 Rheb[T23M] or [E40K] some degree of resistance to metabolic stress.

25 We show that this mechanism of mTORC1-driven activation of the UPR is required for increased
26 secretion of the reporter *Gaussia* luciferase from CHO cells stably expressing GLuc when grown in
27 monolayer. This hypothesis is supported by the observation that both inhibition of mTOR and
28 inhibition of the ATF4 arm of the UPR decreased GLuc secretion. We also observed a significant
29 upregulation of ATF4 in cells expressing Rheb[T23M] or [E40K]. We show that this is also true for CHO
30 cells grown in suspension in chemically-defined, serum-free medium. We found that stable expression
31 of Rheb[T23M] or [E40K] resulted in an increase in the specific productivity compared to cells
32 expressing Rheb[WT] or [Y35N] and a 2-3 fold increase in IgG1 secretion compared to Rheb[WT]
33 overexpression or cells expressing only their endogenous Rheb.

34 These results clearly point to a potential approach to significantly increasing the efficiency of production
35 of commercially valuable proteins. A further development of this approach would be to generate cell
36 lines stably expressing Rheb[T23M] or with the chromosomal copies of the Rheb gene mutated
37 appropriately. Since every cell in the population would then be expressing mutant Rheb (which is not
38 the case for transient transfection), an even greater augmentation of protein output would be
39 expected. The ability to double product output over the same time-frame without increasing nutrient
40 input promises to make production of valuable recombinant proteins such as expensive therapeutic
41 agents more efficient and may therefore lead to significant reductions in the cost of treatment, thus
42 allowing more people being able to benefit from these highly effective treatments and/or at a lower
43 cost. Our findings may thus be of direct value to the burgeoning 'biological drug' sector.

44

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46

1 **Figure 1: Overexpression of Rheb promotes protein synthesis and increases ER stress markers. (A)**
2 HEK293 cells were transfected with indicated vectors (EV = empty vector) and grown for 48 h prior
3 to harvest. Lysates were harvested for SDS-PAGE western blot analysis with the indicated antibodies.
4 SuNSET assays were then performed; the growth medium was changed for EBSS 90 min prior to
5 addition of 30 μ M puromycin for 30 min. Samples indicated 'CHX' were pre-treated with
6 cycloheximide, to inhibit ongoing protein synthesis. **(B)** Quantification of data from (A). **(C)** Lysates of
7 cells that had been transfected with the indicated vectors were analysed by SDS-PAGE and
8 immunoblot using the indicated antibodies. **(D-G)** RT-qPCR analysis for known ER stress markers was
9 performed on cDNA extracted from HEK293 cells expressing the indicated Rheb mutants or an
10 empty vector (EV). Figures represent mean \pm s.d for 3 independent experiments. All graphs
11 represent mean \pm s.d for 3 independent experiments. The data were analysed via Student's t-test
12 compared to EV. *: P < 0.05; ** P < 0.01; *** P < 0.001.

13 **Figure 2: Rheb-T23M and E40K drive increased ER volume. (A)** Immunofluorescence of HEK293 cells
14 transiently expressing the indicated Rheb mutants or EV. Cells were transfected with indicated
15 vectors 24 h prior to fixation and addition of the indicated primary antibodies. **(B)** Quantification of
16 (A) for the ratio of area containing Calnexin : area containing actin. Graph represent mean \pm s.d for 3
17 independent experiments. The data were analysed via Student's t-test. *: P < 0.05; ** P < 0.01.

18 **Figure 3: Rheb mutants drive constitutive mTORC1 signalling in GLuc-CHO cells. (A)** SDS-PAGE and
19 western blot analysis of lysates harvested from GLuc-CHO cells transiently expressing the indicated
20 Rheb mutants or transfected with an empty vector (EV); some were co-transfected with FLAG-TSC1/2,
21 as indicated. The growth medium was replaced with medium lacking FBS for 16 h followed by D-PBS
22 for 60 min immediately prior to harvesting. **(B)** As in (A) for FLuc-CHO cells. **(C)** GLuc-CHO cells
23 expressing the indicated Rheb mutants or an empty vector (EV) were grown in fully supplemented
24 media with cell number recorded every 24 h for 7 days. **(D)** As in (C) with cells grown in media lacking
25 FBS. Figures are representative of mean \pm S.D for 3 independent experiments. **(E)** BrdU incorporation
26 assay of FLuc-CHO cells expressing the indicated Rheb mutants or EV. Cells were allowed to grow in
27 fully supplemented media for 24 h before addition of BrdU for 1 h. **(F)** As in (E) for GLuc-CHO cells. All
28 graphs represent mean \pm s.d for 3 independent experiments. The data were analysed via Student's t-
29 test compared to EV. *: P < 0.05; *** P < 0.001.

30 **Figure 4: Rheb mutants promote increased secretion of Gaussia Luciferase. (A)** CHO cells stably
31 expressing Firefly luciferase (FLuc) were transfected with the indicated Rheb mutants or an empty
32 vector (EV); FLuc assays were then performed every 48 h for 7 days. Results are normalized to cell
33 number. **(B)** Quantification of data in (A) for day 7. **(C)** CHO cells stably expressing Gaussia Luciferase
34 (GLuc) were transfected with the indicated Rheb mutants or an empty vector (EV) and treated with
35 either DMSO (solid line) or 1 μ M AZD8055 (dashed line) for the duration of the experiment. GLuc
36 assays performed on growth media every 48 h for 7 days. Results are normalized to cell number. **(D)**
37 Quantification of data in (C) for day 7. **(E)** As in (C), but cells were treated with a cocktail of 1 μ M
38 iPERK and 1 μ M ISRIB (dashed line). Experiments (C) and (E) were performed simultaneously but are
39 presented separately for clarity. **(F)** SDS-PAGE western blot analysis of cell lysates harvested from
40 GLuc-CHO cells transiently transfected with the indicated Rheb mutants or an empty vector (EV). All
41 graphs represent mean \pm s.d for 3 independent experiments. The data were analysed via Student's t-
42 test. *: P < 0.05; ** P < 0.01; *** P < 0.001.

43 **Figure 5: Rheb T23M and E40K drive increased secretion of mAb from CHO-S cells. (A)** SDS-PAGE
44 western blot analysis of lysates harvested from Expi-CHO cells stably over-expressing the indicated
45 Rheb mutant or transfected with empty vector (EV). **(B)** SDS-PAGE western blot analysis of lysates
46 harvested from Expi-CHO cells stably over-expressing the indicated Rheb mutant or transfected with

1 empty vector (EV). **(C)** EXPI-CHO cells stably expressing the indicated Rheb mutant or endogenous
2 Rheb (Endo) were grown for 48 h prior to treatment with 30 μ M puromycin for 30 min. Lysates were
3 then harvested for SuNSET assays. **(D)** Expi-CHO cells stably expressing the indicated Rheb mutants
4 or endogenous Rheb (Endo) were allowed to proliferate with cell number recorded every 24 h for 10
5 days. **(E)** BrdU incorporation assay of Expi-CHO cells stably expressing the indicated Rheb mutants or
6 endogenous Rheb (Endo). Cells were allowed to proliferate for 24 h before addition of BrdU for 1 h.
7 **(F)** Transient IgG yield from Expi-CHO cells stably expressing the indicated Rheb mutant or
8 endogenous Rheb after 10 days of expression. **(G)** Specific productivity (Qp) of Expi-CHO cells stably
9 expressing the indicated Rheb mutant. Figure represents n=1. **(H)** SDS-PAGE western blot analysis of
10 cell lysates from (G). All figures other than (G) are representative of 3 independent experiments. All
11 graphs represent mean \pm s.d for 3 independent experiments. The data were analysed via Student's t-
12 test compared to Endo. *: P < 0.05; ** P < 0.01; *** P < 0.001.

13 **Supplementary Figure 1: Mutant Rheb drives an increase in levels of proteins of the ER stress**
14 **response. Related to Figure 1** Quantification of SDS-PAGE western blot data for Fig. 1a. All graphs
15 represent mean \pm s.d for 3 independent experiments. The data were analysed via Student's t-test
16 compared to Endo. *: P < 0.05.

17 **Supplementary Figure 2: Rheb-T23M displays increased proliferation on media containing reduced**
18 **FBS. Related to Figure 3. (A)** GLuc-CHO cells expressing the indicated Rheb mutants or an empty
19 vector (EV) were grown in media supplemented with 1% FBS with cell number recorded every 24 h
20 for 7 days. **(B)** f-CHO cells expressing the indicated Rheb mutants or EV were grown in media
21 supplemented with 0.5% FBS with cell number recorded every 24 h for 7 days. All graphs represent
22 mean \pm s.d for 3 independent experiments. The data were analysed via Student's t-test compared to
23 Endo. *** P < 0.001.

24 **Supplementary Figure 3: Characterization of Expi-CHO cells stably over expressing Rheb mutants.**
25 **Related to Figure 5.** Sanger sequencing results of Expi-CHO monoclonal cell lines transfected with
26 vector encoding both the indicated Rheb mutant and NeoR to grant resistance to G-418. Cells were
27 grown for 6 weeks in the presence of 1 μ M G-418 before DNA was extracted and sequenced using
28 primers specific to the inserted plasmid.