IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

GENENTECH, INC. and CITY OF HOPE, *Plaintiffs*, v. AMGEN INC., *Defendant*.	)	C.A. No. 17-1407-CFC (CONSOLIDATED)
GENENTECH, INC., *Plaintiff and Counterclaim Defendant*, v. AMGEN INC., *Defendant and Counterclaim Plaintiff*.	)	C.A. No. 18-924-CFC

APPENDIX TO GENENTECH'S LETTER-BRIEF CONCERNING CONSTRUCTION OF "FOLLOWING FERMENTATION" AND SUPPORTING DECLARATION OF DR. HANSJÖRG HAUSER

Bödeker et al., Production of recombinant factor VIII from perfusion cultures: I. Large-scale fermentation, Animal Cell Technology (1994) ("Bödeker 1994")	Appx234
Ozturk et al., Real-time Monitoring of Protein Secretion in Mammalian Cell Fermentation: Measurement of Monoclonal Antibodies Using a Computer-Controlled HPLC System (BioCad/RPM), *Biotechnol Bioeng*., 48:201-206 (1995) ("Ozturk 1995")	Appx240
Kemp G., O'Neil P., *Large-Scale Production of Therapeutic Antibodies: Considerations for Optimizing Product Capture and Purification*, in: Subramanian G. (eds) Antibodies (2004) ("Kemp 2004")	Appx246
Dwivedi, *Validation of Cell Culture-Based Processes and Qualification of Associated Equipment and Facility*, in: Ozturk, Cell Culture Technology for Pharmaceutical and Cell-Based Therapies ("Dwivedi 2006")	Appx272
US 2007/0141687 (Porro)	Appx307
Kaufmann et al., *Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells*, Biotechnol Bioeng., 63(5): 573-582 (1999)	Appx344
Matijasevic et al., *Hypothermia causes a reversible, p53-mediated cell cyle arrest in cultured fibroblasts*, Oncol Res., 10(11-12): 605-610 (1998)	Appx354
Roobol et al., *ATR (ataxia telangiectasia mutated- and Rad3-related kinase) is activated by mild hypothermia in mammalian cells and subsequently activates p53*, Biochem J., 435(2):499-508 (2011)	Appx360
Hunt et al., *Low-Temperature Pausing of Cultivated Mammalian Cells*, Biotechnol Bioeng., 89(2):157-63, (2004)	Appx374
Yoon et al., *Effect of Low Culture Temperature on Specific Productivity and Transcription Level of Anti-4-1BB Antibody in Recombinant Chinese Hamster Ovary Cells*, Biotechnol Prog., 19(4):1383-6 (2003)	Appx381
Roobol et al., *Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming*, FEBS J., 276(1):286-302 (2009)	Appx385

is consistent with the response to hypothermia being due to stabilization of p53 consequent to its phosphorylation at Ser^{15} and this increased level of p53 then inducing p21 expression.

The ATR protein kinase regulates phosphorylation of p53 at Ser^{15} upon exposure to mild hypothermia

Having established that p53 is phosphorylated at Ser^{15} in response to mild hypothermia, we set out to establish the kinase(s) responsible for this phosphorylation. Phosphorylation at Ser^{15} of p53 can be mediated by several protein kinases, including ATM, ATR, DNA-PK and the stress response signalling pathway protein kinase $p38^{MAPK}$ (p38 mitogen-activated protein kinase) [26]. To determine whether any of these was effecting p53 phosphorylation during mild hypothermia, we used a combination of general and specific protein kinase inhibitors and siRNA knockdown. Initially, we used caffeine, a well known, although not very specific, inhibitor of the PIKK family of protein kinases [27]. In the concentration range usually employed (low millimolar) it inhibits both ATM and ATR, but DNA-PK is relatively resistant. However, another PIKK family member, mTOR (mammalian target of rapamycin), a protein kinase that positively regulates protein synthesis in response to nutrient availability and growth factor signalling, is also inhibited by low millimolar concentrations of caffeine [27]. This must be taken into account when assessing the effect of caffeine on hypothermia-induced p21 expression. In the short term, 2.5 mM caffeine inhibited phosphorylation of p53 at Ser^{15} when cells were transferred to 32 °C, but had little effect when cells were transferred to 27 °C (Figure 2A). During longer-term exposure to caffeine, phosphorylation of p53 at Ser^{15} was less sustained than in the absence of caffeine and p21 expression was reduced, under both hypothermic conditions investigated (32 and 27 °C) (Figure 2B). When compared with the inhibition of general protein synthesis by caffeine (due to mTOR inhibition), the inhibition by caffeine of p21 expression was greater (Figure 2D), consistent with either ATM or ATR being involved in hypothermia-induced expression of p21. More specific inhibition of DNA-PK with NU7441 [28] had no effect on either hypothermia-induced phosphorylation of p53 at Ser^{15} or induction of p21 (Figures 3A and 3B). Thus, of the potential PIKK kinases that could phosphorylate p53 at Ser^{15} upon mild hypothermia, these results suggested that either ATM or ATR is responsible.

The fungal metabolite wortmannin is a widely used, irreversible, inhibitor of phosphoinositide 3-kinases, and treatment of cells with micromolar concentrations of this compound causes inhibition of ATM, DNA-PK and mTOR [29]. However, ATR is relatively resistant to wortmannin, and cells require exposure to concentrations in excess of 100 μM before ATR is inhibited [29]. In agreement with the results from the caffeine studies, which suggested that ATR might phosphorylate p53 at Ser^{15}, 20 μM wortmannin had no effect on hypothermia-associated phosphorylation of p53 at Ser^{15} and marginally inhibited p21 induction (Figure 2C). However, in contrast with inhibition by caffeine, inhibition of general protein synthesis by wortmannin was not significantly different from inhibition of hypothermia-induced p21 expression by wortmannin (Figure 2E). We then used a specific inhibitor of ATM, KU0055933 [28], and this inhibited neither hypothermia-associated phosphorylation of p53 at Ser^{15} nor induction of p21 (Figures 3A and 3B). Therefore, using specific inhibitors to DNA-PK and ATM, we were able to demonstrate that neither is the primary kinase involved in the hypothermia-induced p53–p21 pathway.

Although these inhibitor data are consistent with a signalling pathway in which ATR is a key kinase in the hypothermia-induced p53–p21 pathway, they are not specific ATR inhibitors, therefore, to test this hypothesis further, siRNA knockdown of *ATR* mRNA was employed. This approach has been shown to effectively reduce ATR protein levels by approx. 70 % 24 h after transfection [30,31] and therefore, although this does not obliterate protein levels, a knockdown would be expected to result in decreased Ser^{15}-phosphorylated p53 in response to mild hypothermia if this kinase is responsible. Two commercial validated siRNAs to human ATR were tested for their ability to knock down CHO-K1 *ATR* mRNA due to the lack of availability of such reagents for CHO ATR. As expected, both siRNAs efficiently decreased HeLa cell *ATR* mRNA over a 48 h period by between 67 and 77 % (Figure 4A). When tested in CHO-K1 cells, exposure to one of these siRNAs for 48 h decreased CHO *ATR* mRNA by 77–87 %. However, knockdown by the second siRNA was less effective and more variable in CHO cells (Figure 4A). Knockdown of ATR mRNA was maintained at 72 h and, to a lesser degree, at 96 h post-transfection (Figure 4A). We confirmed that knockdown of *ATR* mRNA resulted in a knockdown in ATR protein levels in both HeLa and CHO cells by Western blotting, which showed that ATR protein levels were reduced by 55–85 % after a 48 h exposure to ATR siRNA (Figure 4B). Following transfection with these siRNAs, cells were maintained at 37 °C for 48 h before transfer to either 32 °C or 27 °C for a further 10 h. The decreases in ATR mRNA and protein observed after a 48 h exposure to ATR siRNA were clearly mirrored by the decrease in the extent of phosphorylation at Ser^{15} of p53 under these mildly hypothermic conditions (Figure 4C). Inhibition of cold-induced phosphorylation of p53 at Ser^{15} was still evident at 72 h, but not at 96 h, post-transfection (Figure 4D), but at this last time point the hypothermia-associated phosphorylation of p53 was already in decline (Figures 1B and 4D). These results are consistent with the inhibitor data indicating that hypothermia induces p53 phosphorylation and p21 activation through the ATR–p53–p21 signalling pathway. Furthermore, the relative longevity (several days) of p53 phosphorylation at Ser^{15} during hypothermia is also consistent with this phosphorylation being regulated by ATR [32]. We note that although knockdown of ATR protein was clearly achieved, ATR protein was still present and some phosphorylated p53 was also present in the knockdown experiments (Figures 4C and 4D). We were unable to ascertain from these results whether the phosphorylated p53 present upon cold shock was due to the residual ATR protein present or as a result of an additional pathway not investigated in the present study. Despite this, when cells were shifted to 27 °C following knockdown of ATR for 48 h at 37 °C by siRNA, those wells in which knockdown had been undertaken initially showed an increased in cell numbers 1 and 2 days after being placed at 27 °C above that observed in the mock knockdown (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/435/bj4350499add.htm). This further suggests that p53 activation through ATR is involved in the inhibition of cell proliferation upon cold shock at 27 °C. This effect was lost after 2 days at 27 °C, probably because, at this stage, the knockdown cells at a higher cell concentration are beginning to experience nutrient and growth stresses that lead to a decrease in cell number as seen in Supplementary Figure S1.

Involvement of the $p38^{MAPK}$ stress kinase signalling pathway in cell cycle arrest during mild hypothermia

Although our results show that ATR is involved in the regulation of p53 Ser^{15} phosphorylation upon mild hypothermia and rule out

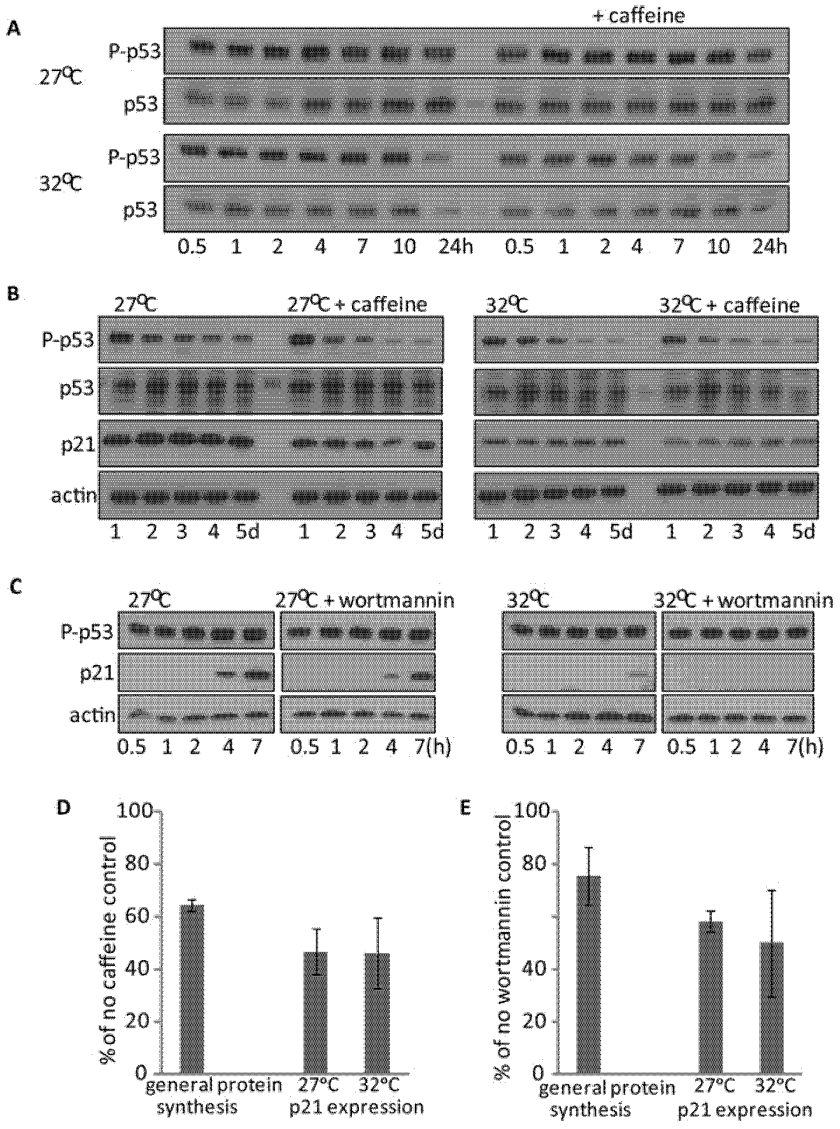

Figure 2 Caffeine inhibits both phosphorylation of p53 at Ser[15] and p21 induction associated with mild hypothermia, but wortmannin does not

(**A**) Immunoblot detection of p53 phosphorylated at Ser[15] and total p53 protein in lysates of CHO-K1 cells, with or without pre-treatment with 2.5 mM caffeine for 30 min at 37 °C immediately prior to exposure to the indicated temperatures for the indicated number of hours. (**B**) Immunoblot detection of p53 phosphorylated at Ser[15], total p53 protein, p21 and β-actin in lysates of CHO-K1 cells, with or without pre-treatment with 2.5 mM caffeine for 30 min at 37 °C immediately prior to exposure to the indicated temperatures for the indicated number of days. (**C**) Immunoblot detection of p53 phosphorylated at Ser[15], p21 and β-actin in lysates of CHO-K1 cells exposed to 20 μM wortmannin for 30 min at 37 °C prior to incubation for the indicated times at 27 °C or 32 °C. The response of total p53 protein levels for these time points at 27 °C and 32 °C are shown in (**B**). (**D**) Quantification of the inhibition by 2.5 mM caffeine of general protein synthesis and of hypothermia-induced p21 expression. (**E**) Quantification of the inhibition by 20 μM wortmannin of general protein synthesis and of hypothermia-induced p21 expression.

ATM and DNA-PK, this phosphorylation could also be effected by the stress response protein kinase p38^{MAPK} (Hog1 in yeast), either directly [33] or through its phosphorylation at Ser[33] and Ser[46] of p53 that, in turn, enhances phosphorylation at Ser[15] [34]. In yeast, this protein kinase is activated by osmotic stress or exposure to cold [35], whereas in mammalian cells, it has also been shown to be activated by osmotic stress [36]. p38^{MAPK} is also activated by hypoxia and it has been reported that mildly hypothermic mammalian cells are hypoxic [37]. Furthermore, ATR can also phosphorylate, and thereby activate, p38^{MAPK} [38]. It was therefore considered important to determine whether the p38^{MAPK} protein kinase was involved, either independently, or through activation by ATR, in the p53–p21 pathway induced by mild hypothermia.

SP203580 is an inhibitor frequently used for assessing involvement of p38^{MAPK} in signalling pathways [39]. Although this inhibitor can also inhibit casein kinase 1 [5], this kinase will not phosphorylate p53 at Ser[15] [40], therefore this inhibitor allowed us to investigate potential p38^{MAPK} involvement in hypothermia-induced phosphorylation of p53 Ser[15]. Treatment of CHO-K1 cells with 10 μM SP203580 for 30 min prior to transfer to 27 °C or 32 °C reduced both phosphorylation at Ser[15] of p53 and expression of p21 at these temperatures (Figure 5A). Since

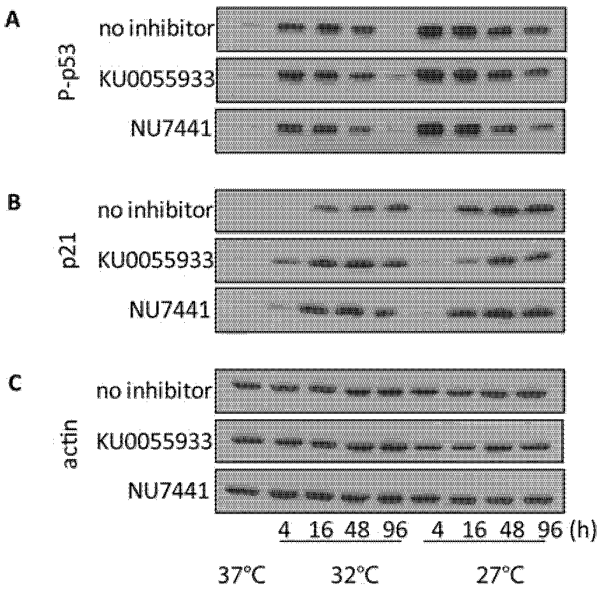

Figure 3 Specific inhibitors of DNA-PK and ATM do not abrogate the cold-induced phosphorylation of p53 and induction of p21

CHO-K1 cells were grown at 37 °C for 24 h, then 1 μM DNA-PK inhibitor NU7441 or 10 μM ATM inhibitor KU0055933 was added as indicated. After a further 30 min of incubation at 37 °C, cells were either maintained at 37 °C or transferred to 32 °C or 27 °C for the indicated number of hours. Immunoblots of cell lysates were probed for Ser^{15}-phosphorylated p53 (**A**), p21 (**B**) and β-actin (**C**). Total p53 protein levels at these temperatures (4, 48 and 96 h) were established previously and reported in Figures 1 and 2.

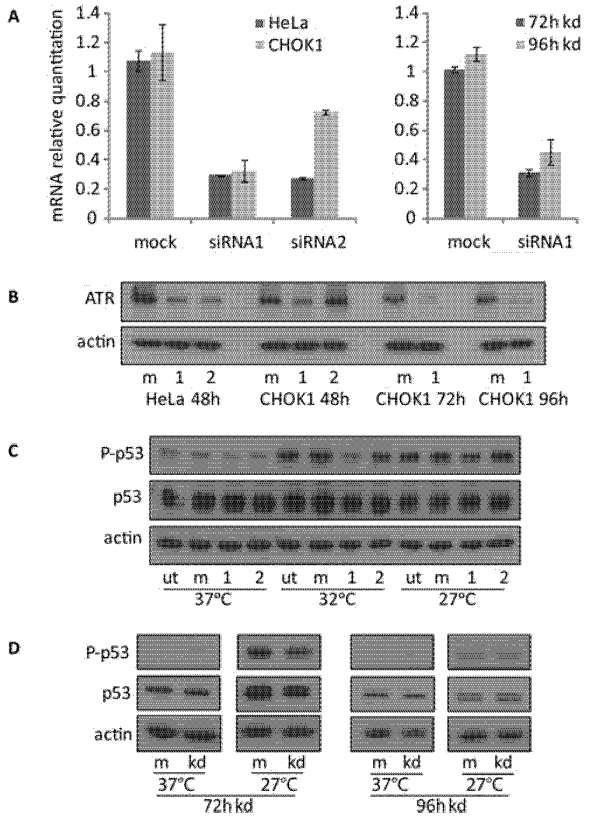

Figure 4 siRNA knockdown of ATR mRNA inhibits hypothermia-induced phosphorylation of p53 at Ser^{15}

(**A**) HeLa and CHO-K1 cells were transfected with 5 nM siRNAs against human *ATR* mRNA and then maintained at 37 °C for 48–96 h before undertaking qRT-PCR analysis of *ATR* mRNA levels in total RNA. (**B**) Immunoblot detection of ATR in HeLa and CHO-K1 cell lysates prepared after 48–96 h of exposure to ATR siRNAs at 37 °C (m, mock transfected; 1, ATR siRNA 1; 2, ATR siRNA 2). (**C**) Immunoblot detection of total p53 protein and p53 phosphorylated at Ser^{15} in cell lysates of CHO-K1 cells 48 h after siRNA knockdown of *ATR* mRNA at 37 °C followed by 10 h at 27 °C or 32 °C (ut, untreated; m, mock transfected; 1, ATR siRNA 1; 2, ATR siRNA 2). (**D**) Inhibition of phosphorylation of p53 at Ser^{15} is maintained over longer periods of siRNA knockdown (kd) of ATR than 48 h. CHO-K1 cells were transfected with siRNA 1 or mock transfected (m), incubated at 37 °C for 72 h or 96 h and then maintained at 37 °C or transferred to 27 °C for a further 10 h prior to extraction for immunoblot detection of the indicated proteins.

SP203580 had no effect on general protein synthesis (Figure 5B), its inhibition of p21 expression suggested involvement of p38^{MAPK} in the hypothermia-induced p53–p21 pathway.

To determine whether this p38 mechanism was a second pathway leading to phosphorylation at Ser^{15} of p53 independently of the ATR route, treatment with SP203580 was combined with siRNA knockdown of ATR. The resulting effects of combined ATR knockdown and SP203580 treatment on hypothermia-induced phosphorylation at Ser^{15} of p53 and the p53 isoform pattern (Figure 6) mirrored those effects observed for ATR knockdown alone (Figure 4). This suggests that the involvement of p38^{MAPK} in hypothermia-induced cell cycle arrest lies within, rather than acts independently of, the ATR pathway; otherwise, the effects of ATR knockdown and SP203580 treatment should have been additive. Therefore we suggest that the p38^{MAPK} protein kinase is involved in phosphorylation of p53 at Ser^{15} upon mild hypothermia through activation by ATR.

How is ATR activated upon exposure of CHO-K1 cells to mild hypothermia?

Our results confirmed that ATR is activated upon CHO-K1 cells being exposed to mild hypothermia, which in turn phosphorylates Ser^{15} of p53 and p21 induction. However, how might ATR itself be activated upon mild hypothermia? We used immunofluorescence to determine whether there was any change in the localization of ATR following cold shock (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/435/bj4350499add.htm). Using this approach, it was found that at 2–48 h after cold shock at 27 °C, ATR appeared to be concentrated into the nucleolus (Supplementary Figure S2). We also noted an overall increase in ATR-associated fluorescence throughout the cell, particularly between 6 and 24 h of exposure to 27 °C.

In addition to localization studies, we investigated changes to the lipid content of cold-shocked cells. When prokaryotic and lower eukaryotic cells are exposed to hypothermic conditions, the unsaturated fatty acyl content of cell membrane lipids has been reported to increase [14]. In mammalian cells, exposure to the Ca^{2+}-independent phospholipase A_2 inhibitor BEL at 37 °C also increases the unsaturated fatty acyl content of phosphatidylcholines and activates ATR [17]. We therefore compared the effect of BEL treatment with that of hypothermia on cellular lipid composition to determine whether a similar effect could be observed that might offer an explanation of ATR activation upon mild hypothermia. To achieve this, MS analysis of total lipids extracted from cells maintained at normal temperature (37 °C), after treatment with BEL, and at mildly hypothermic temperatures was performed. Multivariate analysis (PC-DFA) was applied to the resulting data with cross-validation as described

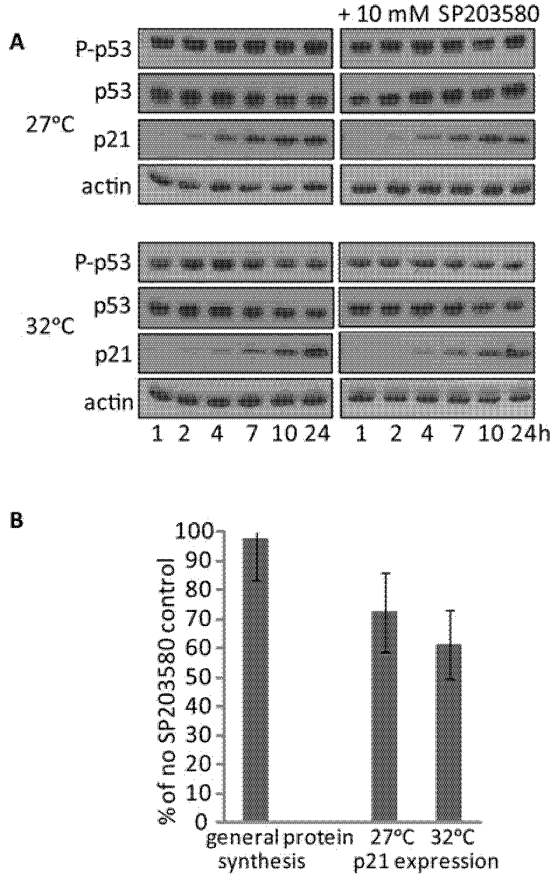

Figure 5 The p38^{MAPK} inhibitor SP203580 attenuates hypothermia-associated phosphorylation of p53 at Ser^{15} and p21 induction

(**A**) CHO-K1 cells were exposed to 10 μM SP203580 for 30 min at 37 °C and then transferred to 27 °C or 32 °C for the indicated times (1–24 h). Immunoblot detection of p53 phosphorylated at Ser^{15}, total p53 protein and p21 with β-actin as an indicator of protein loading is shown. (**B**) Quantification of the effects of 10 μM SP203580 on general protein synthesis and hypothermia-induced p21 expression.

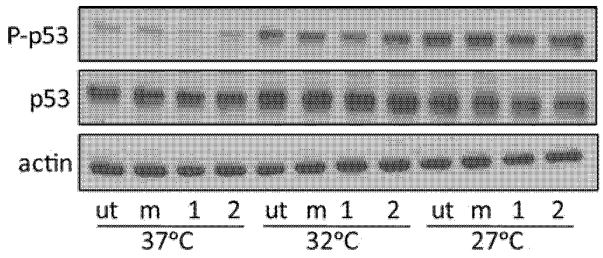

Figure 6 Inhibition of hypothermia-induced phosphorylation of p53 at Ser^{15} by ATR knockdown is not increased by additional inhibition of p38^{MAPK}

At 48 h after siRNA-mediated *ATR* mRNA knockdown, CHO-K1 cells were additionally exposed to SP203580 for 30 min at 37 °C then transferred to 27 °C or 32 °C for a further 10 h. Immunoblot detection of total p53 protein, p53 phosphorylated at Ser^{15} and β-actin in lysates of CHO-K1 cells treated in this way is shown. ut, untreated; m, mock transfected; 1, ATR siRNA 1; 2, ATR siRNA 2 as in Figure 4.

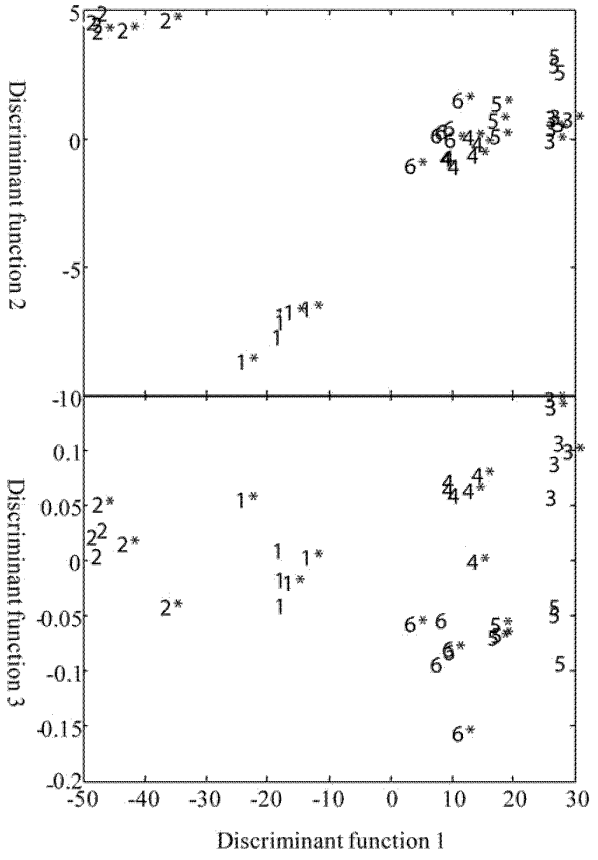

Figure 7 Exposure of CHO-K1 cells to mild hypothermia is associated with changes in the cellular lipid profile

PC-DFA of all samples. Upper panel: PC-DF1 plotted against PC-DF2. Lower panel: PC-DF1 plotted against PC-DF3. The first ten PCs were used by the DFA algorithm and this accounted for 99.8% of the total explained variance. The multivariate model was constructed using three of six samples in each class (no asterisk) and cross-validated by projection of the remaining three samples (shown with an asterisk). The level of agreement of the samples projected with those used to construct the model highlight that the model is validated. Class 1, control maintained at 37 °C for 6 h with no treatment; Class 2, control maintained at 37 °C for 6 h with BEL treatment; Class 3, maintained at 27 °C for 6 h; Class 4, maintained at 32 °C for 6 h; Class 5, recovery at 37 °C for 2 h after a temperature of 27 °C for 6 h; Class 6, recovery at 37 °C for 2 h after a temperature of 32 °C for 6 h.

in the Experimental section and shown in Figure 7. The results show that BEL-treated and 37 °C control cells were different from all cells cultured at reduced temperatures, and the chemical treatment and control cells dominated the separation of the second canonical variate (Figure 7, upper panel). When PC-DF1 was plotted against PC-DF3 (Figure 7, lower panel), each class was biologically distinct from other classes, highlighting the fact that the detectable lipid profile of each of the six classes was different and perturbations (chemical or temperature-based) resulted in distinct phenotypic changes.

Furthermore, univariate analyses using Kruskal–Wallis analysis of variance to define the lipids that were statistically significantly changing (see Supplementary Table S1 at http://www.BiochemJ.org/bj/435/bj4350499add.htm) revealed that the positive control (treatment with BEL) showed a different relative change to the control in the PC-DFA model when compared with the temperature-treated cells. Ten lipids were statistically different ($P < 0.05$) and all showed an increase in

their relative concentration in the BEL-treated cells compared with the control. Cells treated at 27 °C and 32 °C (mild hypothermia) for 6 h showed a similar trajectory from the control samples, with the 27 °C samples showing a greater biological difference in multivariate space compared with the samples perturbed at 32 °C. However, more statistically significant changes were observed between control and 32 °C samples in the univariate analysis (37 compared with four changes for 32 °C and 27 °C respectively). All of the changes showed an increase in concentration of a range of lipids, predominantly phospholipids (diacylglyceroserines, diacylglyceroinositols and diacylglycerophosphocholines). In most cases, although not exclusively, an increase in the unsaturated double-bond content was present in the lipids of increased abundance. This highlights a definitive increase in the production of a specific class of lipids in response to temperature-based perturbations. The increase in temperature after hypothermia perturbation (recovery) provides a change in the lipid profile from that at reduced temperature, but this lipid profile is distinct from all other samples. This shows that an increase in temperature changes the lipid profile, but not to a normal profile at 2 h after the return to 37 °C. Decreases in the relative concentration of lipids were observed in the change from lower to higher temperature, of the same classes of lipids that were observed to increase as the temperature was decreased. This highlights the specific role of these lipids in the response to temperature perturbation and how their relative concentration is temperature dependent. Although many of the lipids were chemically identified, we were unable to show significant changes in the overall unsaturated fatty acyl content of cell membrane lipids. However, we have shown an increase in polyunsaturated lipids upon mild hypothermia consistent with a previous study showing that an increase in phosphatidylcholines containing polyunsaturated fatty acids activates ATR–p53 signalling at 37 °C [17].

DISCUSSION

Although we [12] and others [10,13] have documented that p53 activation of p21 is a key mechanism by which mammalian cells initiate cell cycle arrest upon being subjected to mild hypothermic temperatures, the mechanism by which p53 is activated and the cellular mechanisms that allow the perception of cold and subsequent activation of p53 have remained undetermined. In the present study, we have shown that the exposure of CHO-K1 cells to mildly hypothermic conditions activates the ATR kinase that subsequently activates p53 by phosphorylation at Ser^{15} and hence the ATR–p53–p21 signalling pathway. We note that although our experiments clearly show ATR regulation of p53 phosphorylation upon cold shock, in our ATR knockdown and inhibitor experiments some ATR protein and phosphorylated p53 still remained and we were unable to ascertain from these results whether the phosphorylated p53 present upon cold shock in these experiments was due to the residual ATR protein present or a result of an additional signalling pathway not investigated in the present work.

We speculate that the primary stimulus for the activation of the ATR–p53–p21 signalling pathway upon mild hypothermia may be changes in membrane rigidity [14] as a direct result of changes in membrane lipid composition (homeoviscous adaptation). Our results show changes in the levels of polyunsaturated fatty acids upon cold shock that are known to influence the fluidity of cellular membranes, and, furthermore, that these changes correlated with the activation of ATR. As described above, a previous study has demonstrated that changes to cell membrane fluidity and increased polyunsaturation activates ATR and the authors of that study suggest that this occurs as a result of ATR 'sensing' the change in the ratio of polyunsaturated to saturated hydrocarbons [17]. The question is how might this change in lipid composition activate ATR? Zhang et al. [17] suggest that this is the result of changes in the fluidity and function of the nuclear envelope whereby the nuclear-localized ATR senses these changes and is activated. We speculate further that this leads to an intranuclear relocalization of ATR upon activation (as shown in Supplementary Figure S2), p53 activation and cell cycle arrest. Such intranuclear relocalization of ATR to nuclear foci has been documented in response to both hypoxia [41] and DNA damage [42]. The overall increase in ATR-associated fluorescence throughout the cell during early exposure to 27 °C without an increase in immunoblot detection of ATR also suggests that, additionally, there may be a conformational change in ATR upon exposure of the cell to cold that renders the protein more accessible to the anti-ATR antibody used.

CHO-K1 p53 carries a single point mutation at codon 211 in exon 6 in the DNA-binding domain of the molecule, although this mutation is not within an evolutionarily conserved region [43]. Furthermore, CHO-K1 p53 is rather more abundant and stable than wild-type p53. At 37 °C, its half-life is 5.2 h [12] compared with the more usual range of 20–60 min for p53 half-lives. Furthermore, CHO-K1 p53 is not stabilized further, and thereby increased in amount, by ionizing radiation, i.e. by the ATM signalling pathway alone [43]. Thus, even though CHO-K1 p53 is relatively abundant, it is not sufficient, under normal conditions, to activate transcription of p21. Even under mildly hypothermic conditions, when p21 transcription is activated, increases in CHO-K1 p53 total protein are very modest (Figures 1, 2, 4 and 6). What does change markedly in response to hypothermia is the phosphorylation status of p53. For wild-type p53, phosphorylation at Ser^{15} enhances p53 transactivation of p21 transcription by increasing the binding of p53 to its transcriptional co-activator, p300/CBP [44]. Furthermore, although phosphorylation at Ser^{15} of p53 is not itself sufficient to disrupt the interaction between p53 and Mdm2 (murine double minute 2) that targets p53 for degradation, phosphorylation at this site is a prerequisite for phosphorylation at Ser^{20} of p53. Ser^{20} phosphorylation inhibits the binding of p53 to Mdm2 [45]. The overall effect of phosphorylation of Ser^{15} of wild-type p53 is therefore 2-fold, i.e. enhanced stability and enhanced transcriptional activation ability. In the context of CHO-K1 cells, this must mean that phosphorylation at Ser^{15} is sufficient to enhance the transcription factor activity of an already abundant p53, even though this transactivation activity might be compromised to some extent by the point mutation in the DNA-binding domain of CHO-K1 p53.

A consistent finding that has emerged from the numerous studies of p53 post-translational modifications is that phosphorylation and acetylation sites are seldom modified alone and that post-translational modification at one site is often a prerequisite for further post-translational modifications elsewhere on p53 [26]. This activation of p53 at more than one site has been termed 'intramolecular phosphorylation site interdependence' [46] and is nearly always required before downstream transcriptional activation takes place. These results suggest that p53 transcriptional activation is tightly regulated by multiple modifications, thus minimizing inappropriate transcriptional activation by p53 and providing a point of integration of signals from multiple protein kinases [46]. This appears to be the case for activation of p53 by mild hypothermia, since we have found that $p38^{MAPK}$ is also involved in the ATR–p53–p21 pathway.

Although it is well established that ATR directly phosphorylates p53 at Ser^{15}, there are conflicting reports regarding the ability of

p38^{MAPK} to directly phosphorylate p53 at Ser^{15} [33,34]. However, some of the transient transfection experiments used to delineate this may have been complicated by the transfection vehicle itself eliciting a stress response involving phosphorylation of p53 and activation of p21 transcription [34]. We too have noted this effect of some transfection reagents on p53 activation (A. Roobol and C. M. Smales, unpublished work) and suggest that this, and indeed the induction of p21 expression by addition of DMSO to the culture medium mentioned earlier, may be consequent to changes in membrane fluidity or composition. Nevertheless, it has been established that p38^{MAPK} phosphorylates p53 at Ser^{33} and Ser^{46} and that, when p53 is doubly phosphorylated at these two sites, phosphorylation at Ser^{15} by other protein kinases is enhanced [34]. It has also been shown that, in mammalian cells, activation of p38^{MAPK} by hypoxia is mediated by ATR [47].

These findings, when combined with the results we have presented here, suggest that mild hypothermia activates the transcription of p21 through ATR activation and subsequent phosphorylation of p53 at Ser^{15}. At the same time, we suggest that ATR activates p38^{MAPK}, resulting in the phosphorylation of p53 at Ser^{33} and Ser^{46} that subsequently enhances Ser^{15} phosphorylation. In this way, activated p53 subsequently activates transcription of the downstream target p21, and induction of p21 is known to lead to cell cycle arrest upon mild hypothermia.

AUTHOR CONTRIBUTION

Mark Smales and Anne Willis conceived the initial study. Mark Smales, Anne Willis, Anne Roobol, Jo Roobol, Amandine Bastide and Martin Carden devised the experimental plans. Anne Roobol and Jo Roobol carried out the majority of the experimental work. Warwick Dunn and Royston Goodacre devised, carried out and completed the lipid analysis. Anne Roobol, Mark Smales, Anne Willis, Warwick Dunn and Royston Goodacre wrote the manuscript. All authors analysed the results and read and approved the manuscript.

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online data

SUPPLEMENTARY ONLINE DATA

ATR (ataxia telangiectasia mutated- and Rad3-related kinase) is activated by mild hypothermia in mammalian cells and subsequently activates p53

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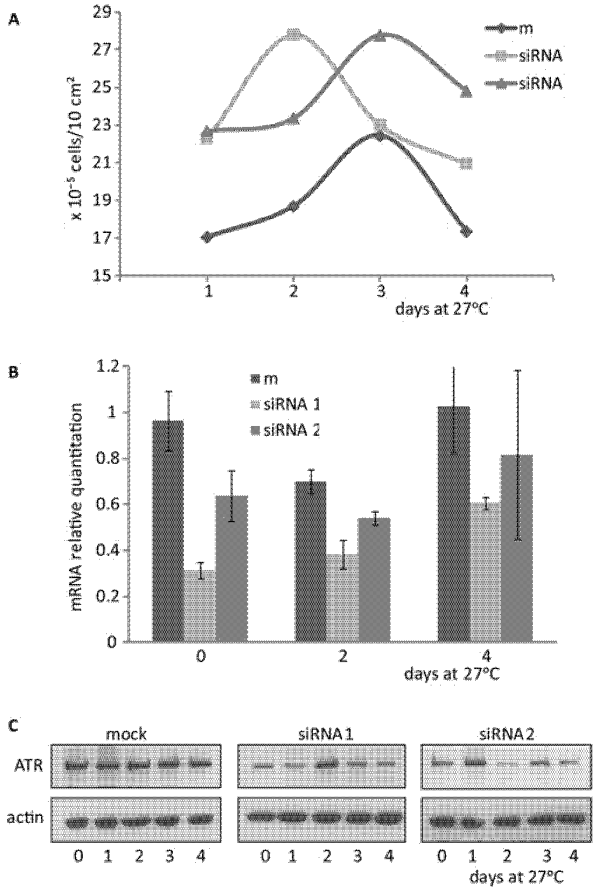

Figure S1 Effect of prolonged siRNA knockdown of ATR on cell proliferation at 27 °C

CHO-K1 cells were transfected with 5 nM ATR siRNA as described in the main text and then maintained at 37 °C for 48 h prior to transfer to 27 °C (day 0 on the indicated time scales). Samples were prepared on the indicated days of maintenance at 27 °C for cell counts (**A**), qRT-PCR quantification of *ATR* mRNA (**B**), and immunoblot detection of ATR protein (**C**). Mock transfections (m), siRNA1 and siRNA2 are as described in the Experimental section of the main text.

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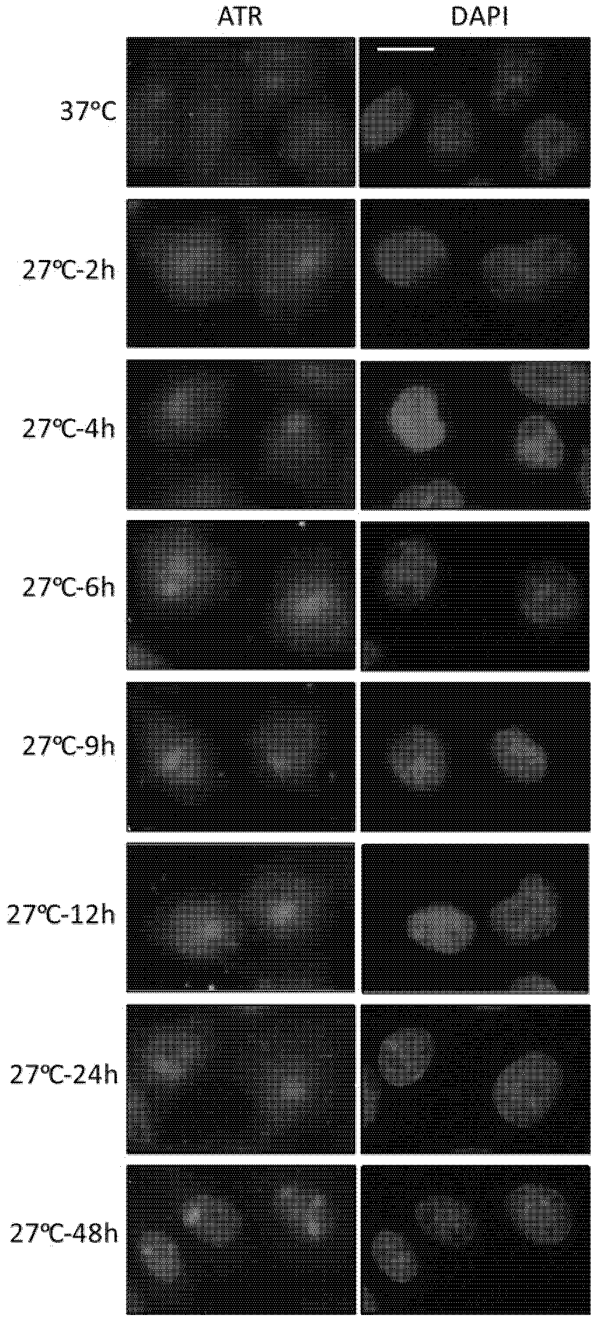

Figure S2 The intracellular localization of ATR changes during the early stages of hypothermia at 27 °C

Immunofluorescence detection of ATR and nuclei [visualized using DAPI (4′,6-diamidino-2-phenylindole)] in CHO-K1 cells maintained at 37 °C and during the first 48 h after transfer to 27 °C. Scale bar, 10 μm.

Table S1 Description of statistically significantly different metabolites

PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, prostaglandin; PI, phosphatidylinositol; PS, phosphatidylserine.

(a) Class 1 (control maintained at 37 °C for 6 h with no treatment) compared with Class 2 (control maintained at 37 °C for 6 h with BEL treatment).

P value	Fold difference	Lipid identification	Lipid class
0.00395	0.33 (lower in control)	PG(18:0/20:4)	Diacylglycerophosphoglycerols
0.00395	0.35 (lower in control)	PG(18:0/22:6)	Diacylglycerophosphoglycerols
0.00395	0.66 (lower in control)	Unidentified	Unidentified
0.00395	0.58 (lower in control)	PI(18:0/18:0)	Diacylglycerophosphoinositols
0.01041	0.66 (lower in control)	Unidentified	Unidentified
0.01041	0.43 (lower in control)	PG(17:0/17:0)	Diacylglycerophosphoglycerols
0.01631	0.73 (lower in control)	Unidentified	Unidentified
0.01631	0.70 (lower in control)	Unidentified	Unidentified
0.01631	0.76 (lower in control)	Unidentified	Unidentified
0.02498	0.69 (lower in control)	Unidentified	Unidentified

(b) Class 1 (control maintained at 37 °C for 6 h with no treatment) compared with Class 3 (maintained at 27 °C for 6 h).

P value	Fold difference	Lipid identification	Lipid class
0.00395	0.32 (lower in control)	Unidentified	Unidentified
0.00395	0.34 (lower in control)	PS(18:0/18:1)	Diacylglycerophosphoserines
0.01041	0.42 (lower in control)	PA(16:0/18:1)	Diacylglycerophosphates
0.02498	0.40 (lower in control)	Unidentified	Unidentified

(c) Class 1 (control maintained at 37 °C for 6 h with no treatment) compared with Class 4 (maintained at 32 °C for 6 h).

P value	Fold difference	Lipid identification	Lipid class
0.00395	5.88 higher in hypothermia	Unidentified	Unidentified
0.00395	2.08 higher in hypothermia	Unidentified	Unidentified
0.00395	2.86 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.00395	3.22 higher in hypothermia	Unidentified	Unidentified
0.00395	1.82 higher in hypothermia	Unidentified	Unidentified
0.00395	2.13 higher in hypothermia	Unidentified	Unidentified
0.00395	2.33 higher in hypothermia	Unidentified	Unidentified
0.00395	2.22 higher in hypothermia	Unidentified	Unidentified
0.00395	2.50 higher in hypothermia	PI(18:0/18:0)	Diacylglycerophosphoinositols
0.00395	2.27 higher in hypothermia	Unidentified	Unidentified
0.00649	3.13 higher in hypothermia	PA(16:0/18:1)	Diacylglycerophosphates
0.00649	1.61 higher in hypothermia	Unidentified	Unidentified
0.00649	1.88 higher in hypothermia	Unidentified	Unidentified
0.01041	1.69 higher in hypothermia	PE(16:0/18:3)	Diacylglycerophosphoethanolamines
0.01041	1.96 higher in hypothermia	1-Tetrahexanoyl-2-(8-[3]-ladderane-octanyl)-*sn*-glycerophosphoethanolamine	1-Acyl,2-alkylglycerophosphoethanolamines
0.01041	1.89 higher in hypothermia	Unidentified	Unidentified
0.01041	1.59 higher in hypothermia	PE(16:0/22:6)	Diacylglycerophosphoethanolamines
0.01041	1.47 higher in hypothermia	Unidentified	Dialkylglycerophosphoglycerols
0.01041	1.75 higher in hypothermia	Unidentified	Unidentified
0.01041	1.92 higher in hypothermia	PI(16:0/18:0)	Diacylglycerophosphoinositols
0.01631	1.82 higher in hypothermia	PA(16:0/16:0)	Diacylglycerophosphates
0.01631	1.85 higher in hypothermia	Unidentified	Unidentified
0.01631	1.54 higher in hypothermia	PS(17:0/20:4)	Diacylglycerophosphoserines
0.01631	1.49 higher in hypothermia	Unidentified	Unidentified
0.01631	1.72 higher in hypothermia	Unidentified	Unidentified
0.01631	1.62 higher in hypothermia	Unidentified	Unidentified
0.02498	1.72 higher in hypothermia	PE(P-16:0/22:6)	1*Z*-alkenyl,2-acylglycerophosphoethanolamines
0.02498	1.54 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.02498	1.47 higher in hypothermia	PS (20:0/18:2)	Diacylglycerophosphoserines
0.02498	1.82 higher in hypothermia	PG(18:0/20:4)	Diacylglycerophosphoglycerols
0.02498	1.82 higher in hypothermia	PG(18:0/22:6)	Diacylglycerophosphoglycerols
0.02498	1.52 higher in hypothermia	PI(16:0/18:1)	Diacylglycerophosphoinositols
0.02498	1.56 higher in hypothermia	PS (22:1/18:3)	Diacylglycerophosphoserines
0.03737	1.92 higher in hypothermia	Uunidentified	Unidentified
0.03737	1.61 higher in hypothermia	PS(16:0/18:1)	Diacylglycerophosphoserines
0.03737	1.92 higher in hypothermia	Unidentified	Unidentified
0.04461	1.89 higher in hypothermia	Unidentified	Unidentified

Table S1 Continued

(d) Class 3 (maintained at 27 °C for 6 h) compared with Class 5 (recovery at 37 °C for 2 h after a temperature of 27 °C for 6 h).

P value	Fold difference	Lipid identification	Lipid class
0.00649	4.23 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.02498	2.48 higher in hypothermia	PA(16:0/18:1)	Diacylglycerophosphates
0.02498	3.23 higher in hypothermia	Unidentified	Diacylglycerophosphoinositols
0.02498	2.55 higher in hypothermia	Unidentified	Unidentified
0.02498	2.63 higher in hypothermia	Unidentified	Unidentified
0.02498	2.98 higher in hypothermia	Unidentified	Unidentified
0.02498	2.37 higher in hypothermia	Unidentified	Unidentified
0.03737	2.97 higher in hypothermia	PI(16:0/18:0)	Diacylglycerophosphoinositols
0.03737	2.25 higher in hypothermia	PS (22:1–18:3)	Diacylglycerophosphoserines
0.03737	2.24 higher in hypothermia	Unidentified	Unidentified
0.03737	2.33 higher in hypothermia	Unidentified	Unidentified
0.03737	2.75 higher in hypothermia	Unidentified	Unidentified

(e) Class 4 (maintained at 32 °C for 6 h) compared with Class 6 (recovery at 37 °C for 2 h after a temperature of 32 °C for 6 h).

P value	Fold difference	Lipid identification	Lipid class
0.003948	2.96 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.006485	2.80 higher in hypothermia	Unidentified	Unidentified
0.006485	2.31 higher in hypothermia	PI(16:0/18:1)	Diacylglycerophosphoinositols
0.006485	2.52 higher in hypothermia	Unidentified	Unidentified
0.006485	2.16 higher in hypothermia	Unidentified	Unidentified
0.006485	2.4 higher in hypothermia	Unidentified	Unidentified
0.006485	2.5 higher in hypothermia	Unidentified	Unidentified
0.006485	2.12 higher in hypothermia	Unidentified	Unidentified
0.006485	2.39 higher in hypothermia	Unidentified	Unidentified
0.006485	2.46 higher in hypothermia	Unidentified	Unidentified
0.006485	2.43 higher in hypothermia	Unidentified	Unidentified
0.006485	2.33 higher in hypothermia	Unidentified	Unidentified
0.016309	1.91 higher in hypothermia	Unidentified	Unidentified
0.016309	1.9 higher in hypothermia	1,2-Dihexadecanoyl-*sn*-glycero-3-phosphosulfocholine	Glycerophospholipids
0.016309	1.80 higher in hypothermia	Unidentified	Dialkylglycerophosphoglycerols
0.016309	2.07 higher in hypothermia	PS(17:0/20:4)	Diacylglycerophosphoserines
0.016309	2.14 higher in hypothermia	PI(16:0/18:1)	Diacylglycerophosphoinositols
0.016309	2.02 higher in hypothermia	Unidentified	Unidentified
0.016309	2.81 higher in hypothermia	PI(16:0/18:0)	Diacylglycerophosphoinositols
0.016309	2.27 higher in hypothermia	PS (22:1/18:3)	Diacylglycerophosphoserines
0.016309	2.20 higher in hypothermia	Unidentified	Unidentified
0.016309	2.07 higher in hypothermia	Unidentified	Unidentified
0.016309	2.24 higher in hypothermia	PI(18:0/18:0)	Diacylglycerophosphoinositols
0.024975	1.96 higher in hypothermia	Unidentified	Unidentified
0.024975	2.00 higher in hypothermia	Unidentified	Unidentified
0.024975	1.91 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.024975	1.70 higher in hypothermia	PE(18:0/22:6)	Diacylglycerophospho-ethanolamines
0.02846	2.11 higher in hypothermia	Unidentified	Unidentified
0.037373	1.68 higher in hypothermia	1-Tetrahexanoyl-2-(8-[3]-ladderane-octanyl)-*sn*-glycerophosphoethanolamine	1-Acyl,2-alkylglycerophosphoethanolamines
0.037373	1.76 higher in hypothermia	Unidentified	Unidentified
0.037373	1.77 higher in hypothermia	PE(16:0/22:6)	Diacylglycerophosphoethanolamines
0.037373	1.72 higher in hypothermia	Unidentified	Unidentified
0.037373	1.68 higher in hypothermia	Unidentified	Unidentified
0.0455	5.73 higher in hypothermia	Unidentified	Unidentified
0.0455	2.38 higher in hypothermia	PS(18:1/18:2)	Diacylglycerophosphoserines
0.0455	2.32 higher in hypothermia	Unidentified	Unidentified

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Low-Temperature Pausing of Cultivated Mammalian Cells

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Abstract: There are currently two methods for maintaining cultured mammalian cells, continuous passage at 37°C and freezing in small batches. We investigated a third approach, the ''pausing'' of cells for days or weeks at temperatures below 37°C in a variety of cultivation vessels. High cell viability and exponential growth were observed after pausing a recombinant Chinese hamster ovary cell line (CHO-Clone 161) in a temperature range of 6–24°C in microcentrifuge tubes for up to 3 weeks. After pausing in T-flasks at 4°C for 9 days, adherent cultures of CHO-DG44 and human embryonic kidney (HEK293 EBNA) cells resumed exponential growth when incubated at 37°C. Adherent cultures of CHO-DG44 cells paused for 2 days at 4°C in T-flasks and suspension cultures of HEK293 EBNA cells paused for 3 days at either 4°C or 24°C in spinner flasks were efficiently transfected by the calcium phosphate-DNA coprecipitation method, yielding reporter protein levels comparable to those from nonpaused cultures. Finally, cultures of a recombinant CHO cell line (CHO-YIgG3) paused for 3 days at 4°C, 12°C, or 24°C in bioreactors achieved the same cell mass and recombinant protein productivity levels as nonpaused cultures. The success of this approach to cell storage with rodent and human cell lines points to a general biological phenomenon which may have a wide range of applications for cultivated mammalian cells. © 2004 Wiley Periodicals, Inc.

Keywords: CHO-DG44 cells; HEK293E cells; green fluorescent protein; IgG antibody; hypothermia; cell storage

INTRODUCTION

Two ways are known to provide cultivated mammalian cells for experimental work. Cells are either continuously propagated under stringent control of temperature and other environmental conditions, requiring constant attention, or limited amounts of cells are stored frozen for extended times (Mazur, 1970). Reestablishing a culture from a stock of frozen cells requires days if not weeks to reach the quantity of cells needed for most applications. Considering these two options, the versatility of mammalian cell culture would be improved if cells could be routinely kept at low temperatures with little or no maintenance and then recovered quickly without a decline in their capacity for growth. Here it is demonstrated that adherent or suspension cultures of mammalian cells can be paused for short periods (days to weeks) in standard media at temperatures between 4°C and 24°C.

The mammalian constraint in body temperature around 37°C has most likely resulted in a large number of diverse molecular adaptations affecting many aspects of cellular activity. However, cells, organs, and even entire organisms can recover from heat and cold stress, seemingly without any long-term consequences. In fact, periodic hypothermic exposure of cultivated mammalian cells can result in cold-tolerant cell lines (Michl et al., 1966; Glofcheski et al., 1993). Mammalian cells are therefore likely to possess a set of natural response mechanisms when diverting from and returning to the 37°C setpoint. Tissues such as skin and testis are normally maintained at temperatures lower than that of the body cavity, and the core temperature of long-term hibernators is allowed to vary during periods of hibernation (Willis, 1987). Hypothermia also has several medical applications. For example, transplantable tissues are often preserved at low temperatures for short periods (Sicular and Moore, 1961; Belzer et al., 1967). Reduction of the heart's temperature during cardiac surgery is known to reduce the risk of myocardial ischemia (Mauny and Kron, 1995), and mild hypothermia is used to limit the severity of traumatic brain injuries (Connolly et al., 1962; Marion et al., 1997).

For cultured mammalian cells, cold exposure reduces the rate of ATP synthesis and alters membrane permeability (Hochachka, 1986; Willis, 1987). Mild hypothermia (25–33°C) reduces the rate of progression through the cell cycle, while moderate (16–20°C) or severe hypothermia (4–10°C) may block the cell cycle in the G_2 phase or at the G_1/S boundary, respectively (Rieder and Cole, 2002). Transcription and translation are reduced by cold exposure, but the expression of a few proteins including p53, WAF1,

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and cold-inducible RNA-binding protein (CIRP) is elevated at temperatures in the range of 25–33°C (Nishiyama et al., 1997; Matijasevic et al., 1998; Ohnishi et al., 1998; Sonna et al., 2002). Interestingly, heat shock proteins are expressed during rewarming to 37°C after hypothermic exposure (Holland et al., 1993; Liu et al., 1994; Kaneko et al., 1997). Cold stress also results in protein denaturation and aggregation and disruption of the cellular cytoskeleton (Fujita, 1999; Sonna et al., 2002). Cold stress can induce apoptosis, but this may be cell-type-specific and dependent on the length and severity of the hypothermic exposure (Soloff et al., 1987; Perotti et al., 1990; Kruman et al., 1992; Gregory and Milner, 1994; Grand et al., 1995; Rauen et al., 2000).

As with other forms of cell stress, mammalian cells can recover from hypothermic exposure. We took advantage of this property to demonstrate that cultivated mammalian cells can be stored for up to 3 weeks at 4–24°C and then recovered by rewarming to 37°C. We use the term pausing to describe this method of cell storage. To be useful, pausing should be possible at any phase of a cell culture and at any scale of operation. Both adherent and suspension cells were paused in commonly used culture vessels such as T-flasks, spinner flasks, and bioreactors. After pausing, viable cells continued to divide and were used for routine applications, including stable and transient recombinant gene expression. These findings suggest that pausing is an attractive alternative for the short-term storage of cultivated mammalian cells.

MATERIALS AND METHODS

Cells

Adherent CHO-DG44 and HEK293 EBNA (HEK293E) cells were maintained in DMEM/F12 medium with 2% fetal calf serum (FCS). For CHO-DG44 cells the medium also contained 0.68 g/l hypoxanthine and 0.194 g/l thymidine (Sigma Chemical, St. Louis, MO). Suspension-adapted HEK293E cells were grown in spinner flasks in serum-free Ex-Cell 293 medium (JRH Biosciences, Lenexa, KS) supplemented with 4 mM glutamine. The agitation speed was 90 rpm. Recombinant CHO-Clone 161 cells expressing the enhanced green fluorescent protein (GFP) were grown as an adherent culture in DMEM/F12 medium in the presence of 2% FCS (Hunt et al., 1999). Suspension cultures of recombinant CHO-YIgG3 cells that express the enhanced yellow fluorescent protein (YFP) and a human IgG were maintained in spinner flasks stirred at 90 rpm in serum-free ProCHO5-CDM medium (Cambrex BioScience, Walkersville, MD) supplemented with 4 mM glutamine (Miescher et al., 2000; Hunt et al., 2002).

Pausing in Microcentrifuge Tubes

CHO-Clone 161 cells at mid-log phase were trypsinized, harvested by centrifugation, washed in phosphate-buffered saline (PBS), resuspended at a density of 1×10^6 cells/ml in fresh DMEM/F12 medium supplemented with 2% FCS and 4 mM glutamine, and transferred as 1-ml aliquots into 1.5 ml microcentrifuge tubes. The cells were incubated in the absence of CO_2 control for various times at the temperatures indicated in the text. Subsequently, 30 or 50 µl aliquots of the paused cells were transferred to 12-well microtiter plates supplied with 0.5 ml of fresh DMEM/F12 medium containing 2% FCS and 4 mM glutamine per well. For assessing cell growth, the samples were incubated at 37°C, and fluorescence was measured at various times with a Cytofluor Series 4000 plate-reading fluorometer (PerSeptive Biosystems; Framingham, MA) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Cell viability was determined by the Trypan blue exclusion method.

Pausing in T-flasks

Adherent CHO-DG44 and HEK293E cells were seeded in 25 cm^2 T-flasks in DMEM/F12 medium supplemented with 2% FCS and incubated at 37°C in 5% CO_2 and 95% humidity. When the cultures reached 80% confluence the caps of the flasks were closed and the cultures were stored at 4°C for 9 days. After pausing, the cells were incubated for 12 h at 37°C under humidity and CO_2 control. Adherent cells were detached with trypsin and 2×10^5 cells were seeded in 25 cm^2 T-flasks in 5 ml of fresh medium with 2% FCS and incubated at 37°C under humidity and CO_2 control. At various times the cells were trypsinized and counted using a CASY1 counter (Schärfe System, Reutlinger, Germany).

DNA Transfections

Adherent CHO-DG44 cells were grown in T-150 flasks to 80% confluence at 37°C in 5% CO_2 and 95% humidity. The cap of the flask was then closed and the cells were stored at 4°C for 2 days. After pausing, the cultures were incubated at 37°C for 5 h. The cells were trypsinized and seeded in 12-well microtiter plates at a density of 4×10^5 cells/ml in 1 ml of modified DMEM/F12 medium with 2% FCS, 4 mM glutamine, 0.68 g/l hypoxanthine, and 0.194 g/l thymidine. Nonpaused control cultures maintained in T-flasks at 37°C were seeded under the same conditions. After 4 h of incubation at 37°C, 660 µl of the medium was removed and 100 µl of a calcium phosphate-DNA coprecipitate containing 2.5 µg of pCMV-DsRed-Express (ClonTech, Palo Alto, CA) was added to each well. After 1 h of incubation at 37°C, the medium was removed and the cells where exposed to an osmotic shock by the addition of 10% glycerol in PBS. After 1 min, the glycerol solution was removed and replaced with fresh medium. After 3 days of incubation at 37°C DsRed expression was determined with a plate-reading fluorometer.

Suspension cultures of HEK293E cells were seeded in spinner flasks at a density of 5×10^5 cells/ml in 300 ml of

Ex-Cell 293 medium. After incubation at 37°C for 1 day, the cultures were incubated at 4°C or 24°C for 3 days. A control culture was maintained at 37°C. Paused and nonpaused cells were then passed to fresh Ex-Cell 293 medium in spinner flasks at a density of 1×10^6 cells/ml and incubated at 37°C for 24 h. Cells were then seeded in 12-well microtiter plates at a density of 5×10^5 cells/ml in 1 ml of modified DMEM/F12 medium with 1% FCS. To each well, 100 µl of calcium phosphate-DNA coprecipitate containing 50 ng pEGFP-N1 (ClonTech) and 2.45 µg calf thymus DNA (Invitrogen, Basel, Switzerland) was added. The plates were agitated at 120 rpm for 4 h at 37°C in 5% CO_2 and 95% humidity. One volume of Pro293s-CDM medium (Cambrex BioScience) was then added to each well. At 3 days posttransfection the cells were lysed by addition of 200 µl PBS with 10% Triton X-100. After a 1-h incubation with agitation at 37°C, the GFP level was determined using a plate-reading fluorometer as described above.

Pausing in Bioreactors

Each 3-L bioreactor (Applikon, The Netherlands) was seeded with CHO-YIgG3 cells at a density of 5×10^5 cells/ml in 1 l of ProCHO5 CDM medium. The cultures were maintained at 37°C at pH 7.1 with the dissolved O_2 maintained at 20%. Agitation was set at 150 rpm. For paused cultures, the temperature was reduced at 6 h postinoculation for a period of 72 h. Pausing at 24°C was accomplished by disconnection of the heating unit. The room temperature during any single experiment fluctuated by ±1°C. Cultures were maintained at either 4°C or 12°C using a 3-L water-jacketed bioreactor (Applikon) connected to a MultiTemp III waterbath (Amersham Biosciences, Uppsala, Sweden). During pausing, the pH was maintained at 7.1. To reduce the risk of cell damage during pausing, the stirring speed was reduced to 90 rpm, the lowest speed that prevented settling of the cells. After pausing the temperature was returned to 37°C and stirring was increased to 150 rpm. Samples were taken once or twice per day. The cell number and viability were determined using the Trypan blue exclusion method. The packed cell volume (PCV) was determined using 1 ml PCV tubes (Techno Plastic Products, Trasadingen, Switzerland). The concentration of fully assembled IgG in the culture medium was determined by sandwich ELISA as previously described (Meissner et al., 2001). Glucose, glutamine, sodium bicarbonate, and sodium hydroxide were added to the cultures as needed.

RESULTS

Pausing in Microcentrifuge Tubes

Our initial studies to investigate the feasibility of pausing utilized recombinant CHO-Clone 161 cells that homogeneously express GFP. For this cell line, a linear correlation between cell number and fluorescence has been observed (Hunt et al., 1999). Therefore, it was possible to use a standard multiwell plate reader to noninvasively monitor growth of these cultures over time. Adherent CHO-Clone 161 cells were trypsinized, suspended in fresh medium, transferred to microcentrifuge tubes, and stored at various temperatures for 4 days without agitation. After pausing, the highest viability was observed in cultures that were maintained at 17°C, but high viability was also seen in cultures stored at 6°C and 22°C (Fig. 1A). In contrast, pausing at 0°C or 37°C resulted in a high percentage of nonviable cells (Fig. 1A). Paused cells were evaluated for growth at 37°C by dilution of 30 µl aliquots of paused cultures into fresh medium in 12-well microtiter plates. The extent of cell growth at 37°C was determined by monitoring the level of GFP expression. Vigorous growth was observed following pausing at 6–22°C, while cells

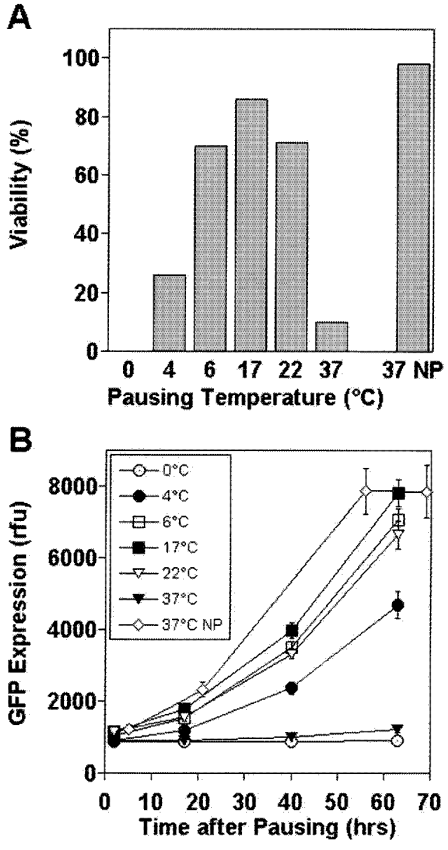

Figure 1. Pausing of CHO-Clone 161 cells in microcentrifuge tubes. **A:** Adherent cells were trypsinized and paused as suspension cultures in microcentrifuge tubes for four days at various temperatures as indicated. The cultures were not agitated during pausing. The nonpaused (NP) culture was maintained in a T-flask at 37°C. The viability of the cultures was determined by the Trypan blue exclusion method. **B:** Cells from paused and nonpaused (NP) cultures were passed in duplicate to fresh medium in 96-well microtiter plates and maintained at 37°C. GFP expression was measured by fluorometry at the times indicated.

paused at 4°C grew more slowly (Fig. 1B). Little growth was observed for cells paused at 0°C or 37°C (Fig. 1B).

The effect of the length of the pausing period on cell viability was investigated by maintaining CHO-Clone 161 cells at 6°C for up to 20 days. The viability of the paused cultures decreased with time, but more than 30% of the cells remained viable after 20 days of storage (Fig. 2A). After pausing, 50 µl aliquots of cultures were diluted in fresh medium in 12-well microtiter plates and incubated at 37°C. All of the cultures resumed growth, albeit with a lag period that was most pronounced for cells stored for 6 or 20 days (Fig. 2B). After the lag period, the paused cells grew at approximately the same rate as the nonpaused cells (Fig. 2B). Although the cultures paused for 6 or more days did not reach the same cell density as the nonpaused control, cells paused for 2 days at 6°C grew at approximately the same rate and to the same cell density as the nonpaused culture (Fig. 2B).

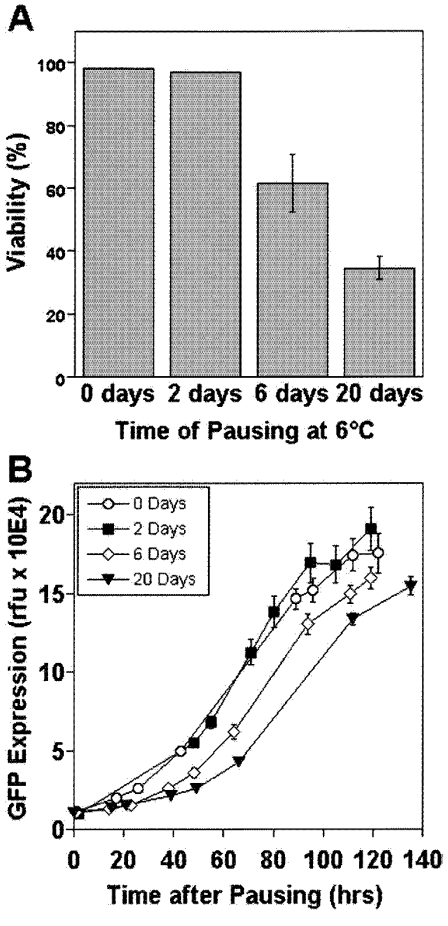

Figure 2. Pausing of CHO-Clone 161 cells in microcentrifuge tubes. **A:** Cells were paused as described in Figure 1 at 6°C for various times as indicated. The nonpaused (0 days) cells were maintained in a T-flask at 37°C. The viability of each culture was determined by the Trypan blue exclusion method. Each bar represents the average of two independent cultures. **B:** Paused and nonpaused (NP) cultures were passed at various times to 96-well microtiter plates and incubated at 37°C. The GFP expression was measured by fluorometry at the times indicated. Each point represents the average of three cultures.

Pausing in T-flasks

To determine if the results observed with CHO-Clone 161 cells applied to other cells, adherent HEK293E and CHO-DG44 cells were paused in T-flasks at 4°C for 9 days. During pausing, the cells became round and detached from the surface even though serum was present in the medium. This may have been due to the disassembly of the cytoskeleton (Sonna et al., 2002). After pausing, the cultures were incubated at 37°C overnight. During this period the viable cells reattached to the plate and regained their fibroblastic appearance. Adherent cells were then trypsinized, replated in fresh medium, and incubated at 37°C. For the paused cells, exponential growth did not begin until about 24 h after plating (Fig. 3). In contrast, the cell number in the nonpaused cultures more than doubled during this period (Fig. 3). By 93 h after plating, the paused cultures had reached about the same cell density as the control cultures (Fig. 3). Similar results were observed with adherent cultures of baby hamster kidney (BHK) cells (data not shown). These results demonstrate that commonly used human and rodent cell lines can be stored at reduced temperatures for several days.

Transfection of Paused Cells

To demonstrate the utility of pausing, we determined if paused cells could be transfected at the same efficiency as nonpaused control cells. Adherent CHO-DG44 cells were grown in T-flasks to 80% confluence at 37°C and then incubated at 4°C for 2 days. After pausing, the cells

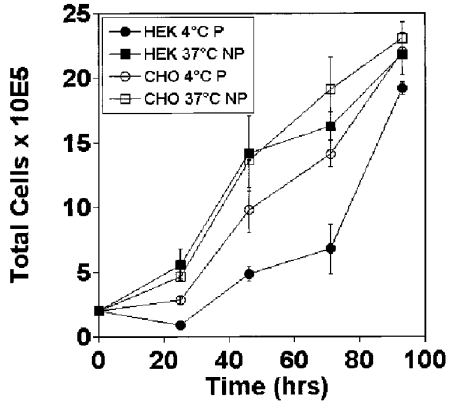

Figure 3. Pausing of adherent cells in T-flasks. Duplicate cultures of HEK293E and CHO-DG44 cells were paused in 25 cm^2 T-flasks at 4°C for 9 days. The viable cells were allowed to reattach by incubation at 37°C for 12 h. Paused (P) and nonpaused (NP) cells were then trypsinized, plated T-flasks, and incubated at 37°C. The cell number was determined with a CASY1 counter at the times indicated.

were incubated at 37°C for 5 h to allow viable cells to reattach to the plate. The viability of the paused culture was 93%. The cells were passed to 12-well microtiter plates and transfected with pCMV-DsRed-Express using the calcium phosphate-DNA coprecipitation method. As shown in Figure 4A, the level of DsRed expression at 3 days posttransfection was slightly higher in the paused culture than in the nonpaused culture (Fig. 4A). These results demonstrated that pausing for 2 days at 4°C did not alter the transfection efficiency of adherent CHO-DG44 cells.

To determine if this observation applied to other cell lines, a similar experiment was performed with suspension-adapted HEK293E cells in spinner flasks. The cultures were incubated at either 4°C or 24°C for 3 days. After pausing, the cells were passed to fresh medium at a density of 1×10^6 cells/ml and maintained at 37°C for 1 day. The cells were passed to 12-well microtiter plates and transfected with pEGFP-N1 using the calcium phosphate-DNA coprecipitation method. At 3 days posttransfection, GFP expression in cells paused at either 4°C or 24°C was similar to the level observed in nonpaused cells (Fig. 4B). Similar results were obtained when the cells were paused for 4 days at either 4°C or 24°C (data not shown). Through 4 days of pausing at either temperature, the viability of the cultures in spinner flasks remained above 85%. After 4 days of pausing, however, the viability decreased to about 75% and continued to decrease with further exposure to low temperature. These results demonstrate that suspension-adapted HEK293E cells paused in spinner flasks retained their capacity for transfection for up to 4 days.

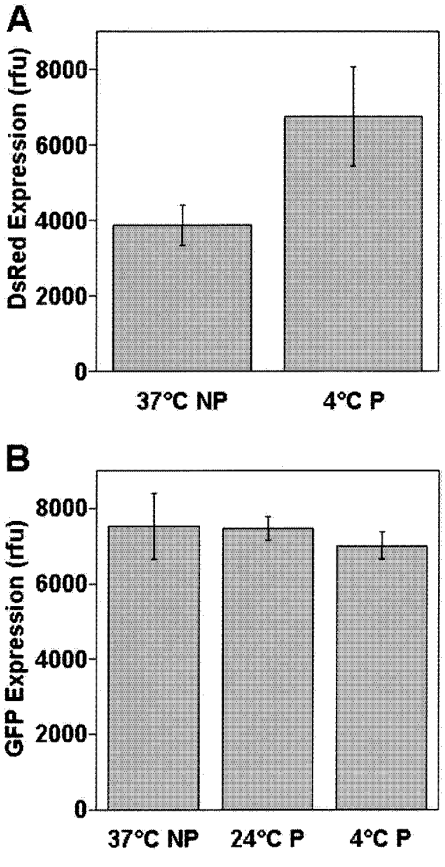

Figure 4. Transfection of paused cells. **A:** Adherent CHO-DG44 cells were paused (P) for 2 days at 4°C or maintained at 37°C (NP), passed to 12-well microtiter plates, and transfected with pCMV-DsRed-Express. DsRed expression was measured at 3 days post-transfection. Each bar represents the average of 11 transfections. **B:** Suspension cultures of HEK293E cells were paused (P) in spinner flasks for 3 days at the temperatures indicated. The nonpaused (NP) cultures were maintained at 37°C. After pausing, the cells were transfected with pEGFP-N1 in microtiter plates. GFP expression was measured at 3 days posttransfection. Each bar represents the average of three transfections.

Pausing in Bioreactors

We also explored the possibility of pausing cells in a bioreactor using a recombinant CHO cell line (CHO-YIgG3) that expresses both YFP and a human IgG (Miescher et al., 2000; Hunt et al., 2002). A single homogenous seed culture was used to inoculate three 3-L bioreactors at a density of 0.5×10^6 cells/ml. One of the reactors was maintained at 37°C and the other two were paused at either 12°C or 24°C beginning at 6 h postinoculation. After 72 h at the low temperature (78 h postinoculation), the temperature was returned to 37°C. The paused cells grew exponentially following a short lag period, but they did

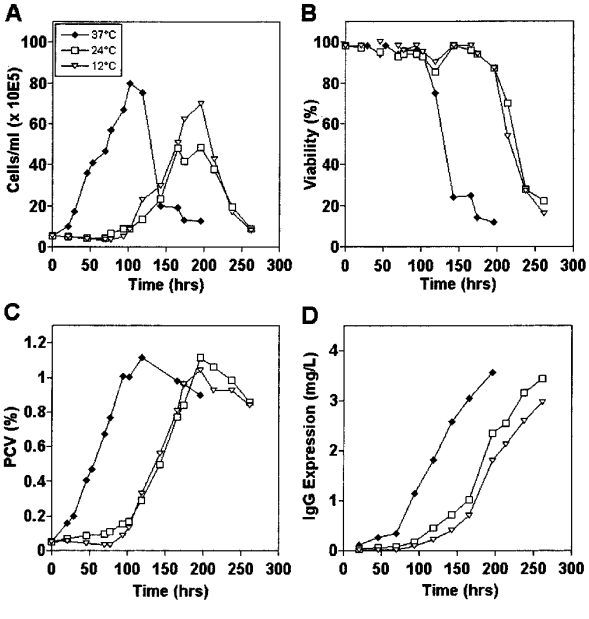

Figure 5. Pausing of CHO-YIgG3 cells in bioreactors. Suspension cultures of CHO-YIgG3 cells in 3-L bioreactors were paused at 12°C or 24°C. Pausing began at 6 h postinoculation and ended at 78 h postinoculation. The control culture was maintained at 37°C throughout the experiment. The viable cell number (**A**), viability (**B**), PCV (**C**), and IgG titer (**D**) were measured at the times indicated.

not achieve the same maximum cell density as the control culture (Fig. 5A). During pausing and after rewarming to 37°C, cell viability did not decrease (Fig. 5B). The onset of the viability decline of the paused cultures occurred ~3 days after that of the control culture (Fig. 5B). The PCV of the two paused cultures reached the same level as that of the control, indicating that the three cultures attained the same cell mass (Fig. 5C). These results suggest that the average cell size in the paused cultures was greater than that in the nonpaused cultures. Finally, the IgG titer in the culture paused at 24°C reached about the same level as in the control culture, while that in the culture paused at 12°C was slightly lower than the control (Fig. 5D).

In a separate experiment CHO-YIgG3 cells were paused in a 3-L bioreactor at 4°C for 72 h. After the temperature was raised to 37°C the paused culture eventually achieved the same maximum cell density as the control (Fig. 6A). The viability of the paused culture was stable during the period of pausing, but a 70% loss in viability was observed after rewarming to 37°C at 78 h postinoculation (Fig. 6B). However, the viability of the culture eventually returned to 90% (Fig. 6B). The PCV of the paused and nonpaused cultures also reached the same level (Fig. 6C). The IgG titer was slightly higher for the paused culture than for the nonpaused culture (Fig. 6D). These results demonstrate that a recombinant CHO cell line can be paused at 4–24°C for up to 3 days without substantial negative effects on cell mass or recombinant protein expression.

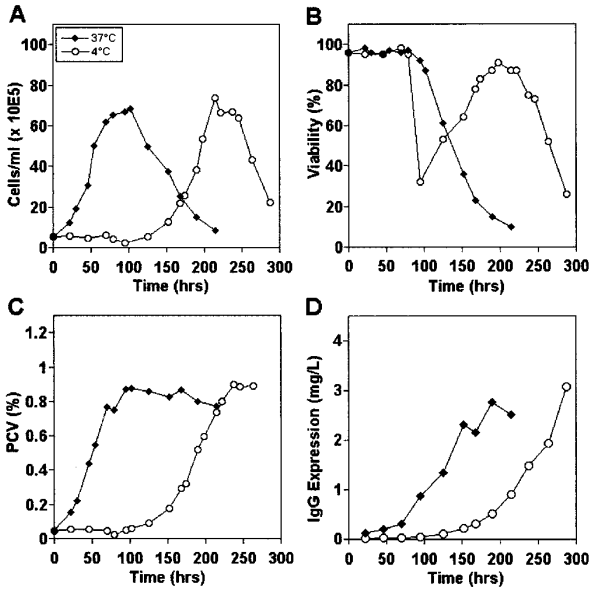

Figure 6. Pausing of CHO-YIgG3 cells in bioreactors. A suspension culture of CHO-YIgG3 cells was paused at 4°C in a 3-L bioreactor. Pausing began at 6 h postinoculation and ended at 78 h postinoculation. The control culture was maintained at 37°C throughout the experiment. The viable cell number (**A**), viability (**B**), PCV (**C**), and IgG titer (**D**) were measured at the times indicated.

DISCUSSION

The experiments described here demonstrate that cultured mammalian cells stored for short times (days or weeks) at low temperatures (4–24°C) resumed exponential growth when rewarmed to 37°C, albeit with a lag period whose duration varied depending on the pausing conditions and the cell line. This represents a new approach to the short-term storage of cultivated mammalian cells. Cells were paused for up to 3 weeks in this range of temperatures, and pausing was performed in a number of different culture vessels including T-flasks, spinner flasks, and bioreactors. For the two applications tested, transient gene expression following transfection and recombinant protein expression from a stable cell line, the paused cells performed as well as the nonpaused control cells in most cases.

As one example of the utility of this method of storage, suspension cultures of CHO-YIgG3 cells were paused for 3 days in bioreactors. During pausing at 4°C, 12°C, or 24°C the cell viability ranged from 93–98%. Rewarming to 37°C was only detrimental to cells paused at 4°C. In this case, the viability was reduced to 30% by 16 h after the return to 37°C. Cell death in this instance may have been due to apoptosis, but this was not confirmed. The paused cells were visibly smaller immediately after rewarming than before, one of the characteristics of apoptotic cells (Hockenberry, 1995). Cold-induced apoptosis has been shown to result from increases in the intracellular pools of chelatable iron and reactive oxygen species (Rauen et al., 1999, 2000). Thus, it may be possible to prevent cell damage due to exposure to 4°C by including antioxidants in the culture medium (Rauen et al., 2000). Clearly, cold-induced apoptosis was not a significant problem with the cultures paused in bioreactors for 3 days at 12°C or 24°C. This is not surprising, since two distinct mechanisms of hypothermic damage have been reported for cells exposed to temperatures above or below the minimum inactivation temperature, which is usually between 5–10°C for cultivated mammalian cells (Kruuv et al., 1995). Below this temperature, direct chilling injury (DCI) caused by hypothermic exposure is linked to thermotropic phase transitions of lipids resulting in loss of membrane integrity (Arav et al., 1996).

Despite the differences in cell viability after rewarming, the cultures paused in bioreactors produced approximately the same level of recombinant antibody as the nonpaused control cultures. These results suggest that stable cell lines can be paused at low temperatures for short periods without compromising their ability to produce recombinant protein. We have also shown that adherent CHO-DG44 cells paused and suspension-adapted HEK293E cells retain the capacity to be efficiently transfected using the calcium phosphate-DNA coprecipitation method. For CHO-DG44 cells, an elevation of reporter gene expression was consistently observed for the paused cells as compared to the nonpaused cells. This may have been a consequence of cell synchronization caused by pausing at 4°C (Rieder

and Cole, 2002). Efficient transfection of adherent CHO-DG44 by this method has been shown to be cell cycle-dependent (Grosjean et al., 2002).

Although the feasibility of pausing has been demonstrated, we have not yet attempted to optimize the method. Preliminary experiments have suggested that the pH and the availability of glucose and glutamine are important parameters for long-term pausing, but pausing for 1–2 days can be performed in PBS (Hunt and Wurm, unpubl. data). Additional experimentation will be necessary to determine how to best maintain cell viability after pausing. Our initial studies, however, do suggest that there are limits to this approach to cell storage. For example, rewarming of cells stored at 4°C had a significant negative effect on cell viability. Despite limitations, low temperature pausing is expected to be beneficial to many users of mammalian cell culture, as it provides a simple and inexpensive method for the short-term storage of cells in a number of different formats. It should be possible to transport cells for several days without temperature control. In addition, the observation that adherent cells detach during pausing suggests that this approach can be used instead of enzymatic or mechanical detachment if these techniques are not feasible.

This article is dedicated to the memory of Dr. Lisa Hunt, who died on September 21, 2002. The authors thank Ilda Tabuas Baieta Muller, Patrizia Tromba, and Hicham El Abridi for technical assistance, Martin Bertschinger for preparation of the figures, and Techno Plastic Products for the gift of the PCV tubes.

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Effect of Low Culture Temperature on Specific Productivity and Transcription Level of Anti-4-1BB Antibody in Recombinant Chinese Hamster Ovary Cells

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Lowering the culture temperature has been suggested as a useful tool for improving the production of recombinant proteins in Chinese hamster ovary (CHO) cells. In an effort to improve anti-4-1BB antibody production in recombinant CHO (rCHO) cells, rCHO cells producing anti-4-1BB antibody (LGA31-56) were cultivated at three different temperatures, 30, 33, and 37 °C. Lowering the culture temperature led to suppressed cell growth, cell cycle arrest in G_0/G_1 phase, and improved cell viability for a longer period. However, antibody production and q_{Ab} were not increased at low culture temperature. The maximum antibody concentration and q_{Ab} at 37 °C were 110.6 ± 2.6 μg mL^{-1} and 0.43 ± 0.03 μg (10^6 cells h)$^{-1}$, respectively, whereas those at 30 °C were 28.3 ± 3.8 μg mL^{-1} and 0.44 ± 0.07 (10^6 cells h)$^{-1}$, respectively. Northern blot analysis revealed that lowering the culture temperature did not increase the transcription level of heavy and light chains. These results were quite in contrast with the improved production of erythropoietin, which is expressed in the same CHO host and driven by the same CMV promoters, by lowering the temperature. Taken together, the results obtained imply that the beneficial effect of low culture temperature on recombinant protein production in rCHO cells is cell-line-specific.

Introduction

Chinese hamster ovary (CHO) cells are being used increasingly in industry to manufacture therapeutic antibodies (*1*). To realize an efficient process for antibody production by CHO cell culture, environmental parameters that affect cell growth and antibody production need to be investigated.

To simulate normal body temperature, recombinant CHO (rCHO) cells are cultivated at 37 °C. Lowering the culture temperature below 37 °C has been recognized as a means to improve culture performance (*2*–*7*). rCHO cell culture at low temperature decreases specific growth rate (μ) but improves cell viability and decreases the rate of releasing cellular proteins for a longer period (*2*). Moreover, lowering the culture temperature may also increase specific productivity (q), though its effect on q is variable among different rCHO cell lines. For example, cultivation of rCHO cells at low temperature resulted in enhanced q of C-terminal α-amidating enzyme (*3*), tissular plasminogen activator (t-PA) (*4*), secreted alkaline phosphatase (SEAP) (*5*), and erythropoietin (EPO) (*6*) but did not affect q of TNK-tPA and decreased q of rhesus thrombopoietin (TPO) (*8*).

Anti-4-1BB monoclonal antibody, a humanized antibody produced from CHO cells, is a potential therapeutic antibody controlling unwanted immune responses in persons with autoimmune diseases (*9*). To improve anti-4-1BB antibody production from rCHO cells (LGA31-56), we investigated the effect of culture temperature (30, 33, and 37 °C) on cell growth and antibody production. Furthermore, to understand the effect of low culture temperature on specific antibody productivity (q_{Ab}), we analyzed the transcription level of immunoglobulin (Ig) and cell cycle of rCHO cells.

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Materials and Methods

Cell Line and Cell Culture. The rCHO cells producing a humanized anti-4-1BB antibody for active suppression of antibody-mediated autoimmune reactions (LGA31-56) were used in this study. Heavy-chain (HC) and light-chain (LC) expression vectors were constructed separately, as described previously (*9*). Then they were cotransfected into dihydrofolate reductase (DHFR)-deficient CHO cells (DUKX-B11, ATCC CRL-9096) and underwent subsequent DHFR/methotrexate (MTX)-mediated gene amplification. The stable rCHO cells (LGA31-56) were selected at 1 μM MTX.

The medium for culture maintenance was Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY) supplemented with 10% (v/v) dialyzed fetal bovine serum (dFBS, Gibco). Cells were maintained as monolayer cultures in 25 cm^2 T-flasks (Nunc, Roskilde, Denmark) in a humidified 5% CO_2 incubator at 37 °C. Exponentially growing cells were inoculated at 0.6×10^5 cells mL^{-1} into 25 cm^2 T-flasks containing 4 mL of IMDM supplemented with 10% dFBS, and the T-flasks were incubated in the humidified 5% CO_2 incubator at 37 °C. When the viable cell concentration reached approximately 1×10^5 cells mL^{-1}, the medium was removed and the cells were washed once with Dulbecco's phosphate-buffered saline (D-PBS, Gibco). The T-flasks were then filled with 4 mL of SF2 serum-free medium (*10*) and were incubated in humidified 5% CO_2 incubators at three

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different temperatures, 30, 33, and 37 °C, respectively. Periodically, T-flasks were sacrificed to determine viable cell concentration. Culture supernatants, after centrifugation, were aliquoted and kept frozen at −70 °C for later analyses. The viable cell concentration was estimated by using the trypan blue dye exclusion method.

Quantitation of Anti-4-1BB Antibody. The secreted anti-4-1BB antibody concentration was quantified by an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Nunc) were coated with bacterially expressed 4-1BB, which is fused to glutathione *S*-transferase (GST-4-1BB) and blocked with bovine serum albumin (BSA) and Tween 20. The human IgG standard (Sigma, St. Louis, MO) and culture supernatants diluted with blocking buffer were loaded on wells and treated with goat anti-human IgG peroxidase conjugate (Sigma) in diluent solution.

Cell Cycle Analysis. To assess the effect of culture temperature on cell cycle distribution, cellular DNA contents were analyzed by flow cytometry, as described previously (*6*).

Northern Blot Analysis. For Northern analysis, total RNA was extracted from the cells using TRI reagent (Sigma) according to the manufacturer's protocol. After total RNA (1.5 μg) was developed on 1.2% agarose formaldehyde gels by electrophoresis, HC and LC mRNAs were characterized by Northern blot hybridization with the respective probes. The HC and LC probes were radioactively labeled by random primed incorporation of [α-^{32}P]dCTP (Amersham, Amersham, U.K.). Membrane transfer, prehybridization, and hybridization were performed using the protocol described previously (*11*). After hybridization, the band intensity was quantitated using PhosphorImager (Molecular Dynamics). Subsequently, the membrane was stripped and rehybridized with a CHO β-actin cDNA probe for band intensity normalization. Hybridization and analysis with the β-actin probe were performed in the same manner.

Evaluation of Specific Antibody Productivity. The q_{Ab} was based on the data collected until the end of stationary phase and was evaluated from a plot of the anti-4-1BB antibody concentration against the time integral values of the growth curve (*12*).

Results and Discussion

To determine the effect of low culture temperature on rCHO cells (LGA31-56) in regard to growth and anti-4-1BB antibody production, cells were cultivated at three different temperatures, 30, 33, and 37 °C. Duplicate cultures were performed two separate times.

Figure 1 shows typical cell growth and viability profiles during culture. When cells were subjected to low temperature, cell growth was suppressed. The maximum viable cell concentration and μ at 37 °C were 2.53 ± 0.20 × 10^6 (av ± SD, n = 2) cells mL^{-1} and 0.022 ± 0.003 h^{-1}, respectively. On the other hand, those at 33 °C were 1.31 ± 0.11 × 10^6 cells mL^{-1} and 0.014 ± 0.004 h^{-1}, respectively. At 30 °C, the maximum viable cell concentration obtained was only 0.31 ± 0.06 × 10^6 cells mL^{-1}.

To further investigate the growth suppression at low culture temperature, cell cycle distribution was analyzed by flow cytometry. As observed in other rCHO cells (*5*, *6*), lowering the culture temperature resulted in rapid reduction of cells in S phase and concomitant accumulation of cells in G_0/G_1 phase. After 78 h cultivation, approximately 77% of cells and 67% of cells accumulated in G_0/G_1 phase at 30 and 33 °C, respectively, while 45% of cells remained in G_0/G_1 phase at 37 °C (Table 1). These

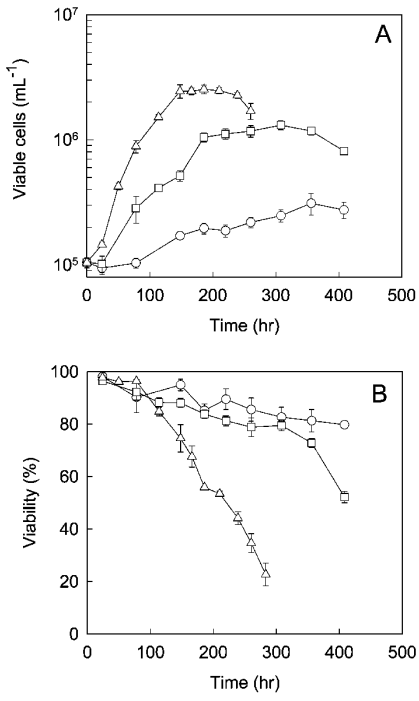

Figure 1. Effect of culture temperature on cell growth and viability: (○) 30 °C; (□) 33 °C; (△) 37 °C. (A) Viable cell concentration. (B) Cell viability. The error bar represents the standard deviations calculated from the data (n = 4).

Table 1. Effect of Culture Temperature on Percent of Cells in Different Phases of the Cell Cycle of rCHO Cells[a]

	culture temperature (°C)								
	30			33			37		
time (h)	G_0/G_1 (%)	S (%)	G_2/M (%)	G_0/G_1 (%)	S (%)	G_2/M (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
24	64.9	20.2	14.9	59.1	28.3	12.6	36.0	52.6	11.4
78	77.4	17.4	5.2	66.6	15.6	17.8	44.8	45.1	10.0

[a] Cell samples were taken during cultures shown in Figure 1.

results agreed that low culture temperature led to suppressed cell growth, cell cycle arrest in G_0/G_1 phase, and improved cell viability for a longer period in batch culture (*2*–*7*).

A cold-inducible RNA-binding protein (CIRP) may play an essential role in suppression of CHO cell growth at low culture temperature. In response to low culture temperature (32 °C), expression of CIRP in mouse fibroblasts was induced, and growth was suppressed. By suppressing the induction of CIRP with antisense oligodeoxynucleotides, this growth suppression was alleviated, while overexpression of CRIP resulted in suppressed cell growth at 37 °C with prolongation of G_0/G_1 phase (*13*). However, the molecular mechanism underlying the cold response in CHO cells needs to be elucidated.

Figure 2A shows antibody concentration in the medium during the cultures shown in Figure 1A. Lowering the culture temperature did not increase antibody production. The maximum antibody concentration at 37, 33, and 30 °C was 110.6 ± 2.6, 106.4 ± 8.6 and 28.3 ± 3.8 μg mL^{-1}, respectively. To determine q_{Ab}, the time integral of viable cells versus antibody concentration was plotted as shown in Figure 2B. The slope of such plots is equal to q_{Ab}, if q_{Ab} is constant. The q_{Ab} was fairly constant until the end of the stationary phase, though the q_{Ab} at 33 and

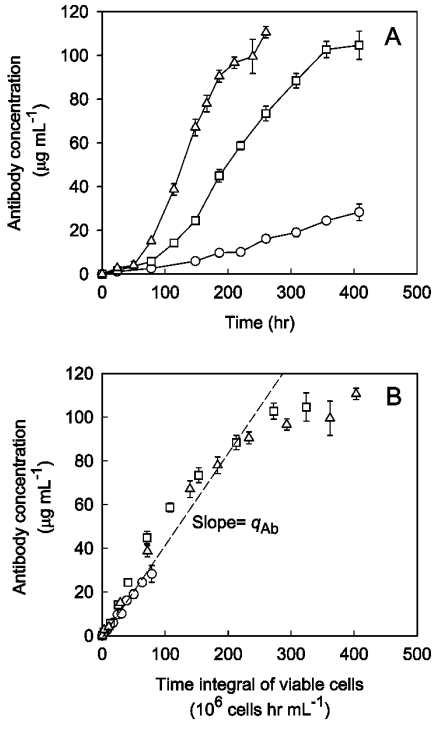

Figure 2. Effect of culture temperature on anti-4-1BB antibody production and q_{Ab}: (○) 30 °C; (□) 33 °C; (△) 37 °C. (A) Antibody concentration. (B) Evaluation of q_{Ab}. The q_{Ab} is equal to the slope of the plot. The error bar represents the standard deviations calculated from the data ($n = 4$).

37 °C decreased during the decline phase of growth. Therefore, the q_{Ab} was calculated until the end of the stationary phase (Table 2). Like the maximum antibody concentration, the q_{Ab} was not enhanced at low culture temperature.

To investigate whether lowering the culture temperature affects the transcription level, Northern blot analysis was performed. Since the expression of β-actin mRNA is often considered as a constant and is not affected by temperature (*2*), β-actin mRNA was used as an internal control for Ig mRNA analysis.

Figure 3 shows the Northern blots of Ig and β-actin mRNAs prepared from cell samples during the cultures shown in Figure 1. The relative mRNA content of HC and LC was estimated as a ratio of the mRNA content of HC and LC to β-actin mRNA content for each sample. Like q_{Ab}, the relative HC and LC mRNA was not increased by lowering the culture temperature. In addition, the mRNA level of HC was higher than that of LC regardless of the culture temperature, and we confirmed that the expression of HC was also higher than that of LC by SDS−PAGE analysis (data not shown).

Table 2. Effect of Culture Temperature on q_{Ab}[a]

culture temperature (C°)	q_{Ab} (μg (10^6 cells h)$^{-1}$)	q_{EPO}[b] (μg (10^6 cells h)$^{-1}$)
30	0.44 ± 0.07	0.49 ± 0.14
33	0.50 ± 0.09	0.35 ± 0.08
37	0.43 ± 0.03	0.09 ± 0.03

[a] Values are means ± SD of two independent experiments. [b] q_{EPO} is extracted from ref 6.

The result that low culture temperature did not increase q_{Ab} of rCHO cells (LGA31-56) was rather surprising to us. Arresting cells in G_0/G_1 phase has been suggested as a useful tool for improving production of recombinant proteins. As observed in other rCHO cells (*5*, *6*), the culture of rCHO cells (LGA31-56) at low temperature resulted in rapid accumulation of cells in G_0/G_1 phase. Furthermore, we previously observed that lowering the culture temperature significantly increased specific EPO productivity (q_{EPO}) of rCHO cells (LGE10-9-27) (Table 2) (*6*). The q_{EPO} at 33 °C was approximately 4-fold higher than that at 37 °C. Like q_{EPO}, the relative EPO mRNA content increased by lowering the culture temperature, indicating that the increased transcription level of EPO is responsible in part for the enhanced q_{EPO} at low culture temperature. Both anti-4-1BB antibody and EPO were expressed in the same CHO host and were driven by the same CMV promoters. Therefore, the beneficial effect of lowering the culture temperature on q of rCHO cells appears to be cell-line-specific and may depend on the integration site of a foreign gene. The efficacy of the simultaneous use of hyperosmotic pressure and glycine betaine as a means to improve TPO production in rCHO cells was variable among clones (*14*, *15*). However, clonal variations of rCHO cells in regard to the effect of low culture temperature on q need to be investigated.

In conclusion, cultivation of rCHO cells producing anti-4-1BB antibody (LGA31-56) at low temperature led to suppressed cell growth, cell cycle arrest in G_0/G_1 phase, and improved cell viability for a longer period. However, antibody production and q_{Ab} were not increased at low culture temperature. These results imply that the efficacy of low culture temperature as a means to improve foreign protein production in rCHO cells depends on the cell line.

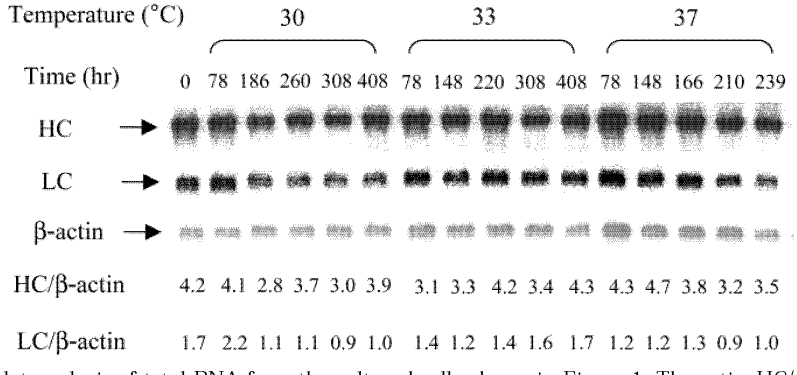

Figure 3. Northern blot analysis of total RNA from the cultured cells shown in Figure 1. The ratio, HC/β-actin and LC/β-actin, shows the relative content of anti-4-1BB mRNA normalized with the internal control, β-actin mRNA.

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Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming

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Mammalian cells cultured *in vitro* are able to recover from cold stress. However, the mechanisms activated during cold stress and recovery are still being determined. We here report the effects of hypothermia on cellular architecture, cell cycle progression, mRNA stability, protein synthesis and degradation in three mammalian cell lines. The cellular structures examined were, in general, well maintained during mild hypothermia (27–32 °C) but became increasingly disrupted at low temperatures (4–10 °C). The degradation rates of all mRNAs and proteins examined were much reduced at 27 °C, and overall protein synthesis rates were gradually reduced with temperature down to 20 °C. Proteins involved in a range of cellular activities were either upregulated or downregulated at 32 and 27 °C during cold stress and recovery. Many of these proteins were molecular chaperones, but they did not include the inducible heat shock protein Hsp72. Further detailed investigation of specific proteins revealed that the responses to cold stress and recovery are at least partially controlled by modulation of p53, Grp75 and eIF3i levels. Furthermore, under conditions of severe cold stress (4 °C), lipid-containing structures were observed that appeared to be in the process of being secreted from the cell that were not observed at less severe cold stress temperatures. Our findings shed light on the mechanisms involved and activated in mammalian cells upon cold stress and recovery.

The heat shock response has been extensively studied in a variety of systems and organisms, and generally involves the conserved and coordinated upregulation of heat shock proteins that act to alleviate the cellular stresses imposed by hyperthermic stress. Our current understanding of the cellular responses to subphysiological temperatures (hypothermia) is less extensive. This is somewhat surprising, because of their relevance in medicine for the storage of cells, organs, and tissues, and the treatment of brain damage; as well as in the biopharmaceutical sector, where reduced culture temperature can sometimes improve recombinant protein yields from mammalian cells cultured *in vitro* [1]. What is clear is that the general response to hypothermia appears to include the global attenuation of transcription and translation, whereas a small group of proteins, termed the cold shock proteins, are selectively induced [2]. However, unlike their heat shock counterparts, these cold shock proteins do not appear to be particularly well conserved between prokaryotic and eukaryotic systems, and their functions, such as have been defined, have to date been described in terms of their RNA rather than their protein biology. Exposure to subphysiological temperature is also known to generally lead to changes in the lipid make-up of membranes, resulting in increased membrane rigidity,

Abbreviations
CCT, chaperonin containing T-complex polypeptide1; Cirp, cold-inducible RNA-binding protein; ER, endoplasmic reticulum; HSF, heat shock factor; NEPHGE, non-equilibrium pH gradient gel electrophoresis; qRT-PCR, quantitative real-time PCR; Rbm3, RNA-binding motif protein 3.

compromised membrane-associated cell functions, and alterations in lipid synthesis and disposition [3].

The most well-characterized cold shock responses to date are those in plants and bacterial systems [3], there being much less information on the molecular mechanisms underpinning the cold shock response in mammalian cells. Our current understanding is that the cold shock response in mammalian cells involves the coordination of transcription, translation, the cell cycle, metabolism, and cell cytoskeleton organization, but the exact mechanisms by which these are modulated remain to be elucidated in most cases. Furthermore, mammalian cells respond to mild hypothermia (25–35 °C) in a different manner to more severely reduced temperatures (0–10 °C). This may largely reflect the fact that at more moderate temperatures, cells can still proliferate and grow, whereas at the severe temperatures, growth is fully arrested. Studies on hibernating animals and various *in vitro* cultured mammalian cell systems have also reported that mammalian cells in general respond to cold shock by disassembly of the cell cytoskeleton, delayed apoptosis, reduced metabolism with reduction of ATP expenditure, reduced protease activity, a reduction in free radical oxygen species, and attenuation of transcription and translation [3]. As a result of such reports, it has been suggested that there are five general mechanisms by which mammalian cells respond to cold shock. These are: (a) a general reduction in transcription/translation; (b) reduction of RNA degradation; (c) increased expression of specific target genes; (d) the generation of alternative mRNAs via presplicing events; and (e) use of internal ribosome entry segments for preferential cap-independent translation of specific mRNAs under cold shock conditions [4].

There have been only two well-characterized mammalian cold shock proteins reported to date, RNA-binding motif protein 3 (Rbm3) [5] and cold-inducible RNA-binding protein (Cirp) [6]. Both of these are induced in response to mild hypothermia (maximal expression around 32 °C) but not severe hypothermia, and are probably general stress response proteins, as they are also induced by a number of other stresses. It is generally thought that Rbm3 and Cirp are involved in the modulation of transcription and translation upon cold stress and function as RNA chaperones, although the exact function of these proteins remains to be elucidated [7]. Cirp and Rbm3 are highly similar proteins that consist of an N-terminal RNA-binding domain and a C-terminal glycine-rich domain, but show no homology to the cold shock proteins found in bacterial systems [3]. Our current understanding of the cold shock response, and the mechanisms involved in coordinating that response, in mammalian cells is therefore extremely limited.

It is interesting to note that although cold stress appears to generally reduce protein synthesis, in recent years recombinant protein production from *in vitro* cultured mammalian cells has been improved by reducing operating temperatures from 37 °C to mildly hypothermic levels (28–34 °C) towards the end of the logarithmic increase in cell number [8]. This strategy has been adopted because although cell division and protein synthesis rates are appreciably slowed, cells show prolonged viability and increased cell-specific productivity under these mildly hypothermic conditions. With regard to the cultivation of mammalian cells at subphysiological temperatures, the prolonged cell viability, delayed apoptosis, decreased glucose and glutamine consumption, decreased waste product release and increased tolerance to shear stress during cultivation under mildly hypothermic conditions are all features likely to extend the productive life of cells in terms of recombinant protein production [1]. However, as the cell division cycle slows and even arrests in G_1 at the lower end of the mildly hypothermic range, and both transcription and translation rates are reduced at subphysiological temperatures, this may offset any potential positive effects of subphysiological culturing.

To date, characterization of the mammalian cold stress response has largely derived from microarray analyses of transcriptional changes and single snapshot proteomic analyses of changes in protein levels upon exposure to subphysiological temperatures [3]. These have been most useful in defining the overall adaptations to growth at subphysiological temperatures and in highlighting areas for further, more detailed investigations into the mechanisms of cold adaptation. Here, we have investigated changes in protein synthesis rates upon cold stress, and show that an examination of changes in the synthesis rates of specific proteins during cold stress, and during recovery, identifies subtle adaptations to growth at subphysiological temperatures, not all of which have been previously uncovered by proteomic analysis of overall protein levels. The proteins identified in this study encompass a wide range of cellular activities, including cell cycle regulation, translation initiation, cytoskeleton organization and, most particularly, molecular chaperone activity. Specific investigation of the roles of a number of the proteins identified leads us to the conclusion that the regulation of p53, Grp75 and eIF3i protein levels may play a key role in the response to, and recovery from, cold stress in mammalian cells. The implications of these findings in terms of the cold stress response in mammalian cells are further discussed.

Results

The cell lines chosen for this study were the commercially relevant CHOK1 cell line and two mouse cell lines, P19 embryonal carcinoma cells and NIH 3T3 fibroblasts. P19 cells were chosen because of their particular sensitivity to cold stress. The temperature ranges investigated covered both severe cold stress (4 and 10 °C) and more mild cold stress (27 and 32 °C), and were considered to be relevant to organ/tissue storage and the subphysiological *in vitro* culturing of mammalian cells for recombinant protein production respectively. The periods of time for which cells were exposed to cold stress (6–30 h) were those sufficient to elicit detectable cold stress responses while still allowing the majority of cells, if not all, to recover upon rewarming.

Severe, but not mild, cold stress results in marked changes in the cellular architecture of *in vitro* cultured mammalian cells

Immunofluorescence studies highlighted changes in the structural architecture of all cell lines investigated upon their exposure to more severe cold stress (Figs 1 and S1). Cells cold-stressed at 27 or 32 °C for 6 h were indistinguishable from those maintained at 37 °C when investigated by immunofluorescence, with the exception that although the microtubule content was unchanged in P19 cells at 27 °C, the microtubule organization appeared to be compromised. Cells cold-stressed at 27 or 32 °C, or maintained at 37 °C, were well spread, and the organelle distribution appeared to be unchanged as exemplified by mitochondrial staining (Fig. 1). Furthermore, when we monitored poly(ADP-ribose) polymerase levels by western blot analysis, there was no evidence of poly(ADP-ribose) polymerase cleavage (Fig. S2A), which is activated upon apoptosis, and therefore we conclude that the cells are not apoptotic at the times and temperatures investigated. At 27 and 32 °C, reduced cell proliferation was observed, and previous reports have suggested that the cold shock protein Cirp may be responsible for cell cycle arrest of mammalian cells at subphysiological temperatures [9]. However, our results suggest that cell cycle arrest at 27 °C is the result of an increase in the overall level (Fig. 2A) and changes to the post-translational modification pattern (Fig. 2B) of p53, which persisted in cells maintained at 27 °C for 6 h to 6 days. The expression of p21, a general inhibitor of cyclin-dependent protein kinases and a downstream effector of elevated p53 levels, was also induced at 27 °C. During recovery from cold stress at 37 °C, the overall amounts of these two proteins, and the isovariants of p53, returned towards their normothermic values and appearance (Fig. 2). The change in isovariant levels of p53 was not due to increased levels of acetylation, as shown by western blot analysis with an antibody specific for acetylated p53 (Fig. S2B). Across the mild cold stress conditions and time periods examined, there was 100% viability and recovery upon returning the cells to 37 °C.

At near-freezing temperatures, the cellular appearance and architecture were profoundly affected, in a cell line-specific manner, as compared with cells maintained at 37 °C. Under conditions of severe cold stress, cells were much more rounded and less well spread, and both nuclear and cellular diameter were decreased. Most cells cold-stressed at 4 °C had translucent, vesicle-like structures that were easily detected by phase contrast viewing, on the surface of the cell (arrowed in Figs 1 and S1). These vesicle-like structures appeared within 2 h of exposure to the severe cell stress but disappeared very rapidly (< 0.5 h) upon rewarming. The content of these vesicles was not revealed by general stains for DNA, RNA or protein, although this last stained the perimeter of the vesicles, but was instead strongly stained by the dye Oil Red O, which preferentially binds to uncharged lipids (Fig. S1). The proportion of cells able to recover from severe cold stress was much smaller than observed for recovery from mild cold stress, and typically 20–30% of cells did not survive upon rewarming from extreme cold (4 °C). Electron microscopy of cold-stressed cells revealed the presence of lipid-containing structures (as stained by osmium tetroxide) corresponding to these vesicles that appeared to be in the process of being extruded from the cell (Fig. 3).

Of the cell lines investigated, CHOK1 cells proved to be the most resilient to subphysiological temperatures, possibly because they contain a sizeable population of cold-stable microtubules and therefore maintain a relatively ordered intracellular organization at low temperatures (Fig. 1). Even at 4 °C, a sizeable population of microtubules persisted in CHO cells, although these appeared to be less ordered than those in cells maintained at 37 °C. Mitotic cells appeared to be particularly vulnerable to cold stress, and below 20 °C cells were observed that contained multiple microtubule asters (Fig. 1; CHOK1 cells at 4 °C, lower section of the upper right panel) that were very similar in appearance to those formed in mitotic cells exposed to the microtubule-stabilizing drug taxol [10]. Such structures are formed when the nuclear envelope breaks down during mitosis and releases proteins (e.g. NuMA) that stabilize microtubule minus-ends and that

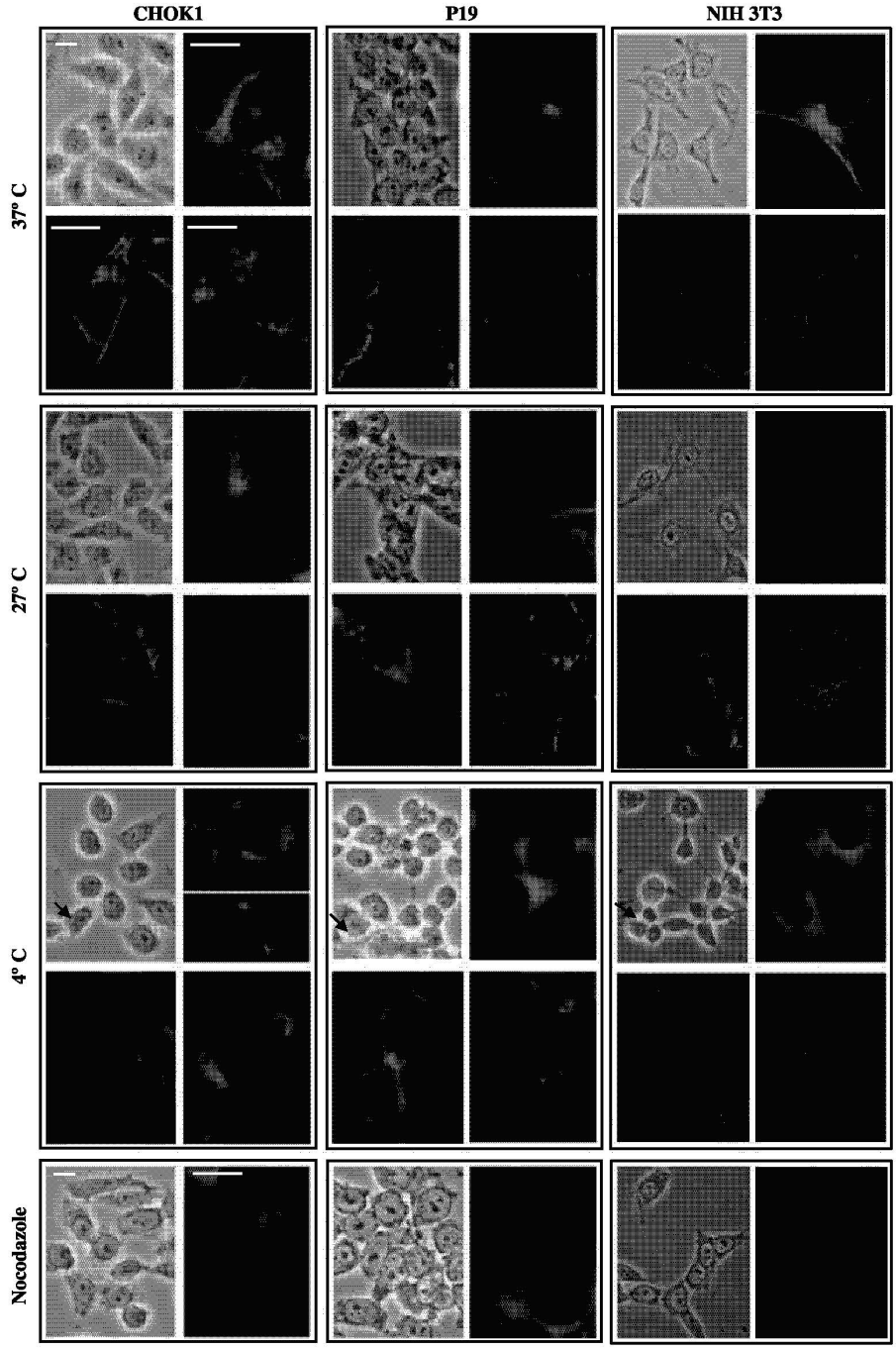

Fig. 1. Structural changes in CHOK1, P19 and NIH 3T3 cells during mild and severe cold stress and after exposure to nocodazole. Cells were maintained at 37 °C, or then transferred to 27 or 4 °C for 6 h, or exposed to growth medium containing 3 $\mu g \cdot mL^{-1}$ nocodazole, prior to fixation for immunofluorescence microscopy. In each panel of four: top left, phase contrast; top right, α-tubulin detection; bottom left, F-actin detection; bottom right, mitochondrial Hsp60 detection. In CHOK1 cells at 4 °C, the lower section of the upper right panel shows the presence of multiple microtubule asters. In each panel of two (nocodazole staining):left, phase contrast; right, α-tubulin detection. Bar: 10 μm. Arrows in the 4 °C phase contrast image point to vesicle-like structures that are also highlighted in Fig. S1.

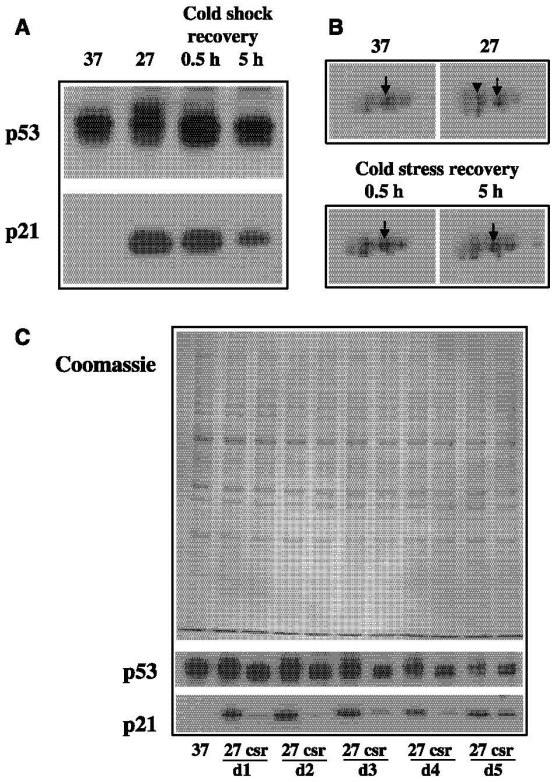

Fig. 2. p53 is elevated and undergoes changes in post-translational modification in CHOK1 cells during maintenance at 27 °C. (A) Twenty micrograms of proteins extracted from CHOK1 cells, maintained at 27 °C or then rewarmed at 37 °C for the indicated times, were resolved by SDS/PAGE and detected by probing immunoblot for p53 or p21. (B) One hundred micrograms of proteins extracted from CHOK1 cells as in (A) were resolved by NEPHGE-SDS/PAGE and immunoblots were probed for p53. Arrows highlight changes in isoform distribution between the 37 and 27 °C samples. (C) As in (A) at 27 °C for up to 5 days (d, day) and upon rewarming (csr, cold shock recovery) for 5 h.

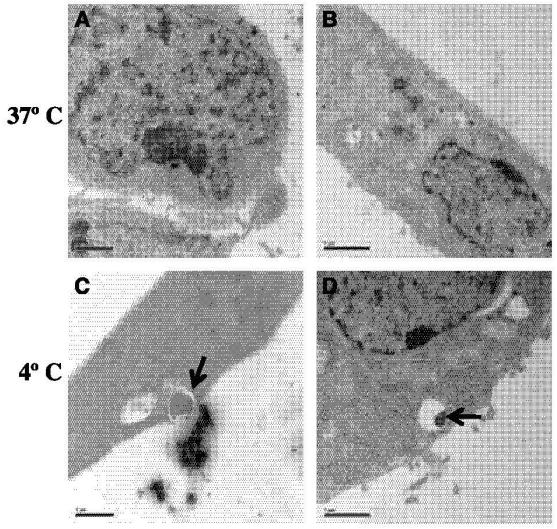

Fig. 3. Electron micrographs of P19 and CHO cells at 37 and 4 °C. P19 cells (A, C) and CHO cells (B, D) at 37 °C or after 6 h at 4 °C. At 4 °C, vesicle-like structures that contain lipids were observed (arrowed) that appeared to be in the process of being extruded from the cell. Bar: 1 μm.

would normally fulfil this function within the mitotic spindle poles. In the presence of taxol, these proteins mis-localize to the cytoplasm, where they stabilize microtubule aster formation [11]. The structures reported here in cold-stressed cells may originate from similar mis-localization of nucleating proteins.

P19 cells had no detectable cold-stable microtubules, and organelle disposition was severely disrupted by temperatures below 20 °C (Fig. 1). The difference in microtubule stability between CHOK1, NIH3T3 and P19 cells was reflected by a relative abundance of STOP proteins (data not shown), splice variants of which stabilize microtubules to cold exposure [12–14]. However, the apparent reduction in cell size and vesicle release observed in cells exposed to severe cold shock is not simply a consequence of a loss of microtubules. Exposure of CHOK1, NIH3T3 and P19 cells to the antimicrotubule drug nocodazole at 37 °C completely depolymerized the microtubule networks in all three cell types and caused retraction of the cytoplasm, but, in contrast to cold-stressed cells, the nuclear size appeared to be unchanged (Fig. 1). Furthermore, no extracellular vesicular structures were observed after nocodazole treatment alone, suggesting that microtubule depolymerization is not the key signal in the formation of the vesicle structures.

Mammalian cells regulate the synthesis rates of specific proteins in response to cold stress and upon recovery at 37 °C

Although there have now been a number of proteomic studies of the cold shock response in various systems that have yielded valuable information, these have been static-based measurement approaches and so do not account for any variation in protein synthesis and turnover rates upon exposure to any given cold-related stress. In order to determine the protein synthesis capacity of *in vitro* cultured mammalian cells at sub-physiological temperatures, newly synthesized proteins were radiolabelled with [^{35}S]methionine/cysteine mix for 1 h at the cold stress temperatures described above.

Scintillation counting was undertaken of samples from cells stressed at different temperatures, to ensure that any difference in the radioactive amino acid uptake did not account for differences in label incorporation into polypeptides (Fig. 4A). The acid-soluble material extracted from CHOK1 cells changed little between 20 and 37 °C, and at 20 °C the acid-soluble/insoluble ratio was actually increased (Fig. 4A). We therefore concluded that methionine/cysteine uptake was not limiting for changes in label incorporated into polypeptide/protein over the temperature range investigated.

SDS/PAGE analysis followed by autoradiography revealed that overall protein synthesis capacity was generally reduced at subphysiological temperatures, although cells at 32 °C, and even at 27 °C, still displayed an appreciable amount of protein synthesis (Fig. 4B), and at this level of resolution the range of proteins being synthesized was similar at 37, 32 and 27 °C. Below 27 °C, protein synthesis was much more severely attenuated, being most affected in the most temperature-sensitive cell line, P19 (Fig. S3), the cell line whose cellular architecture was also most compromised upon severe cold stress. Protein synthesis rates were fully restored upon rewarming to 37 °C, and close examination of the SDS/PAGE analyses revealed that changes in protein synthesis rates were discernible between control cells maintained at 37 °C and those subjected to cold stress and then rewarming, particularly in the 50–75 kDa range (Fig. 4B).

To examine changes in the synthesis rates of individual proteins at subphysiological temperatures and upon recovery more closely, proteins were resolved by 2D non-equilibrium pH gradient gel electrophoresis (NEPHGE)-SDS/PAGE (pI > 4.5 and size 20–150 kDa; Fig. 5A). Owing to this size range limitation, we did not detect the two well-characterized mammalian cold shock-inducible proteins Cirp and Rbm3 (< 20 kDa). However, changes in the synthesis rate (up or down) for 25 newly synthesized polypeptides at 32 °C (Fig. 5B) and for 16 at 27 °C (data not shown) relative to those at 37 °C was observed. The synthesis rates of rather more (31) polypeptides changed upon rewarming after cold stress relative to their rates during continuous growth at 37 °C (Fig. 5C). The synthesis rates of a similar number of polypeptides were observed to change for the other rewarming conditions examined: 4–37 °C (27); 10–37 °C (33); and 20–37 °C (23). The range of polypeptides showing altered synthesis rates during cold shock recovery was similar regardless of whether the recovery was from severe or mild cold stress. Most of the changes in synthesis rates upon cold stress or recovery observed in CHOK1 proteins were observed in similar experiments with P19 and 3T3 cells (Fig. S3).

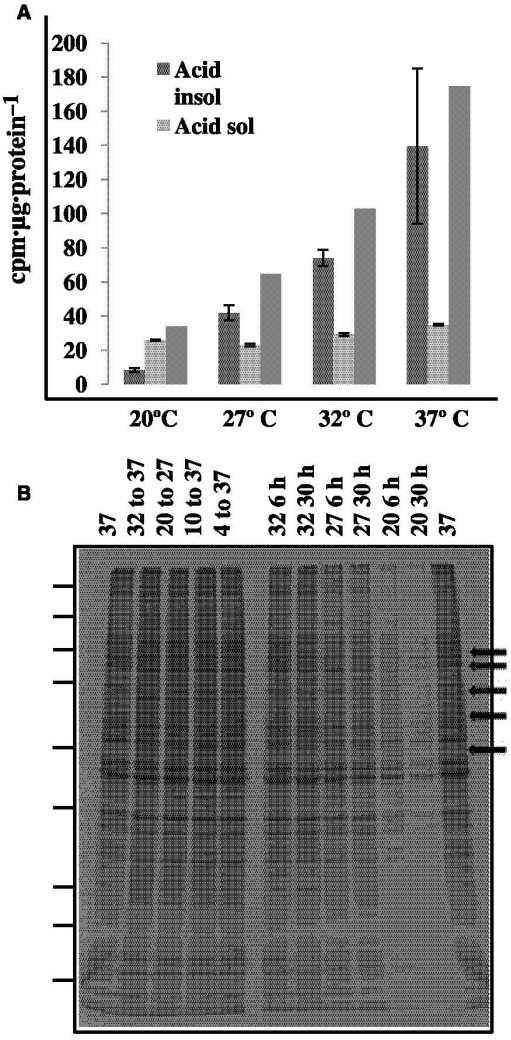

Fig. 4. Amino acid uptake and protein synthesis in CHOK1 cells exposed to, and recovering from, cold stress. (A) Cells maintained at 37 °C, or then exposed to the indicated temperatures for 6 h, were radiolabelled in methionine/cysteine-deficient growth medium supplemented with 1770 kBq·mL^{-1} [^{35}S]methionine/cysteine cell labelling mix for 10 min at the indicated temperature prior to extraction into ice-cold 0.6 M trichloracetic acid. (B) Cells maintained at 37 °C, or then exposed to the indicated temperatures for the indicated times, were radiolabelled as in (A) but for 1 h at the indicated temperatures. Thirty micrograms of extracted proteins were resolved by SDS/PAGE and detected by autoradiography. Molecular mass markers were 205, 150, 100, 75, 50, 37, 25, 20 and 15 kDa, and are indicated by lines on the left-hand side of the figure.

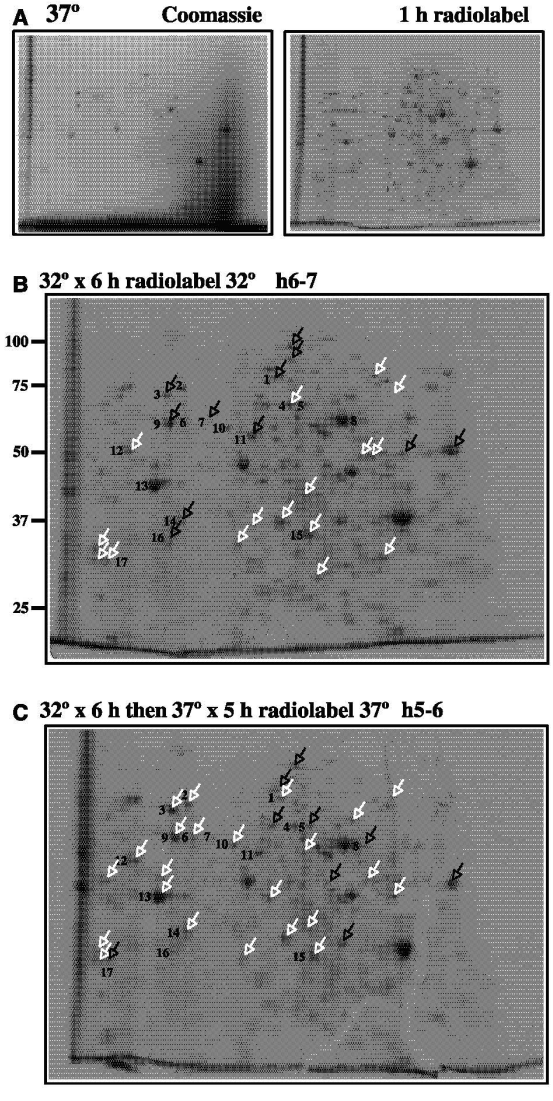

Fig. 5. Changes in protein synthesis rate in CHOK1 cells during exposure to, and recovery from, cold stress. CHOK1 cells were radiolabelled for 1 h under the indicated conditions, and then 100 μg of extracted proteins were resolved by two-dimensional NEPHGE followed by SDS/PAGE. (A) Proteins extracted from cells maintained at 37 °C, detected by Coomassie staining (left panel) or by autoradiography (right panel). (B) Proteins extracted from cells held at 32 °C for 6 h. (C) Proteins extracted from cells held at 32 °C for 6 h and then transferred to 37 °C for 5 h. In (B) and (C), white arrows identify increased synthesis relative to 37 °C, black arrows identify decreased synthesis relative to 37 °C, and numbers identify polypeptides referred to in Table 2.

The more abundant CHOK1 polypeptides (i.e. those readily visible by Coomassie staining) showing altered synthesis rates during cold stress or during recovery were excised and subjected to in-gel tryptic digestion followed by MS analysis by MALDI-TOF MS for their identification. In a number of cases, the identity was confirmed by immunoblot, and in a few cases [subunits of the cytoplasmic molecular chaperone chaperonin containing T-complex polypeptide 1 (CCT)], by a combination of immunoblot and previously identified positions on the NEPHGE-SDS/PAGE system used, and with this approach, 17 CHOK1 polypeptide spots were identified (Table 1). All identified proteins are relatively abundant proteins, but they cover a broad spectrum of functional activities in cells, including energy metabolism, cytoskeleton organization, protein synthesis, protein secretion and purine biosynthesis. The majority, however (9/17), were molecular chaperones deriving from at least three subcellular compartments, the cytoplasm (CCT subunits, Hsc73, and HOP p60), the mitochondrion (Grp75, Hsp60), and the endoplasmic reticulum (ER) (ERp57).

Protein degradation is generally attenuated upon cold stress in mammalian cells

The overall abundance of a polypeptide, and any change in it, depends not only on its rate of synthesis but also its degradation, and so protein degradation rates were also examined at subphysiological temperatures. Examples of the protein half-life determinations for some of the specific CHO proteins investigated are shown in Fig. 6. Interestingly, at subphysiological temperatures relevant to bioprocessing (32 °C), protein degradation was severely curtailed at a global level, being undetectable for all proteins examined at 27 °C over the time period (12 h) investigated. These, remarkably, included the normally very short-lived cell cycle regulator p53. It is notable that the half-life measured for CHO p53 at 37 °C (5.2 h) was longer than reported for this protein in many other cell lines (20–60 min), a fact attributed to the CHOK1 p53 gene having a point mutation in exon 6 sufficient to compromise the normal function; that is, CHOK1 cells fail to arrest in G_1 after radiation-induced DNA damage, and the mutant protein is present at high spontaneous levels in these cells [15].

When the effect of cold stress and recovery on overall protein levels was examined by immunoblot, the changes detected were, with a few exceptions, much more subtle than might have been anticipated from the observed changes in their synthetic rates, particularly as their degradation rates were negligible at 27 °C. This is most likely because the polypeptides chosen for identification were relatively abundant proteins, so that

Table 1. Identification of CHOK1 proteins showing above-average temperature-dependent changes in synthesis rate. NC, no change; ↑, increased abundance; ↓, decreased abundance.

No.	Common name	Method	Identifier, Swissprot	*m* (Da)	pI	Cold stress (°C)		Recovery at 37 °C from (°C):			
						27	32	4	10	20	32
1	NEM-sensitive fusion protein	MS	X15652	83 811	6.38	NC	↓	↑	↑	↑	NC
2	Grp75	MS/blot	U92313	73 970	5.87	NC	NC	↑	NC	↑	↑
3	Hsc73	MS/blot	M34561	70 989	5.24	↑	↓	↑	↑	↑	↑
4	HOP	MS	NM_138911	63 158	6.40	↑	NC	NC	NC	NC	NC
5	IMP cyclohydrolase	MS	D89514	64 705	6.72	NC	NC	↓	NC	NC	↓
6	CCTθ	Blot	Z37164	59 600	5.43	NC	NC	↑	↓	NC	↑
7	CCTα	Blot	M34665	60 339	5.71	NC	↓	NC	NC	NC	NC
8	CCTδ	Blot	Z31554	58 100	8.24	NC	NC	NC	↓	↓	NC
9	Hsp60	MS/blot	M22383	61 122	5.83	NC	↓	↑	NC	NC	↑
10	ERp57	MS	Q91Z81	57 217	5.98	↑	NC	↑	NC	↑	↑
11	CCTβ	MS/blot	Z31553	57 753	5.91	NC	↓	↓	NC	↓	NC
12	β5-Tubulin	MS/blot	NM_011655	50 095	4.78	↑	↑	↑	NC	NC	↑
13	Actin	MS	AB013098	42 087	5.30	↑	NC	↑	↑	↑	↑
14	eIF3i	MS	U39067	36 878	5.38	NC	↓	NC	NC	NC	↑
15	Lactate dehydrogenase A	MS	DQ912661	36 781	7.01	↑	↑	↑	↑	NC	↑
16	β-Tubulin fragment	MS	AJ717320	28 874	4.86	NC	↓	NC	↓	NC	NC
17	Tropomyosin 3	MS	XM_860687	24 918	4.88	NC	↑	NC	NC	NC	↓

a small change in amount due to increased synthesis rate might be difficult to detect by immunoblot against a background of the total polypeptide. Furthermore, the time periods investigated were short (6 h of cold shock, 5 h of recovery), which would make detection of small changes in the total amount of an abundant protein difficult by this method. An exception to this was the θ-subunit of the cytoplasmic chaperonin CCT. The level of this particular subunit was particularly sensitive to both hypothermic and hyperthermic stress (Fig. 7). Comparatively, significant changes in the levels of other subunits of this molecular chaperone were not detectable by immunoblot (data not shown), even though the synthesis rates of several changed during cold shock and/or recovery from cold stress (Table 1).

mRNA degradation is also attenuated during cold stress in mammalian cells

Quantitative real-time PCR (qRT-PCR) was used to ascertain the levels of mRNAs encoding selected proteins for which synthesis rates changed in response to temperature variation. As it was unclear what would be a suitable mRNA to standardize the data to, the data shown in Fig. 8 have been standardized to the respective values at 37 or 27 °C. The mRNAs monitored showed appreciable degradation rates at 37 °C but were found to be more stable at 27 °C. This agrees with a previous study [8] showing increased mRNA levels at reduced temperature, although the study did not analyse synthesis rates. We note further that the more labile a specific mRNA is at 37 °C, the greater are the changes observed in its levels during exposure to, and recovery from, cold stress.

Recovery of cold-stressed cells at 37 °C does not induce a full classical heat shock response

It has been reported that rewarming cold-stressed cells of human origin at 37 °C initiates a heat shock response [16]. Although we also observed that the synthesis rates of several constitutively expressed heat shock proteins increased during recovery from hypothermia, these changes were rather modest when compared with the changes in synthesis rates of these same proteins during recovery from a classical heat shock (hyperthermia; Fig. 9A). Furthermore, upon recovery from heat shock, an increase in the total amount of heat shock proteins could be detected by immunoblot (Fig. 7). Interestingly, Hsp72, which is strictly inducible in rodent cells [17], was not detectable during cold stress or recovery, even though it was clearly induced in the same cells during their recovery from hyperthermic heat shock (Fig. 7, arrowed, Fig. 9A). These observations suggested that the increased expression of constitutive heat shock proteins during recovery from cold stress might be regulated by

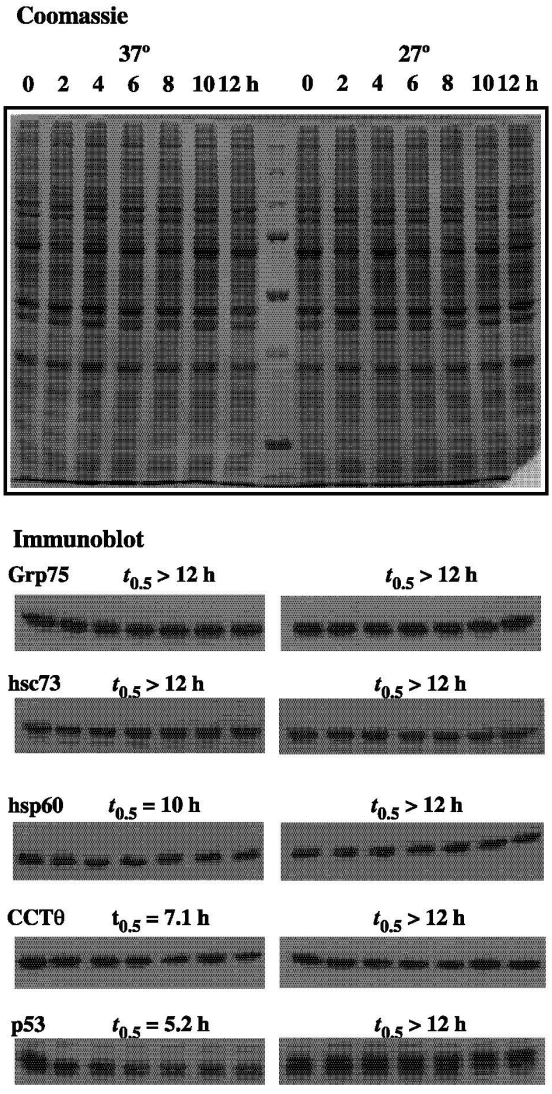

Fig. 6. The half-life of proteins is increased upon exposure to mild cold stress. CHOK1 cells maintained at 37 or 27 °C were then exposed to growth medium containing 50 μg·mL^{-1} cycloheximide. At the indicated times, cells were extracted, and 20 μg of protein was resolved by SDS/PAGE, and then detected either by Coomassie stain (upper panel) or by probing immunoblots for the indicated proteins (lower panel). Molecular mass markers as in Fig. 4.

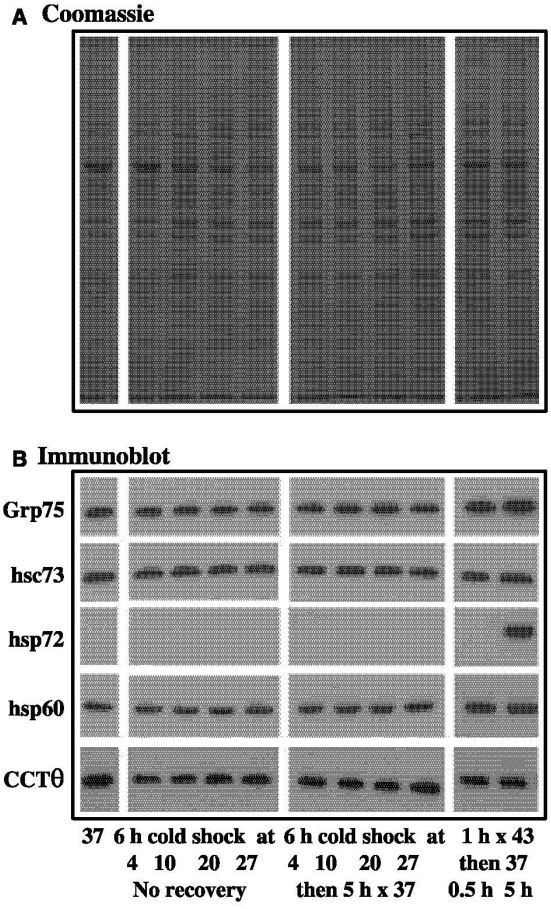

Fig. 7. Marked changes in synthesis rate do not correlate with large changes in overall amounts of relatively abundant proteins. CHOK1 cells maintained at 37 °C, or exposed to the indicated temperature changes, were extracted, and 20 μg of protein was resolved by SDS/PAGE and then detected by Coomassie stain (A) or by probing immunoblots for the indicated proteins (B).

a different mechanism from that involved during a 'classical' recovery from heat shock, during which the inducible form of Hsp70 is robustly expressed.

Transcription of inducible heat shock genes is activated by the binding of heat shock factors (HSFs) to heat shock elements in their promoter-proximal regions [18,19], the best understood being that of HSF1. In unstressed cells, HSF1 exists as a constitutively phosphorylated monomer in the cytoplasm, but during heat stress, HSF1 undergoes trimerization [20] and becomes hyperphosphorylated [21]. It is this hyperphosphorylated, trimeric form that accumulates in the nucleus and binds to heat shock elements, thereby activating transcription [21]. Figure 9B shows the basal level of constitutive phosphorylation of HSF1 determined using immunoblots of HSF1 in cell extracts prepared in the presence of protein phosphatase inhibitors (Fig. 9B, as a cluster of bands ~ 85–90 kDa). The hyperphosphorylation occurring during heat shock could also be readily demonstrated (Fig. 9B). Cold shock produced a much more subtle change in the HSF1 banding pattern, evident immediately after cold shock and then slowly returning to the

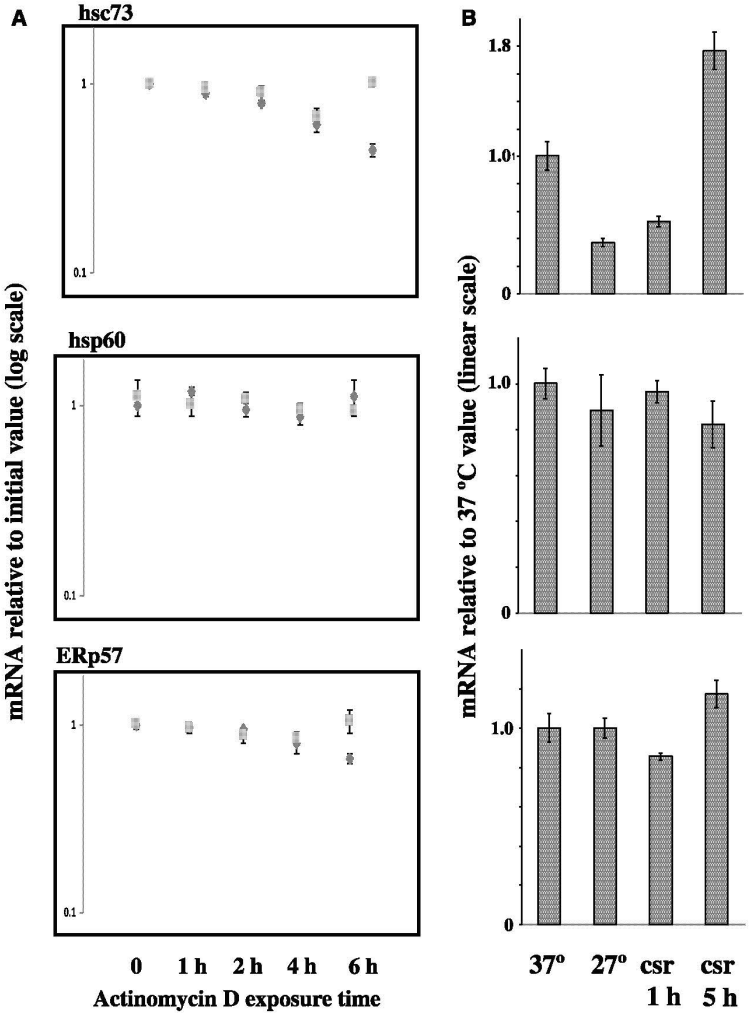

Fig. 8. Specific mRNAs are longer-lived at 27 °C than at 37 °C. (A) CHOK1 cells maintained at 37 °C (squares) or 27 °C (diamonds) were then exposed to growth medium containing 2 $\mu g \cdot mL^{-1}$ actinomycin D at the same temperatures. (B) CHOK1 cells maintained at 37 °C or held at 27 °C for 6 h without or with a recovery period (crs, cold shock recovery) at 37 °C for 1 h or 5 h. At the indicated times, total RNA was extracted from the cells and the indicated mRNAs were quantified by qRT-PCR. Data are normalized to the initial mRNA content at 37 or 27 °C.

constitutive pattern during a subsequent 5 h recovery. This cold shock-induced phosphorylation change in HSF1 was more pronounced with increasing hypothermia, and was most evident in the very cold-sensitive P19 cells.

Trimerization was assessed by chemical cross-linking analysis, using ethylene glycol *bis*(succinimidylsuccinate), to stabilize the trimer for SDS/PAGE resolution prior to immunoblot detection of HSF1. Heat shock-induced trimerization of HSF1, i.e. the hyperthermic response, was extensive, so that immediately after heat shock, almost all HSF1 was in the hyperphosphorylated, trimeric form (Fig. 9C, lower panel). In contrast, little trimeric HSF1 was evident immediately after cold (hypothermic) shock, and only modest amounts were present during recovery from this cold stress, even though the synthesis rates of constitutive heat shock proteins were increased at this time. Once again, although this response was stronger in the most cold-sensitive cell line, P19, it was still weak in comparison to that observed upon heat stress. These findings collectively suggest that the recovery from cold stress, at least in rodent cells, does not initiate a classical heat shock response, and that any response initiated through HSF1 is comparatively weak or restricted in comparison to a classical heat shock response.

Discussion

Here we report changes in the cellular architecture, and the synthesis and degradation rates, of specific

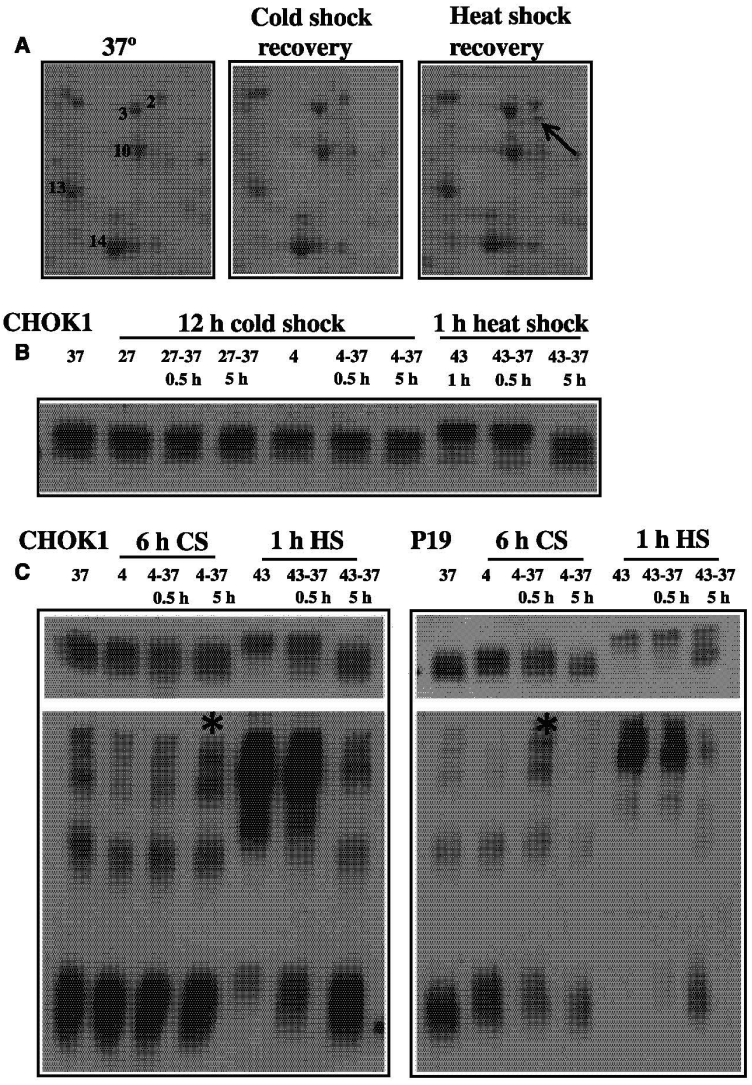

Fig. 9. A classical heat shock response is not initiated upon recovery of cold-stressed cells at 37 °C. (A) Proteins extracted from CHOK1 cells that had been maintained at 37 °C, or held at 27 °C for 6 h and then transferred to 37 °C for 5 h (cold shock recovery), or held at 43 °C for 1 h and then transferred to 37 °C for 5 h (heat shock recovery), and then radiolabelled for a further 1 h at 37 °C, were resolved and detected as in Fig. 5. Only the area including Grp75 (spot 2) to actin (spot 14) is shown; the spot numbers refer to the proteins listed in Table 2. Hsp72 is arrowed. (B) An immunoblot of proteins extracted from CHOK1 cells maintained at 37 °C, or held at 4 or 27 °C for 12 h, or held at 43 °C for 1 h, with or without subsequent recovery at 37 °C for 0.5 h or 5 h, probed for HSF1. (C) Immunoblots of SDS/PAGE resolutions (upper panels) or of nondenaturing gel resolutions (lower panels) of proteins extracted from CHOK1 and P19 cells maintained at 37 °C, or held at 4 °C for 6 h, or held at 43 °C for 1 h, with or without subsequent recovery at 37 °C for 0.5 h or 5 h, probed for HSF1. Trimerization of HSF1 upon recovery from cold stress is indicated by an asterisk. CS, cold stress; HS, heat stress.

proteins in mammalian cells subjected to both mild and severe cold stress, and during recovery from hypothermic shock. Collectively, they help to define the specific cellular responses and protein players during cold stress and recovery. The changes identified here in the synthesis and turnover rates reveal that adaptations are easy to miss when comparing total protein levels monitored either by densitometry-based studies (typically, global proteomic 'snapshot' studies) or by immunoblot. Our studies have shown that subphysiological temperatures induce specific changes in synthesis rates for proteins involved in a wide spectrum of cellular activities, including energy metabolism, cytoskeletal organization, protein synthesis, purine biosynthesis, secretion and, most particularly, molecular chaperone function.

Representative molecular chaperones from three intracellular compartments, the cytoplasm, the mitochondrion, and the ER, were all detected as part of the adaptive changes of cells exposed to mild hypothermia and, more especially, in cells recovering from this state. It is of particular interest that the synthesis rates of the cytoplasmic molecular chaperones Hsc73 and HOP/p60, and of the ER chaperone ERp57, were increased upon cold stress at 27 °C but not at 32 °C. The strength of hydrophobic interactions decreases with decreasing temperature, and so higher orders of protein structure become less stable at subphysiological

temperatures [22]. Thus, at 27 °C, this must become problematic and generate unfolding of existing proteins and/or compromise the folding of newly synthesized proteins, as appreciable protein synthesis is still taking place at this temperature. Furthermore, as protein degradation becomes undetectable at 27 °C, the cell responds to the unfolded protein load by increasing the synthesis of selected molecular chaperones to sequester unfolded proteins until more favourable conditions, including revival of turnover apparatus, are restored.

Rapid recovery of protein synthesis capacity upon rewarming after cold stress would be expected to increase the requirement for molecular chaperones involved in protein folding, particularly in the cytoplasm. However, we also observed increases in the synthesis rates of chaperones in the mitochondrion and the ER after restoration to normothermic conditions. This will undoubtedly be, in part, a response to the overall increase in protein synthesis activity, but the fact that two of these chaperones, Grp75 and ERp57, are redox-sensitive chaperones indicates that the resumption of metabolic activity upon rewarming increases the free radical load on the cell, as might be expected. As the synthesis of the mitochondrial chaperones did not increase during cold shock at 27 °C, this further supports the idea that it is a change in the redox state upon rewarming that is the main stimulus for the increased synthesis rate of the mitochondrial chaperones during recovery from cold stress.

During recovery from cold stress, we also detected increased synthesis of several constitutive heat shock proteins but not of the classical heat shock protein, inducible Hsp72. Kaneko *et al.* [23] also reported no increase in Hsp72 mRNA upon rewarming NIH 3T3 cells from 32 to 37 °C. Earlier studies using human cell lines did detect increased amounts of Hsp72 upon rewarming after cold shock [16]. An explanation for this discrepancy is that in human cells, Hsp72 is constitutively expressed, whereas in rodent cell lines it is strictly inducible [17]. It would appear, then, that the heat shock proteins induced during recovery from cold stress are the constitutive heat shock proteins, not the strictly inducible ones. Specifically with regard to heat shock protein induction, our findings show that the HSF1 activation process during recovery from cold stress is different from that induced during the classical heat shock response. The degree of HSF1 hyperphosphorylation varies from robust in the normal heat shock response to only a partial response as reported here for cold shock recovery, but also following exposure to certain antimicrotubule drugs used in cancer chemotherapy [24]. Under these latter circumstances, not only HSF1 hyperphosphorylation but also HSF1 trimerization occurred at a reduced level, and only induction of the constitutive heat shock proteins Grp75 and Hsp60, not of inducible Hsp72, was detected.

It is generally accepted that cold stress results in the attenuation of mRNA translation, although we show here that at mildly hypothermic temperatures (27 and 37 °C), protein synthesis is active, although reduced, and that both the banding pattern and relative intensity of polypeptides synthesized at these lower temperatures remain very similar to those observed at 37 °C. Translation is a tightly controlled process, modulated greatly by the (de)phosphorylation of key initiation and elongation factors. Previous studies have shown that mutant initiation factors can elevate the effects of such a slowdown in mRNA translation upon cold stress [25]. Here, we observed that cold stress at 32 °C results in reduced levels of newly synthesized eIF3i, a subunit of initiation factor 3. Upon recovery, this is reversed and eIF3i levels are increased. Although eIF3i is essential for mRNA translation *in vivo* [26–28], it is not essential for the reconstruction of initiation complexes that can scan and find the AUG start codon [29]. Therefore, its role *in vivo* is likely to be related to regulation of initiation. Furthermore, overexpression of eIF3i has been shown to be associated with increased cell proliferation, an accelerated cell cycle, and an increase in cell size, whereas the knockdown (by RNA interference) of eIF3i resulted in the reverse of these effects [30,31]. These opposing consequences of eIF3i knockdown or overexpression are mirrored in the observations here of the cellular responses to cold stress at 32 °C and recovery, respectively. It is therefore likely that eIF3i plays a pivotal role in directing cell growth and proliferation upon cold stress and subsequent recovery.

It has been reported elsewhere that cells cultivated under mildly hypothermic conditions undergo cell cycle arrest, predominantly in G_1, but also in G_2/M [32], and it has recently been suggested that this is in part due to expression of the RNA-binding cold shock proteins Cirp and Rbm3, as their overexpression under normothermic conditions can lead to cell cycle arrest [9]. Previous reports, however, have shown that p53-deficient mammalian cells do not show cell cycle arrest at mildly hypothermic temperatures [33,34], and that at 4–20 °C, p53 induces p21 (WAF1) expression [34]. Our results support this mechanism of p53-mediated cell cycle arrest. p53 in CHOK1 cells has a point mutation that confers unusual stability on this protein and prevents these cells undergoing a normal response to DNA damage, i.e. induction of p21 and consequent cell cycle arrest in G_1 [15]. Nevertheless, during mild cold stress, we observed

an increase in the level of p53 in CHOK1 cells, a change in p53 isoform pattern due to post-translational modification, and induction of p21 expression. Furthermore, re-entry of cells into the cell cycle upon return to normothermic conditions could be mediated by the increased synthesis of Grp75 that we observed under these conditions. Expression of Grp75 has a two-fold positive effect on cell cycle progression. When present in the cytoplasm, it sequesters p53 [35], thereby preventing entry into the nucleus and subsequent activation of p21 transcription. Furthermore, p53 binding to the centrosome [36,37], which is inhibitory to centrosome duplication, is antagonized by Grp75 [38]. Additionally, Grp75 itself binds to the centrosome, thereby activating Mps1 protein kinase, the activity of which is essential for the initiation of centrosome duplication [39]. Under this model, cell cycle arrest upon cold stress and then re-entry upon recovery is modulated and controlled via the balance of p53 and Grp75 levels.

Finally, our electron microscopy studies and Oil Red O staining show the presence of lipid-containing vesicle-type structures under conditions of severe cold stress. These vesicle-like structures may be the result of lipid material being secreted from the cell, or alternatively, these vesicles may only be observed under severe cold stress because the membrane rigidity and/or membrane-associated cell functions are so severely compromised at very low temperatures that this results in the arrest of the vesicles before secretion, whereas at higher temperatures these are secreted efficiently and hence not observed. It is well known that cold stress results in membrane rearrangements [40], and changes in cellular lipids have been linked to the heat shock response in yeast [41]. More recent research has shown that changes in the lipid composition of the cell membrane induce the phosphorylation of p53 by the ataxia–telangiectasia and Rad-3 related kinase [42], and we are now investigating whether cold stress-induced cell cycle arrest is due to this ataxia–telangiectasia and Rad-3 related kinase activation of the p53–p21 signalling pathway.

In conclusion, we have here identified a number of mechanisms involved in the response of *in vitro* cultured mammalian cells to mild and severe cold stress, and in recovery from such stress. In addition to a global decrease in mRNA and protein turnover, the synthesis of specific proteins involved in regulating cell growth, proliferation and mRNA translation are upregulated or downregulated during cold stress and recovery. Furthermore, changes in the lipid composition of the cell may underpin these responses, especially upon severe cold stress. On the basis of the results presented here, we suggest that the cytoskeleton, and the balance in the levels of p53, Grp75 and eIF3i, are likely to be of particular importance during the response to, and recovery from, cold stress that allows mammalian cells to survive and recover from low-temperature stress.

Experimental procedures

Cell lines, routine culture conditions, and treatment conditions

CHOK1 cells were sourced from the European Collection of Cell Cultures and P19 cells from P. Andrews, University of Sheffield, UK. Cells were routinely cultured in DMEM/F12 (Invitrogen, Paisley, UK) supplemented with 200 mM L-glutamine, 500 μM glutamic acid, 500 μM asparagine, 30 μM adenosine, 30 μM guanosine, 30 μM cytidine, 30 μM uridine, 10 μM thymidine, 1% nonessential amino acids (Invitrogen, Paisley, UK), and 10% (v/v) heat-inactivated fetal bovine serum (PAA Laboratories Ltd, Yeovil, UK) at 37 °C in a 5% CO_2 atmosphere. NIH 3T3 cells were also sourced from the European Collection of Cell Cultures and maintained as above, except that DMEM was used in place of DMEM/F12. For radiolabelling, the routine maintenance media were replaced with cysteine/methionine-deficient DMEM (Sigma-Aldrich, Poole, UK) supplemented with 10% (v/v) dialysed, heat-inactivated fetal bovine serum, 2 mM glutamine and 1770 kBq·mL^{-1} Pro-Mix L-[^{35}S] cell labelling mix (GE Healthcare, Chalfont St Giles, UK), and then incubated for 1 h at the indicated temperature. Uptake and incorporation of the ^{35}S-labelled amino acids was as previously described [43]. Cold shock was undertaken in routine medium for 6 h or 30 h at 4, 10, 20, 27 and 32 °C in appropriately regulated incubators. Heat shock was also undertaken in the routine culture medium for 1 h by flotation in a water bath at 43 °C. Treatment of cells with the antimicrotubule drug nocodazole was performed in routine medium at 1–3 μg·mL^{-1} for 2 h at 37 °C. Recovery incubations were undertaken in routine culture medium at 37 °C for 0.5, 1.5 and 5 h. For the determination of mRNA half-lives, cells were incubated in routine culture medium containing 2 μg·mL^{-1} actinomycin D. For protein half-life determinations, cells were incubated in routine culture medium containing 50 μg·mL^{-1} cycloheximide.

Extraction of RNA and protein from cell pellets

Total RNA was prepared from intact cells using the commercially available RNeasy kit (Qiagen). Cell extracts for protein analyses were prepared by lysing cells into ice-cold extraction buffer [20 mM Hepes/NaOH, pH 7.2, containing 100 mM NaCl, 1% (w/v) Triton X-100, protease inhibitors (10 μL·mL^{-1} leupeptin, 2 μg·mL^{-1} pepstatin, 0.2 mM phen-

ylmethanesulfonyl fluoride) and protein phosphatase inhibitors (50 mM NaF, 1 mM activated Na_3VO_4)]. Cell lysates were then centrifuged at 16 000 ***g*** for 2 min at 4 °C, and the resulting supernatants were retained for further analysis. For the determination and detection of HSF1 trimer formation levels, cell extracts were cross-linked with ethylene glycol *bis*(succinimidylsuccinate) (Sigma) at room temperature for 30 min, and then blocked with 50 mM Tris/HCl (pH 7.5) at room temperature for 15 min.

Gel electrophoresis analysis of protein extracts

For SDS/PAGE analysis, 10% separation gels were utilized according to the procedure of Laemmli [44]. Prior to NEPHGE-SDS/PAGE, the proteins in cell extracts were precipitated overnight with four volumes of acetone at −20 °C. Following NEPHGE-SDS/PAGE, resolved proteins were detected by Coomassie staining and/or autoradiography using Hyperfilm MP (GE). Gel images were analysed using the commercially available PROGENESIS PG200 software package (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) to determine spots that had changed in abundance. Spot detection was undertaken using the spot detection wizard with the parameters set as follows: minimum spot area, 16; split factor, 7; peak location, use centre of mass as peak. Manual splitting of nonsplit spots and deletion of noise were then undertaken. Following spot detection, background subtraction was achieved using the mode of nonspot option with a margin of 45. In-gel tryptic digestion of excised spots and protein identification by MALDI-TOF MS were undertaken according to Smales *et al.* [45]. Analysis was undertaken on triplicate biological samples, and only spots whose abundance was changed at the 95% confidence level ($P < 0.05$) relative to the 37 °C control were considered to show significant changes in polypeptide synthesis rates.

Determination of mRNA levels by qRT-PCR

qRT-PCR was used to determine the relative mRNA levels of target genes using the commercially available BioRad iScript qRT-PCR kit according to the manufacturer's instructions, and the appropriate primers to amplify CHO sequences as listed in Table 2. All reactions were performed using a BioRad DNA Engine Chromo4 Continuous Fluorescence Detector thermocycler (BioRad, Hemel Hempstead, UK). Cycling conditions included a reverse transcription step by incubation at 50 °C for 20 min, followed by heating at 95 °C for 15 min. Sequentially, the target templates were amplified using 39 cycles (30 s at 98 °C, 15 s at 55 °C). The fluorescence threshold value (C_t) was calculated using OPTICON MONITOR software (version 3.1; BioRad). For normalization purposes, all levels were normalized to control levels at 37 °C.

Table 2. Primers used for qRT-PCR experiments described in this article.

Name	Sequence (5′- to -3′)
Hsc73F	CGACAAGAAGGACATCAGCGAG
Hsc73R	GAATCGAGCACGGGTAATGGAG
Hsp60F	TGCTCATCGTAAGCCCTTGGTC
Hsp60R	TTCTCCAAACACCGCACCAC
ERp57F	AACTACAGATTTGCACACACC
ERp57R	CAGTATATACCACAGTTTTGTC

Immunoblot analysis

PAGE-resolved polypeptides were transferred to nitrocellulose using standard procedures, and then blocked with 5% (w/v) nonfat milk in NaCl/P_i or for phosphorylation-dependent epitopes in 0.2% (w/v) Tween-20. Antibody probes against α-tubulin (TAT) and β-tubulin (KMX) [46] were gifts from K. Gull (University of Oxford, UK), and the antibody 23c against STOP [13] was a gift from C. Bosc and D. Job (Commisariat A L'Energie Atomique, Grenoble, France). Affinity-purified rabbit polyclonal antibodies against the C-termini of CCT subunits and against Hsc70 were as described elsewhere [47]. Commercial antibodies against Grp75 (clone 30A5), Hsp60 (clone LK-2) and HSF1 (rabbit polyclonal) were from Stressgen, antibody against p53 (clone DO-7) was from Dako, and antibody against p21 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibodies were detected by enhanced chemiluminescence using Hyperfilm ECL (GE). Images were analysed using KODAK GEL LOGIC 100 imaging system software. The linearity of antibody response over the concentration range of target protein used is shown in Fig. S4.

Immunofluorescence microscopy

For immunofluorescence microscopy studies, cells were grown on 13 mm glass coverslips and then fixed with methanol at −20 °C for 5 min, or with 4% (w/v) paraformaldehyde in NaCl/P_i; this was followed by permeabilization with 0.1% (w/v) Triton X-100 in NaCl/P_i. Cells were rehydrated after methanol fixation for 5 min in NaCl/P_i. All coverslips were then blocked for 15–30 min in 3% (w/v) BSA in NaCl/P_i. Incubation with primary antibodies diluted in blocking solution (TAT, 1 : 100; anti-hsp60, 1 : 100) was performed overnight at 4 °C. The appropriate secondary antibodies (anti-mouse tetramethyl rhodamine iso-thiocyanate; Sigma) were diluted 1 : 100 before use. F-actin staining with rhodamine–phalloidin (Molecular Probes, Invitrogen, Paisley, UK) was achieved according to the manufacturer's instructions. Cells were counterstained with 4′,6-diamidino-2-phenylindole, and coverslips were then mounted in

Mowiol containing *p*-phenylenediamine as antifade. Cells were then examined under a Leica DMR fluorescence microscope, and images were captured with a Leica DC300F digital camera.

Electron microscopy

For electron microscopy cells, were grown at 37 °C or in the cold as described, and then fixed with 2.5% glutaraldehyde in NaCl/P_i, postfixed with 1% osmium tetroxide, and dehydrated with a graded series of alcohols. After two changes of 100% ethanol, they were detached from the flasks by agitation in ethoxypropane, and then embedded in Agar Low Viscosity Resin. Sections were cut at 60–90 nm, stained with uranyl acetate and lead citrate, and examined in a Jeol 1230 transmission electron microscope (Jeol UK, Welwyn Garden City, UK) operating at 80 kV. Images were recorded with a Gatan Multiscan 600CW camera (Gatan UK, Oxford, UK).

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Supporting information

The following supplementary material is available:

Fig. S1. The small vesicles associated with severely cold-stressed cells do not stain for protein or RNA but do stain for uncharged lipid.

Fig. S2. (A) Mild hypothermia does not initiate apoptosis in CHOK1 or P19 cells. (B) Mild hypothermia is not associated with acetylation of p53.

Fig. S3. Protein synthesis in P19 and 3T3 cells exposed to, and recovering from, cold stress.

Fig. S4. Antibody response to a concentration range of proteins identified by immunoblot.

This supplementary material can be found in the online version of this article.

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BIOCHEMICAL INSIGHTS INTO THE MECHANISMS CENTRAL TO THE RESPONSE OF MAMMALIAN CELLS TO COLD-STRESS AND SUBSEQUENT REWARMING

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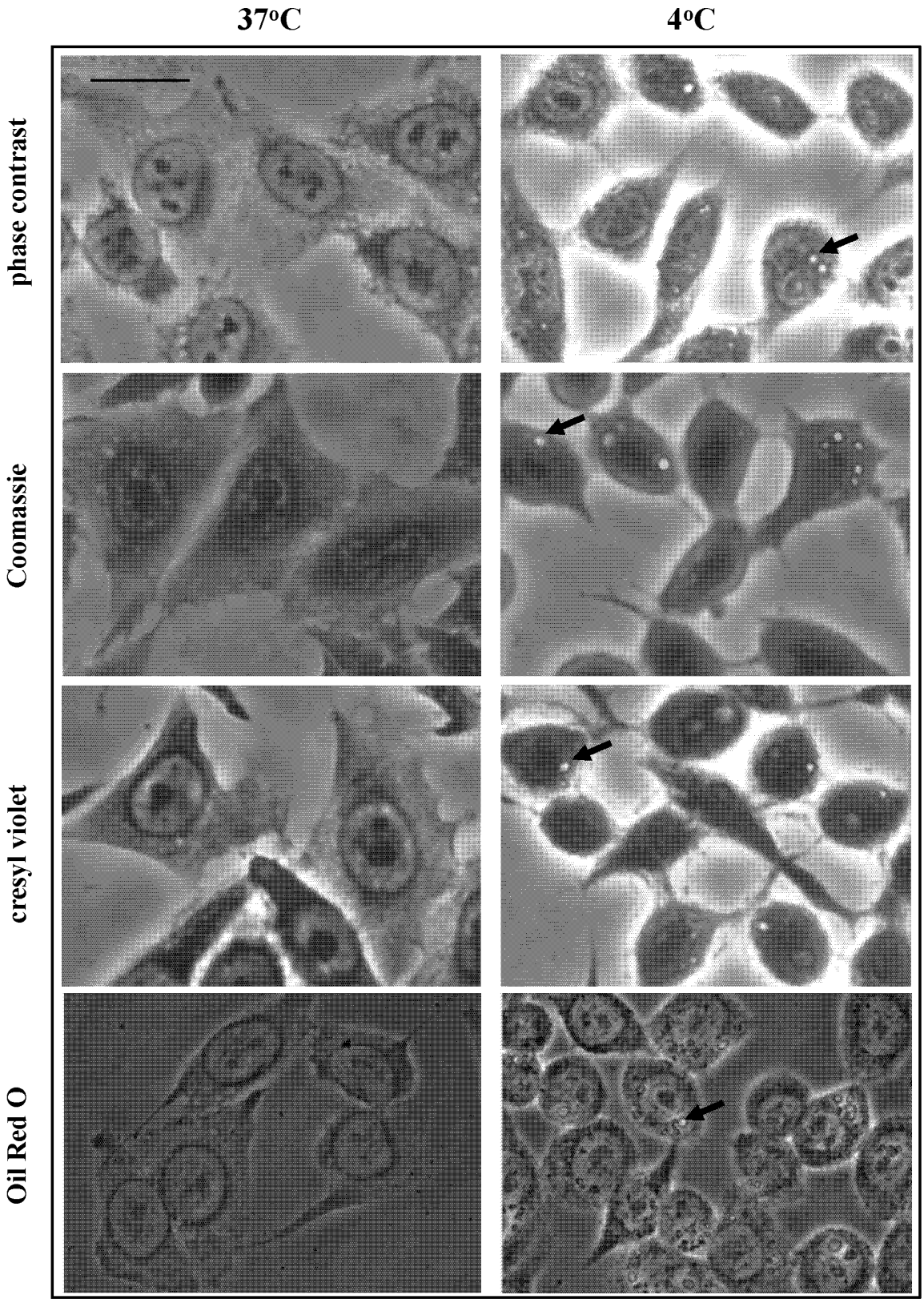
Titles of Supplementary Material

Supplementary FIGURE S1. The small vesicles associated with severely cold-stressed cells do not stain for protein or RNA but do stain for uncharged lipid. CHOK1 cells, growing on 13 mm glass coverslips, were maintained at 37°C or transferred to 4°C for 6 h prior to fixation with -20°C methanol, then left unstained (phase contrast) or stained with 0.1% (w/v) Coomassie blue G250 in 50% methanol-10% acetic acid, or with 0.5% (w/v) cresyl violet acetate in H_2O, both for 2 min or with a saturated solution of Oil Red O in 60% (v/v) isopropanol for 10 min, before washing with phosphate buffered saline or, in the case of Oil Red O, first with 60% isopropanol and then with water. Arrows point to the vesicles observed at 4°C. Bar = 10 μm.

Supplementary FIGURE S2. (A) Mild hypothermia does not initiate apoptosis in CHOK1 or P19 cells. Cells were maintained at 37°C for 24 h then transferred to the indicated temperatures. At the indicated times, cells were washed once with PBS then lysed into SDS-PAGE sample buffer. Lysates were passaged 6 times through a 19 gauge syringe needle then held at 100°C for 2 min. Immunoblots of 20 μg samples resolved over 10% resolution gels were probed with anti-PARP rabbit polyclonal (Stressgen AAP-250). Note: Early apoptosis is characterized by the appearance of an 85 kDa cleavage product of PARP which is absent in the blots. **(B) Mild hypothermia is not associated with acetylation of p53.** Immunoblots of cells treated as in (A), or extracted into a buffer containing the deacetylase inhibitors trichostatin A (1 μM) and nicotinamide (5 mM, not shown) were probed for acetylated p53 (Cell Signaling #2570). Note: Increased acetylation of p53 was detected as cells became confluent as exemplified by the later time points for cells held at 37°C and 32°C.

Supplementary FIGURE S3. Protein synthesis in P19 and 3T3 cells exposed to, and recovering from, cold-stress. Cells maintained at 37°C, or then exposed to the indicated temperatures for the indicated times, were radiolabelled in a methionine-cysteine deficient growth medium supplemented with 1770 kBq/ml [^{35}S] methionine-cysteine cell labelling mix for 1 further hour at the indicated temperature. 30 μg of extracted proteins were resolved by SDS-PAGE and detected by autoradiography. MW markers were 205, 150, 100, 75, 50, 37, 25, 20 and 15 kDa.

Supplementary FIGURE S4. Antibody response to a concentration range of proteins identified by immunoblot. (A) 30 – 1 μg of proteins extracted from CHOK1 cells were resolved by SDS-PAGE and detected by immunoblot with the indicated antibodies. (B) shows the densitometric analysis of the data in (A).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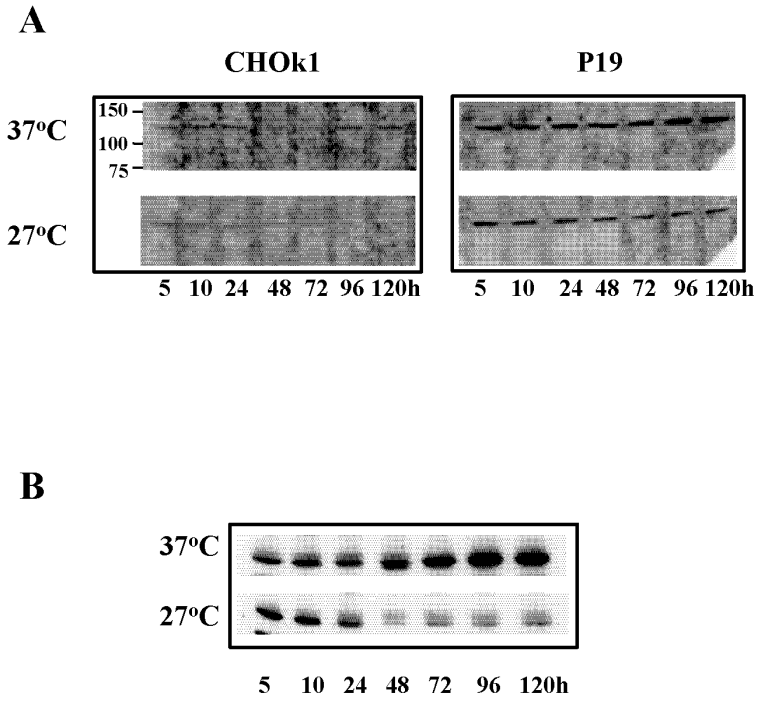
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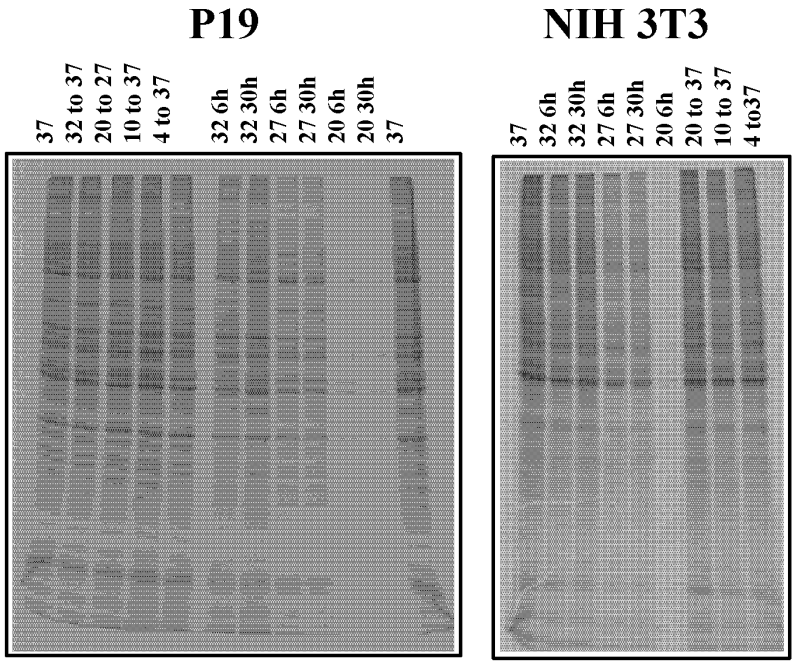
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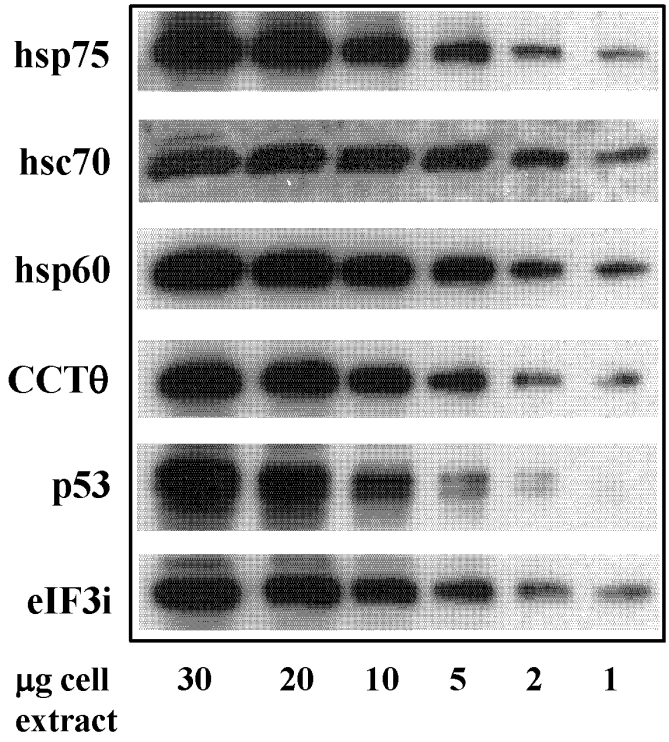
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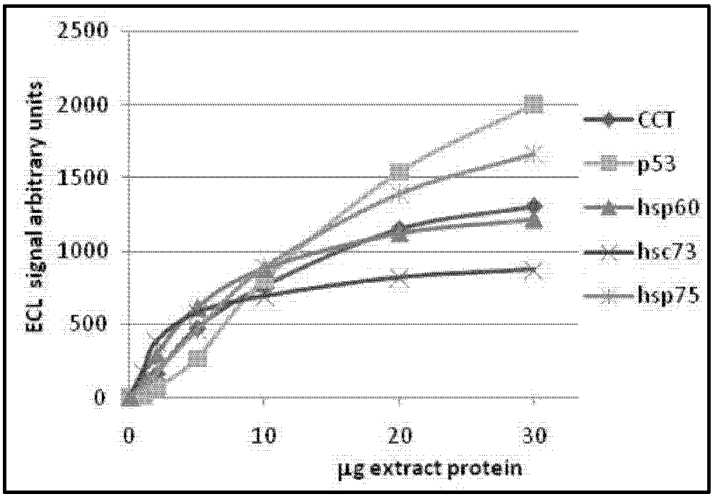
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On the cover: Representation of a fullerene molecule with a noble gas atom trapped inside. At the Permian-Triassic sedimentary boundary the noble gases helium and argon have been found trapped inside fullerenes. They exhibit isotope ratios quite similar to those found in meterorites, suggesting that a fireball meteorite or asteroid exploded when it hit the Earth, causing major changes in the environment. ***(Image copyright © Dr. Luann Becker. Reproduced with permission.)***

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FENNEL

Fennel (*Foeniculum vulgare*). (*USDA*)

fenestrated membrane [HISTOL] One of the layers of elastic tissue in the tunica media and tunica intima of large arteries. { 'fen·ə,strād·əd 'mem,brān }

fenestration [ARCH] The arrangement of openings, especially windows, in the wall of a building. [BIOL] **1.** A transparent or windowlike break or opening in the surface. **2.** The presence of windowlike openings. { ,fen·ə'strā·shən }

fenitrothion [ORG CHEM] $C_9H_{12}NO_5PS$ A yellow-brown liquid, insoluble in water; used as a miticide and insecticide for rice, orchards, vegetables, cereals, and cotton, and for fly and mosquito control. { ,fen·ə·trō'thī,ān }

fennel [BOT] *Foeniculum vulgare*. A tall perennial herb of the family Umbelliferae; a spice is derived from the fruit. { 'fen·əl }

fennel oil [MATER] The essential oil obtained from fennel; a colorless liquid with aromatic scent and bitter taste, insoluble in water and boiling at 160–220°C; used in medicine, perfumes, and liqueurs. Also known as oil of fennel. { 'fen·əl ,ȯil }

fen peat *See* low-moor peat. { 'fen ,pēt }

Fenske equation *See* Fenske-Underwood equation. { 'fen·skē i,kwā·zhən }

Fenske-Underwood equation [CHEM ENG] Equation in plate-to-plate distillation-column calculations relating the number of theoretical plates needed at total reflux to overall relative volatility and the liquid-vapor composition ratios on upper and lower plates. Also known as Fenske equation. { ¦fen·skē 'ən·dər,wůd i,kwā·zhən }

fenster *See* window. { 'fen·stər }

fensulfothion [ORG CHEM] $C_{11}H_{17}S_2O_2P$ A brown liquid with a boiling point of 138–141°C; used as an insecticide and nematicide in soils. { ,fen,səl·fō'thī,ān }

fentinacetate [ORG CHEM] $C_{20}H_{18}O_2Sn$ A yellow to brown, crystalline solid that melts at 124–125°C; used as a fungicide, molluscicide, and algicide for early and late blight on potatoes, sugarbeets, peanuts, and coffee. Also known as triphenyltinacetate. { ,fent·ən'as·ə,tāt }

fenuron [ORG CHEM] $C_9H_{12}N_2O$ A white, crystalline compound with a melting point of 133–134°C; soluble in water; used as a herbicide to kill weeds and bushes. { ,fen'yů,rān }

fenuron-TCA [ORG CHEM] $C_{11}H_{13}Cl_3N_2O_3$ A white, crystalline compound with a melting point of 65–68°C; moderately soluble in water; used as a herbicide for noncrop areas. { ,fen'yů,rān ¦tē¦sē¦ā }

FEP resin *See* fluorinated ethylene propylene resin. { ¦ef¦ē¦pē 'rez·ən }

ferbam [ORG CHEM] $C_9H_{18}FeN_3S_6$ [iron(III) dimethyldithiocarbamate] A fungicide for protecting fruits, vegetables, melons, and ornamental plants. { 'fər·bəm }

ferberite [MINERAL] $FeNO_4$ A black mineral of the wolframite solid-solution series occurring as monoclinic, prismatic crystals and having a submetallic luster; hardness is 4.5 on Mohs scale, and specific gravity is 7.5. { 'fər·bə,rīt }

ferghanite [MINERAL] $U_3(VO_4)_2·6H_2O$ Sulfur-yellow mineral composed of hydrated uranium vanadate, occurring in scales. { fər'gä,nīt }

fergusonite [MINERAL] $Y_2O_3·(Nb,Ta)_2O_5$ Brownish-black rare-earth mineral with a tetragonal crystal form; it is isomorphous with formanite. { 'fər·gə·sə,nīt }

Fermat numbers [MATH] The numbers of the form $F_n = (2^{(2^n)}) + 1$ for $n = 0, 1, 2, \ldots$. { 'fer·mä ,nəm·bərz }

Fermat's last theorem [MATH] The proposition, proven in 1995, that there are no positive integer solutions of the equation $x^n + y^n = z^n$ for $n \geq 3$. { fer'mäz ¦last 'thir·əm }

Fermat's principle [OPTICS] The principle that an electromagnetic wave will take a path that involves the least travel time when propagating between two points. Also known as least-time principle; stationary time principle. { fer'mäz 'prin·sə·pəl }

Fermat's spiral [MATH] A plane curve whose equation in polar coordinates (r,θ) is $r^2=a^2\theta$, where a is a constant. { fer'mäz ,spī·rəl }

Fermat's theorem [MATH] The proposition that, if p is a prime number and a is a positive integer which is not divisible by p, then $a^{p-1} - 1$ is divisible by p. { 'fer,mäz ,thir·əm }

ferment [BIOCHEM] An agent that can initiate fermentation and other metabolic processes. { ¦fər¦ment }

fermentation [MICROBIO] An enzymatic transformation of organic substrates, especially carbohydrates, generally accompanied by the evolution of gas; a physiological counterpart of oxidation, permitting certain organisms to live and grow in the absence of air; used in various industrial processes for the manufacture of products such as alcohols, acids, and cheese by the action of yeasts, molds, and bacteria; alcoholic fermentation is the best-known example. Also known as zymosis. { ,fər·mən'tā·shən }

fermentation accelerator [MATER] Substance that speeds chemical fermentation (as for wines) without participating in the resulting chemical changes; can be an enzyme or other catalytic agent. { ,fər·mən'tā·shən ak'sel·ə,rād·ər }

fermentation tube [MICROBIO] A culture tube with a vertical closed arm to collect gas formed in a broth culture by microorganisms. { ,fər·mən'tā·shən ,tüb }

fermenter [FOOD ENG] A vessel used for fermenting, such as a vat for fermenting mash in brewing. { fər'ment·ər }

ferment oil [MATER] A volatile oil formed by the fermentation of plant material in which the oil was not present originally. { ¦fər¦ment ,ȯil }

fermi *See* femtometer. { 'fer·mē }

Fermi age [NUCLEO] The value calculated for the slowing-down area in the Fermi age model; it has the dimensions of area, not time. Also known as age; neutron age; symbolic age of neutrons. { 'fer·mē ,āj }

Fermi age equation [NUCLEO] An equation in the Fermi age model which states that the Laplacian of the slowing-down density equals the partial derivative of the slowing-down density with respect to the Fermi age. { 'fer·mē ¦āj i,kwā·zhən }

Fermi age model [NUCLEO] A model used in studying the slowing down of neutrons by elastic collisions; it is assumed that the slowing down takes place by a very large number of very small energy changes. { 'fer·mē ,āj ,mäd·əl }

Fermi beta-decay theory [NUC PHYS] Theory in which a nucleon source current interacts with an electron-neutrino field to produce beta decay, in a manner analogous to the interaction of an electric current with an electromagnetic field during the emission of a photon of electromagnetic radiation. { 'fer·mē ¦bād·ə di¦kā ,thē·ə·rē }

Fermi constant [NUC PHYS] A universal constant, introduced in beta-disintegration theory, that expresses the strength of the interaction between the transforming nucleon and the electron-neutrino field. { 'fer·mē ,kän·stənt }

Fermi derivative [RELAT] A generalization of covariant differentiation along a curve that reduces to covariant differentiation when the curve is geodesic; an orthonormal tetrad constructed at each point along a timelike curve such that the Fermi derivative of the tetrad along the curve is zero has (1) its timelike basis vector equal to the curve's unit tangent vector and (2) its spatial basis vectors nonrotating along the curve. { 'fer·mē də,riv·əd·iv }

Fermi-Dirac distribution function [STAT MECH] A function specifying the probability that a member of an assembly of independent fermions, such as electrons in a semiconductor or metal, will occupy a certain energy state when thermal equilibrium exists. { ¦fer·mē di¦rak ,dis·trə'byü·shən ,fəŋk·shən }

Fermi-Dirac gas *See* Fermi gas. { ¦fer·mē di¦rak ,gas }

Fermi-Dirac statistics [STAT MECH] The statistics of an assembly of identical half-integer spin particles; such particles have wave functions antisymmetrical with respect to particle interchange and satisfy the Pauli exclusion principle. { ¦fer·mē di¦rak stə'tis·tiks }

Fermi distribution [SOLID STATE] Distribution of energies of electrons in a semiconductor or metal as given by the Fermi-Dirac distribution function; nearly all energy levels below the Fermi level are filled, and nearly all above this level are empty. { 'fer·mē ,dis·trə,byü·shən }

Fermi energy [STAT MECH] **1.** The average energy of electrons in a metal, equal to three-fifths of the Fermi level. **2.** *See* Fermi level. { 'fer·mē ,en·ər·jē }

Fermi gas [STAT MECH] An assembly of independent particles that obey Fermi-Dirac statistics, and therefore obey the Pauli exclusion principle; this concept is used in the free-electron theory of metals and in one model of the behavior of the nucleons in a nucleus. Also known as Fermi-Dirac gas. { 'fer·mē ,gas }

Fermi hole [SOLID STATE] A region surrounding an electron in a solid in which the energy band theory predicts that the

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Date: February 6, 2019
Case: Genentech, Inc., et al. -v- Amgen, Inc.

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IN THE UNITED STATES DISTRICT COURT

FOR THE DISTRICT OF DELAWARE

- - - - - - - - - - - - - - - - x

GENENTECH, INC., and :

CITY OF HOPE, :

Plaintiffs, : CA No. 17-1407-CFC

v. : (CONSOLIDATED)

AMGEN, INC., :

Defendant. :

- - - - - - - - - - - - - - - - x

Videotaped Deposition of JEFFREY JOHN CHALMERS, PhD

Chicago, Illinois

Wednesday, February 6, 2019

8:36 a.m.

Job No.: 227140

Pages: 1 - 278

Reported by: Melanie L. Humphrey-Sonntag,

CSR, RDR, CRR, CRC, FAPR

Videotaped deposition of JEFFREY JOHN CHALMERS, PhD, held at the location of:

PROSKAUER ROSE, LLP
70 West Madison Street
Suite 3800
Chicago, Illinois 60602
(312) 962-3550

Pursuant to notice before Melanie L. Humphrey-Sonntag, a Certified Shorthand Reporter, Registered Diplomate Reporter, Certified Realtime Reporter, and a Notary Public in and for the State of Illinois.

"following fermentation" but -- yeah, you're asking me to ask what one word means. It's taken out of context. 08:54:45 08:54:46 08:54:49

Q So let's talk about the second term, "fermentation," and I would direct you to paragraph 48 of your declaration. 08:54:50 08:54:54 08:54:56

A Yes. 08:54:56

Q You state that, quote, "In the -- the ordinary meaning of 'fermentation' relates to activities such as making beer or wine, in which there is a chemical breakdown of a substance, e.g. sugar, usually under anaerobic conditions, by bacteria or yeast." 08:55:01 08:55:04 08:55:08 08:55:10 08:55:14 08:55:18

Did I read that correctly? 08:55:19

A Yes. 08:55:20

Q Is it your understanding that the Kao patent relates to a process for making beer or wine? 08:55:20 08:55:22 08:55:24

A It does not. 08:55:24

Q Then why did you believe that was the definition that was appropriate to apply in coming up with your opinion in this case? 08:55:25 08:55:28 08:55:30

MR. GUTMAN: Objection; mischaracterizes his declaration, mischaracterizes his testimony. 08:55:32 08:55:33

A The word "fermentation" is traditional, 08:55:38

referring to the metabolic -- not just this but if 08:55:44
you want to start with -- let's start -- getting 08:55:48
down further, it has "metabolic breakdown." It 08:55:50
refers to the metabolism from an engineering 08:55:52
biological point of view, so it's -- it's talking 08:55:56
about that whole process. 08:56:02
And "fermentation" can be used -- to this 08:56:04
day it's -- it's referred to in making biofuels. 08:56:07
THE COURT REPORTER: I'm sorry. 08:56:07
"Making" -- 08:56:07
THE WITNESS: "Biofuels," alternative 08:56:16
energy. 08:56:18
A (Continuing.) People will quite often use 08:56:18
the word "fermentation," but even then they get a 08:56:19
little iffy on what you're calling it. 08:56:24
So it is a firm and fast concept among our 08:56:26
community, and it's considered sloppy to call -- 08:56:30
to even use the term "cell" -- "fermentation" in 08:56:34
the cell culture world. It's like you're -- "Why 08:56:37
are you doing that? Call it 'cell culture.'" 08:56:41
Q In the context of making beer and wine, 08:56:44
what is the process of fermentation, just briefly? 08:56:53
A I completely stand by what's here. But 08:56:55
then to go the next step, which I think you're 08:57:03
asking -- so I want to be on record that I agree 08:57:05

with what's here.
And then go the step further, in the traditional sense, it's in an anaerobic environment. The sugars are broken down; the organism gets its energy from that. The waste products, instead of us, as humans, making -- just using -- taking oxygen and making CO2, the organisms are dumping electrons onto molecules that then become ethanol, lactic acid, acetic acid, all array of different compounds.
When we go out and exercise, don't get enough oxygen in our body and our muscles ache, that's anaerobic fermentation going on in our body. That is -- they're making lactic acid.
Q So if I could simplify, it's the process of the cells taking sugars and producing other chemicals with them?
MR. GUTMAN: Objection; mischaracterizes his testimony, asked and answered.
A Not other specific chemicals, very specific chemicals.
Q So it's a process of cells breaking down sugars and producing specific chemicals?
A That's right.
Q Okay.

A Very specific. And the reason I say that, it's the dream of many of my colleagues to be able to do it better because we could solve our energy problems. 08:58:15 08:58:17 08:58:21 08:58:24

So there's very specific chemicals these organisms make, and we'd like them to be certain kinds of fuels. So it's not a random thing at all. It's very specific. 08:58:26 08:58:29 08:58:33 08:58:35

Q And while the cells are producing these specific chemicals, are they growing? 08:58:38 08:58:40

A Yes, almost always. 08:58:43

There could be a few exceptions, but it's very much considered classified in my community as a growth-associated product. 08:58:45 08:58:47 08:58:50

Q So in -- in the context of "fermentation" as discussed in paragraph 48 of your declaration, cells are both growing and producing; is that right? 08:58:54 08:58:56 08:59:00 08:59:02

A That is correct. 08:59:03

MR. GUTMAN: Objection; vague, lacks foundation. 08:59:04 08:59:07

Q Does the term "fermentation" have any meaning in the context of antibody production? 08:59:09 08:59:11

A In a sloppy manner. It should be saying "cell culture" because, bringing this back, you're 08:59:14 08:59:20

growing the cells -- these are genetically engineered cells to produce a specific product. That engineering is what makes the antibody. 08:59:29 08:59:31 08:59:33

Normal cells that make beer and wine or make yeast don't make antibodies. 08:59:38 08:59:41

Q So I think I've understood your testimony to be that the term "fermentation" does have a meaning in the context of antibody production but it's a sloppy meaning. Is that right? 08:59:46 08:59:48 08:59:50 08:59:52

MR. GUTMAN: Objection; mischaracterizes his testimony, asked and answered. 08:59:54 08:59:55

A If you get into the very -- again, the fundamental definition of "fermentation" is as I wrote in here. It is the process anaerobically by which sugars are converted to ethanol, lactic acid, acetic acid, butanol, and it's a process by which energy is generated by the organisms. 08:59:57 09:00:01 09:00:05 09:00:09 09:00:16 09:00:20

In an antibody production of CHO cells, they are not getting any energy by making antibodies, none, zero. In fact, that's a drain of energy. So it's a fundamentally different metabolic process. Fundamentally different, not comparable at all. 09:00:24 09:00:28 09:00:30 09:00:33 09:00:35 09:00:38

Q And let me ask my question a different way. 09:00:39 09:00:41

If you could look -- I believe it's the second sentence of paragraph 48 of your declaration. You say, "'Fermentation' is not typically used to refer to culturing mammalian cells." 09:00:41 09:00:44 09:00:46 09:00:49 09:00:52

Do you see that? 09:00:53

A Yes. 09:00:54

Q I want to ask you a couple questions about that statement. 09:00:56 09:00:59

Is it your understanding that if a term has multiple meanings, the person of ordinary skill has to determine which one is most common to figure out the ordinary meaning? 09:01:00 09:01:01 09:01:04 09:01:07

MR. GUTMAN: Objection; calls for a legal conclusion, foundation. 09:01:08 09:01:10

A As someone skilled in the art, we're immediately going to go, "Why is that -- why are they using the word 'fermentation'"? 09:01:13 09:01:21 09:01:24

If read the patent -- which I did, as we discussed at the beginning -- my first question was, "Why are they saying 'fermentation'? Why are they saying 'fermentation' in the claim?" Doesn't make sense to me. It adds indefiniteness, as I keep coming back to that concept. It made it indefinite to me. 09:01:27 09:01:29 09:01:32 09:01:35 09:01:39 09:01:42 09:01:46

But if you push me hard enough, I'm going to have to say, "All right. They have -- they have not defined it in the patent, but they do have a clear statement saying, 'Following fermentation proteins are purified.'" 09:01:47 09:01:49 09:01:51 09:01:54 09:01:56

So now if I also go back to the prosecution history -- in fact, you could make the argument that at least one of the papers that the examiner pulled was definitely much more in the tradition of fermentation. And for Genentech to get their claim, they had to associate fermentation with cell growth and the production which was going on in those vessels. And so Genentech then said "Okay. We're going to use the word 'following fermentation' to -- to construct our claim around." And that meant after cell growth. 09:02:00 09:02:05 09:02:10 09:02:13 09:02:18 09:02:25 09:02:27 09:02:32 09:02:36 09:02:39 09:02:41 09:02:44

So you just asked me a minute ago "fermentation." That right there -- if I look at the whole prosecution history, they're locking fermentation into this more traditional view of a metabolic process of taking a sugar or some compound and breaking down, getting energy from. 09:02:44 09:02:48 09:02:53 09:02:54 09:02:57 09:03:00

CHO cells that are used -- and in Claim 10 of the patent we've got potential prokaryotes. 09:03:06 09:03:10

It's not -- the patent is not just about animal cells. Those prokaryote cells, when they make antibody, they are using energy from sugar, kicking off CO2. They're not making ethanol. They're -- you do not want ethanol when you're doing those kind of cultures. You need oxygen. 09:03:14 09:03:17 09:03:20 09:03:24 09:03:27 09:03:28

So, again, when I read it, I go, "Okay. The fermentation here sounds like they're trying to link it to the cell growth, that production, but definitely not making -- they're -- they're definitely not making ethanol. They're not making a product where they're dumping their electrons onto it." 09:03:31 09:03:34 09:03:38 09:03:43 09:03:45 09:03:47 09:03:49

Q Thank you. 09:03:51

I noted in the sentence we just read that you use the word "typically." You said "Fermentation is not typically used to refer to culturing mammalian cells"? 09:03:51 09:03:53 09:03:54 09:03:58

A Yeah. 09:03:58

Q So I take it that it's fair to say that "fermentation" sometimes is used to refer to culture in mammalian cells. 09:03:59 09:04:02 09:04:03

MR. GUTMAN: Objection. Is -- is that a question or -- 09:04:04 09:04:06

Q Is that correct? 09:04:07

MR. GUTMAN: -- or testimony? 09:04:07

A Sometimes. Sometimes it's used. 09:04:08

That's where I -- when I'm using the word 09:04:14
"sloppy" -- again, my community is not using -- 09:04:16
words are precise to us but not in the same legal 09:04:23
sense. And I'm not a lawyer. 09:04:26

But we turn our nose up at that. We would 09:04:31
say, "That's sloppy. You shouldn't say that 09:04:34
because it's misleading. It leads you somewhere 09:04:37
else." 09:04:39

I have a PhD student currently who -- who 09:04:40
is working with a contract manufacturing 09:04:42
organization that makes -- does biosimilar cells 09:04:46
and other things. I was recently there and I -- 09:04:51
they showed me their process, and I asked them, 09:04:54
"Do you use the word 'fermentation'?" And he just 09:04:58
turned his nose up, just as I'm saying. No, we 09:05:02
would not call it that. It's a cell culture 09:05:05
process. 09:05:07

Q What are some of the times that 09:05:07
"fermentation" is used to refer to culturing 09:05:10
mammalian cells? 09:05:12

A Obviously, this patent, '69, does. That's 09:05:13
why we're talking about it. 09:05:17

Q Other than this patent have you ever heard 09:05:18

the term "fermentation" used for -- for culturing mammalian cells? 09:05:19 09:05:23

A Very rarely. 09:05:25

And the Cell Culture Engineering Award I talk about is, which is highlighted -- 4. 09:05:27 09:05:32

Why I keep highlighting that -- excuse me for my voice cracking -- it is -- this is -- this meeting has been ongoing for 30 years. There's really two meetings, that meeting and a European meeting, where all of us -- most of us that work in this field that do what I am talking about here, we come every two years, and we discuss all -- so much of this. 09:05:37 09:05:39 09:05:44 09:05:47 09:05:55 09:05:57 09:06:00 09:06:03

No one -- I mean, has it happened? Yeah. In 30 years, every two years, I'm sure there's been some. But Cell Culture Engineering -- that's the name of the meeting. We speak of it as cell culture. We don't have sessions on fermentation of CHO cells, no. You wouldn't do it. 09:06:05 09:06:10 09:06:13 09:06:16 09:06:20 09:06:22

So that's why I say "typically." I'd probably say it's rarely done. I'm sure if you looked hard, you could find examples. But it's not typical. 09:06:24 09:06:26 09:06:28 09:06:34

Q But you have never personally used the term "fermentation" to refer to culturing 09:06:34 09:06:37

mammalian cells? 09:06:41

A I might have been sloppy once myself. But 09:06:41

the vast majority of the time I haven't. Yeah, 09:06:43

you can probably find somewhere in 30 years that 09:06:46

I've used that word. Maybe. 09:06:48

Q The Cell Culture Engineering conference 09:06:49

you talked about earlier, are you involved in 09:06:52

running that conference in any respects? 09:06:56

A Yes. 09:06:58

Q How so? 09:06:58

A I chaired the meeting twice in the '90s -- 09:06:59

well, cochaired it -- and I cochaired it 09:07:03

four years ago. 09:07:07

Q What were your responsibilities as chair 09:07:07

of the meeting? 09:07:12

A Picking the session chairs, picking the 09:07:13

location. 09:07:26

We have an advisory board. It's approving 09:07:28

that, in a sense, because, you know, there's a 09:07:32

tradition of how that continues. So we -- we 09:07:34

agree with -- pick the leadership team of the 09:07:40

meeting itself who's overseeing the meeting, like 09:07:42

I said, the location, the price, how much we're 09:07:45

charging. It gets down into the weeds as much as 09:07:46

even menus. 09:07:49

But more -- more globally here with our scientific advisory panel, we'll figure out what's the focus of the meeting, what are the important topics. I think this is really important. It's made up of half academics, half industrialists, and we make a real goal of being in touch with what the issues are. 09:07:50 09:07:55 09:08:00 09:08:02 09:08:04 09:08:08 09:08:13

Q Is this a prestigious conference? 09:08:14

A Yes. 09:08:19

Q Are papers presented at this conference? 09:08:20

A Pretty much so. 09:08:22

Q And I imagine it's hard to get your paper presented at the cell culture conference. 09:08:25 09:08:28

A Very hard. 09:08:30

Q What is the screening process like for papers presented at the cell culture conference? 09:08:31 09:08:33

A Well, to answer your question -- let me -- let's go back for a second. 09:08:36 09:08:38

As of now it's very hard. If you go back into the earlier days, the late '80s, not as hard. Because in the late '80s, it was a dream to see cell culture be the way it is today. The -- the market size was not anticipated. 09:08:40 09:08:43 09:08:49 09:08:58 09:09:00

As the market grew, got bigger and bigger, then it became really prestigious and hard to get 09:09:03 09:09:10

a paper accepted. 09:09:13

So there's a progression. I want to get 09:09:14

that straight. 09:09:16

Q Okay. 09:09:16

A What other -- okay. 09:09:20

And -- so I probably didn't answer your 09:09:22

question. Go back, please. I wanted to back up 09:09:24

for a minute so -- 09:09:26

Q Yeah, I don't think -- I -- I don't think 09:09:27

you did. 09:09:28

A So -- 09:09:28

Q My question was, what is the process for 09:09:29

getting a paper selected at the Cell Culture 09:09:30

Engineering conference? 09:09:34

A As I mentioned, as a chair, as chair of 09:09:35

the meeting, we pick our cochairs. 09:09:38

We, in general, do not -- and I didn't 09:09:41

micromanage it -- so the actual picking of the 09:09:48

papers are the responsibility of the cochairs -- 09:09:50

I mean, the -- sorry -- the session chairs. I've 09:09:55

got to make sure I get the word right. The -- the 09:09:57

session chairs. 09:10:00

So we have -- I'm -- we have cochairs 09:10:01

that oversee it, then we'll have eight, 09:10:04

nine different sessions. We pick the chairs of 09:10:07

the sessions, and those are usually an industrial and an academic. 09:10:13 09:10:16

They -- we then have a call for papers. They bring in people or they invite people as well as we have a call for papers. 09:10:17 09:10:20 09:10:27

They sit down -- "they" being the session chairs -- they decide. Very rare does a meeting chairperson override the choices of the session chairs. That would be a -- it might happen, but that's basically implying you didn't do your job of picking the proper session chairs. 09:10:29 09:10:31 09:10:36 09:10:42 09:10:48 09:10:49

So, you know, it's possible there could be some sloppiness at that point even. 09:10:52 09:10:57

In terms of terms being used, would we override it on that? Probably not unless it was really bad. 09:11:04 09:11:07 09:11:10

And, you know, we basically, after the meeting, get our buddies together -- people we trust -- and we ask, "Okay. Who did a good job running the session? Papers good? Not so good?" 09:11:12 09:11:14 09:11:17 09:11:21

I was just in a meeting last week, did -- we exactly did that. We got aside and said, "Yeah, they shouldn't have invited these people. That was not good." 09:11:26 09:11:29 09:11:32 09:11:35

Q And when you decide that a paper is good, 09:11:36

is there a collection or anything done after the conference of the best papers? 09:11:38 09:11:40

A Yes. Not always. It's less -- it's less common now. 09:11:41 09:11:48

So in the early days there was. However, the journal -- so they would be collected -- we call them proceedings. 09:11:52 09:11:56 09:11:59

And as the years have gone by -- now -- again, now I'm speaking purely as an academic here, which you're expecting of me, but I want to preface that that -- there's been a fundamental change over the years that now people are really worried -- researchers are much more worried about the prestige of the journal. 09:12:01 09:12:05 09:12:08 09:12:11 09:12:13 09:12:16 09:12:18

So nowadays, the most recent decade, the journals -- the collection -- a lot of people elect not to do it, elect not to put a paper that they presented into these journals that are proceedings because these were not considered prestigious. 09:12:20 09:12:26 09:12:29 09:12:32 09:12:36 09:12:37

Q I see. But at the time that you were cochairing the meeting in the '90s, it was still prestigious to have your paper selected for inclusion? 09:12:38 09:12:40 09:12:43 09:12:44

A To be selected for presentation is 09:12:45

prestigious. Even then you'd opt out. If you had a really great paper, you wouldn't put it in the proceedings. You would try to go for a great name journal, which typically has better peer review. 09:12:49 09:12:52 09:12:54 09:12:59

But that's -- we can go down the road of peer review, but that's a different -- I don't think we're here to discuss the merits of peer review. 09:13:02 09:13:05 09:13:08 09:13:09

Q No. I'm sure you could talk all day about the merits of peer review. 09:13:10 09:13:13

A That's exactly right. 09:13:14

Q I'm -- I'm going to hand you what's going to be marked as Exhibit 63. 09:13:16 09:13:18

(Deposition Exhibit 63 marked for identification and attached to the transcript.) 09:13:30 09:13:32

MR. GUTMAN: Thank you. 09:13:32

MR. MC CLOUD: You're welcome. 09:13:32

Do you guys want one? 09:13:34

MS. GETTEL: Thanks. 09:13:36

A Yep, I remember this meeting. 09:13:37

This was one of the ones I hosted. You're right. 09:13:40 09:13:42

BY MR. MC CLOUD: 09:13:42

Q So just so the record is clear, this is an exhibit titled "Cell Culture Engineering VI," and 09:13:45 09:13:48

I take it from the statement you just made this is the proceedings of the conference that you cochaired. Is that right? 09:13:51 09:13:53 09:13:57

A Yes. 09:13:58

Q And I see your name is listed on the cover here. What role did you have in putting together this publication? 09:13:58 09:14:06 09:14:08

A Let me make sure. 09:14:10

This was one of the two meetings that I was cochairing with Rob Arathoon, who was -- I forget his title. He was at Genentech. I don't -- you know, I'd have to go back and check his title. 09:14:14 09:14:20 09:14:29 09:14:32 09:14:34

Q Okay. It says that you were one of the editors of this publication on the cover page. Is that correct? 09:14:35 09:14:37 09:14:39

A Technically Mike Betenbaugh was. We delegated it to Mike Betenbaugh and Alison. So it's the -- how do I want call it? 09:14:39 09:14:44 09:14:49

Protocol, the etiquette at the time would list Rob and I because we ran the meeting. And then these -- the people that were the editors -- as I mentioned before, we delegated it. So we would delegate the authority to that, to the chairs. 09:14:53 09:14:55 09:14:58 09:15:01 09:15:03 09:15:07

Because to be presented here, you had to be a -- to get a -- these are representative of some of the papers that were presented, not all. 09:15:08 09:15:10 09:15:14

These were -- these had -- to be in here, they had to be picked by one of the cochairs -- I mean -- cosession leaders. I keep misspeaking here. 09:15:17 09:15:22 09:15:25 09:15:28

So there's a co- -- there's a chair and cochair on top, then we have session chairs. The session chairs pick the papers that are presented. The papers that are presented have the option to -- to be published in this and get peer reviewed. 09:15:29 09:15:31 09:15:34 09:15:40 09:15:43 09:15:46

And this journal is an example of what I said, that people don't let -- this is not considered that prestigious of a journal anymore so nowadays people don't. 09:15:47 09:15:49 09:15:53 09:15:56

Q So the papers in this publication were selected by the session chairs -- 09:15:58 09:16:01

A That's correct. 09:16:03

Q -- at the conference? 09:16:04

A That's correct. 09:16:05

Q Can you turn to page 19? 09:16:05

A Yes. 09:16:13

Q Are you there? 09:16:20

A Yes. 09:16:20
Q Okay. And you see there's an article 09:16:22
entitled "Effects on Growth Behavior in Continuous 09:16:23
Hybridoma Cell Cultures: The Role of Viral 09:16:26
Contamination"? 09:16:30
Do you see that title? 09:16:31
A "Hybridoma Cell Cultures, the Role of 09:16:32
Viral Contamination," that's correct. 09:16:41
Q What is a hybridoma? 09:16:42
A Hybridoma is a fusion of an antibody- 09:16:45
producing cell with a type of cancer cell that 09:16:52
allows them to be immortalized. 09:16:55
Q And could you turn to page 20, please. 09:16:57
Are you on page 20? 09:17:09
A I am. 09:17:10
Q And you see there's a section entitled 09:17:11
"Materials and Methods"? 09:17:13
Do you see that? 09:17:14
A Yes. 09:17:15
Q And under "Materials and Methods" there's 09:17:15
a subheading for "Cell Cultures"? Do you see 09:17:18
that? 09:17:21
A Yes. 09:17:21
Q Okay. Could you read the first sentence 09:17:22
under the subheading "Cell Cultures" out loud, 09:17:25

please? 09:17:29
A "The cell line used in the present study 09:17:29
was the rat-mouse hybridoma cultured in a 09:17:33
serum-free medium based on DMEM/F12" -- keep 09:17:38
reading? -- "with human transferrin and bovine 09:17:43
insulin. The amino acids were supplemented to 09:17:50
necessary concentrations." 09:17:52
Q Thank you. 09:17:53
A rat-mouse hybridoma, that is a mammalian 09:17:54
cell; is that correct? 09:17:58
A Correct. 09:17:59
Q And I saw a reference there to bovine 09:18:00
insulin. What is insulin? 09:18:03
A A growth hormone -- well, it's not just a 09:18:04
growth hormone. It is a hormone that is involved 09:18:07
in uptake and regulation of sugar, among other 09:18:12
things, in animal cells. 09:18:17
Q And it said "bovine insulin." Is that cow 09:18:18
insulin? 09:18:22
A Correct. 09:18:23
Q Are there different kinds of insulin? 09:18:24
A There is. 09:18:27
Q So if you look further down the page, 09:18:28
you'll see another subheading that says 09:18:30
"Fermentation Process." 09:18:32

Do you see that? 09:18:33
A Yes, I do. 09:18:34
Q And the first sentence under fermentation 09:18:34
process says, "After inoculation the perfusion 09:18:36
fermentation process, Figure 2, was started with a 09:18:38
dilution rate of D equals 1.75D to the minus 1." 09:18:40
Do you see that? 09:18:46
A Yes, I do. 09:18:47
Q Is the fermentation process that's being 09:18:48
referred there a process for making beer and wine? 09:18:50
A It is not. 09:18:52
Q It's a process for making -- for culturing 09:18:53
mammalian cells; correct? 09:18:59
A That is what's being described here. And 09:19:01
it's also very sloppy. And the reviewers should 09:19:03
have clipped that. 09:19:06
I did not deny earlier that those terms 09:19:12
get in. This is why it's sloppy. 09:19:14
Q Do you recall anyone, when this article 09:19:20
was being reviewed, commenting that the use of 09:19:23
"fermentation" here was incorrect? 09:19:27
A I was not one of the reviewers of this 09:19:28
article. I can honestly say that. 09:19:32
Q Do you recall anyone commenting, when this 09:19:35
article was presented at the conference, that they 09:19:41

didn't understand what "fermentation" meant in this context? 09:19:44 09:19:46

A This would be an example of a lot of us shaking our head, going, "They shouldn't be saying that." 09:19:47 09:19:50 09:19:54

Q Would someone in your field understand what authors of this paper were referring to when they said "fermentation process"? 09:19:55 09:19:58 09:20:00

A As it's shown here, yeah. And it's also very poor because you're still going, "All right. What point is metabo- -- metabolism going on and when is product being made?" 09:20:02 09:20:07 09:20:11 09:20:18

It causes a vagueness. 09:20:20

So it's sloppy, imprecise. 09:20:23

Q You can put that to the side. 09:20:37

I'll hand you what is going to be marked as Exhibit 64. 09:20:39 09:20:41

(Deposition Exhibit 64 marked for identification and attached to the transcript.) 09:20:49 09:20:51

Q Do you recognize Exhibit 64? 09:20:51

A I need -- I would need to spend some time looking this over. 09:21:06 09:21:09

I know Sadettin Ozturk, and I know he has edited a number of collections like this. In fact, I've been involved in some of his 09:21:14 09:21:18 09:21:20

collections. 09:21:23

Whether -- I need to figure out which one this is and when it was, so you need to give me time to look it over. 09:21:24 09:21:26 09:21:28

Q Sure. I think if you turn to the contents table, page 6, you'll see that you were involved in this collection. 09:21:30 09:21:33 09:21:35

A Yeah. That's what I say, I could have been. 09:21:37 09:21:39

Q Okay. 09:21:39

A But there's -- he's done some that I haven't and some that I have so -- 09:21:39 09:21:42

Q And you said that you know Dr. Ozturk; is that right? 09:21:45 09:21:50

A Yeah. 09:21:51

Q What is his reputation in the field? 09:21:51

A Well respected. 09:21:53

Q Who is the audience for the -- a book like this? 09:21:57 09:22:01

MR. GUTMAN: Objection; lacks foundation, vague. 09:22:06 09:22:10

A Actually, a way to find out who the audience is is probably very -- for me to sit down and read the preface. That's usually where you list that. 09:22:19 09:22:21 09:22:24 09:22:27

So -- I mean, I could spend some time now if you want me to read it. 09:22:28 09:22:31

Q Please go ahead. 09:22:32

A But -- okay. 09:22:33

It doesn't really say at that point what they're addressing it to in general, so I wasn't really correct in saying it usually does. 09:23:51 09:23:52 09:23:54

But it does sort of cover what's going on, which it should. Yes. Okay. 09:23:56 09:23:59

Q So what is going on with this book? 09:24:01

A I -- 09:24:02

MR. GUTMAN: Objection; vague, calls for a narrative. 09:24:02 09:24:06

MR. MC CLOUD: Let me withdraw that. I'll ask a different question. 09:24:10 09:24:11

Q You submitted a paper for inclusion in this book; is that right? 09:24:12 09:24:15

A Yeah. Let me see which one it is. 09:24:15

Yep, down here, 225. Yep. 09:24:18

I did. Chapter 7. Or -- I think it's a chapter -- yep. 09:24:20 09:24:25

Q And this was not a chapter that you submitted for the general population; right? 09:24:27 09:24:29

A No. 09:24:31

Q This is for -- 09:24:35

A Well, what -- what do you mean by "general population"? 09:24:36 09:24:37

Q The average person on the street. This is a chapter that you wrote for your colleagues in the industry; right? 09:24:38 09:24:39 09:24:42

A That would be a better description than "a person on the street." That would be better. 09:24:44 09:24:46

Q Okay. So this is a resource for individuals in industry who want to learn about new topics in cell culture technology; is that right? 09:24:48 09:24:51 09:24:53 09:24:55

A And summarize, correct. 09:24:55

Q Okay. Could you turn to Chapter 15 starting on page 523? 09:24:57 09:25:01

A Chapter 15? 09:25:05

525? 09:25:11

Q 523. 09:25:12

A 523. 09:25:13

Yes. 09:25:28

Q Okay. And you see there's a chapter entitled "Validation of Cell Culture-Based Processes and Qualification of Associated Equipment and Facility"? 09:25:28 09:25:30 09:25:32 09:25:34

Do you see that? 09:25:36

A Correct. 09:25:36

Q And this chapter's authored by a Chandra M. Dwivedi. Do you know Mr. Dwivedi? 09:25:37 09:25:40

A No. I'm not familiar with the name. 09:25:43

Q Okay. And if you look, there is an asterisk after his name. Do you see that? 09:25:45 09:25:48

A Yes. 09:25:51

Q If you look down at the bottom of page 523, it says Mr. Dwivedi is currently at Biogen Idec, Inc., Cambridge, Massachusetts, USA. 09:25:51 09:25:54 09:25:58

A Correct. 09:25:58

Q Do you see that? 09:26:02

A Yes. 09:26:02

Q Do you know what Biogen Idec is? 09:26:03

A Yes, I do. 09:26:06

Q What is Biogen Idec? 09:26:07

A A biotech -- biotechnology company. 09:26:11

Q And they make therapeutic antibodies? 09:26:13

A They do. 09:26:15

MR. GUTMAN: Objection; lacks foundation. 09:26:16

Q Do they make their therapeutic antibodies using mammalian cells? 09:26:17 09:26:21

MR. GUTMAN: Lacks foundation, vague. 09:26:21

A I believe so. 09:26:23

Q Could you turn to page 529, please? 09:26:24

A Yes. 09:26:26

Q Okay. And you see there's a section titled "Cell Culture and Fermentation Process"? 09:26:34 09:26:36

Do you see that? 09:26:39

A I do. 09:26:40

Q Okay. And the third sentence of that paragraph states, "Commercial fermentation processes and bioreactor technologies have been developed in the last several decades to state-of-the-art production of pharmaceutical agents of interest." 09:26:41 09:26:46 09:26:48 09:26:52 09:26:53 09:26:56

Do you see that? 09:26:57

A Yeah. Took me a minute. You were a little fast there. But -- so I -- I caught on, though. I got it, yeah. 09:26:57 09:27:01 09:27:04

Q Okay. And is it your understanding that the fermentation processes being referred to in that sentence are processes for making beer and wine? 09:27:05 09:27:07 09:27:09 09:27:11

A It is a sloppy use of the word, and I'm consistent with what I said earlier. They pushed -- "You will, by association, say it's cell culture" -- in this case but it's sloppy. It should not have been used. 09:27:12 09:27:17 09:27:20 09:27:24 09:27:27

Q But you understand that the process of fermentation that's being referred here -- to here 09:27:29 09:27:30

is a process for making therapeutic antibodies? 09:27:33
MR. GUTMAN: Objection; lacks foundation, 09:27:36
calls for speculation. 09:27:39
A By inference, that's what they're 09:27:40
referring to. I'm capable of inferring it. But 09:27:42
as we have discussed previously, if I go look up 09:27:46
the word -- and as I understand it fully well -- 09:27:50
it is sloppy. It should have been edited out. 09:27:57
Someone shouldn't have -- should have edited that. 09:28:01
I did not edit that chapter. 09:28:02
And as I said, it could -- it can slip 09:28:06
through. You might be able to find it in my work, 09:28:08
but it will slip through. It's -- it's sloppy. 09:28:11
Q Can you turn to page 531, please? 09:28:14
A Yes. 09:28:18
Q Okay. And you'll see at the bottom of the 09:28:20
page it says "Production Scale Fermentation." 09:28:21
Do you see that? 09:28:24
A Yes. 09:28:24
Q And the first sentence after that heading 09:28:25
says, "Fermentation at the production scale may be 09:28:29
carried out in a vessel (fermentor or bioreactor), 09:28:32
bottle, or a bag depending on the product type." 09:28:40
Do you see that? 09:28:43
A Yes, I do. 09:28:43

Q Is it your understanding the production scale fermentation being referred to in that sentence is a process for making beer and wine? 09:28:43 09:28:46 09:28:48

A It is my -- my answer has not changed. It's sloppy. 09:28:50 09:28:52

Q I'm going to ask my question again. 09:28:53

Is it your understanding that the production scale fermentation being referred to in that sentence is a process for making beer and wine? 09:28:57 09:28:58 09:29:01 09:29:03

MR. GUTMAN: Objection; lacks foundation. 09:29:03

A And I'm saying that that is a sloppy, imprecise use of the word and should have been edited out. 09:29:05 09:29:09 09:29:11

Q You understand that the fermentation process being referred to in that sentence is a process for making a therapeutic antibody; correct? 09:29:13 09:29:15 09:29:17 09:29:24

MR. GUTMAN: Objection; lacks foundation, calls for speculation. 09:29:24 09:29:26

A I know that Biogen Idec does not make beer and wine. So by inference -- I have to infer that they mean "of." They should have said -- they actually didn't say "bio." Why they used "fermentor" -- there -- there's an inference there 09:29:28 09:29:33 09:29:37 09:29:39 09:29:41

and it's sloppy. It shouldn't have been used. 09:29:44

Q I wanted to ask you about the term 09:29:46

"fermentor." What is a fermentor? 09:29:48

A Historically a fermentor was what they 09:29:51

made moonshine in. 09:29:57

Then, as the field became more commercial, 09:30:00

people started using -- like, for instance, 09:30:05

decades ago New Brunswick Scientific made 09:30:09

fermentors for bio- -- for not just 09:30:13

fermentation but bioenergies. 09:30:18

In the 1970s they were doing it, and then 09:30:19

they would make it for bacterial work. And then 09:30:22

it started getting a little back and forth -- 09:30:24

"Well, we should start calling -- stop calling 09:30:26

them fermentors and calling them bioreactors." 09:30:28

So there was an evolution because these 09:30:31

vessels went from just doing -- making beer and 09:30:34

wine and traditional and -- traditional metabolic 09:30:36

by-products to genetic engineering, which started 09:30:39

in the late '70s, early '80s. 09:30:42

And people then began to start saying, "We 09:30:45

should call them bioreactors because we're not 09:30:47

fermenting it now. We are doing -- even bacterial 09:30:50

work is making a recombinant protein." So there 09:30:53

began to be a division. 09:30:57

And we want to call that fermentors that does fermentation and it -- bioreactors here. So this is actually kind of representing that breaking apart. 09:30:58 09:31:00 09:31:03 09:31:05

And they should have did -- like I said, that shouldn't have been used but it is. We just kind of go, "Oh, well, being sloppy again." So it's by inference. 09:31:06 09:31:08 09:31:11 09:31:17

Q And, again, you're inferring, based on the context, that this is referring to fermentation being a process for making antibodies? 09:31:19 09:31:21 09:31:24

A That's right. 09:31:27

MR. GUTMAN: Objection; mischaracterizes his testimony, lacks foundation. 09:31:28 09:31:29

A So bring -- it's bringing us back to the claim. And you asked me -- let me read my -- No. 47 here. 09:31:33 09:31:36 09:31:40

"I also understand that Amgen has proposed that 'following fermentation' is indefinite, which I am informed means that the scope of the claim could [sic] not be reasonably certain to a person skilled in the art, such that it would not be reasonably certain to a person skilled in the art when fermentation ends and steps following fermentation begin. I agree that 'following 09:31:43 09:31:45 09:31:48 09:31:52 09:31:55 09:31:56 09:31:58 09:32:02

fermentation' is indefinite. However, if a person skilled in the art were to seek an interpretation of this term despite its indefiniteness, a person skilled in the art would find Amgen's construction to best reflect the limited guidance given in the patent." 09:32:05 09:32:08 09:32:12 09:32:16 09:32:17 09:32:21

So when I read this statement you're saying, it's the exact same kind of thinking -- or at least a very similar one. I shouldn't be so exact. It's very similar. I'm inferring it. 09:32:21 09:32:23 09:32:26 09:32:29

I'm having to interpret it and go with -- and they're helping me here by saying "fermentor or bioreactor." So in this context they're trying to equate it. 09:32:33 09:32:36 09:32:39 09:32:43

I -- you didn't ask me but I was deposed eight years ago on an import/export dispute from a -- a biotech company that makes bioreactors. They were explicitly clear they make bioreactors. They don't make fermentors. 09:32:45 09:32:47 09:32:54 09:32:59 09:33:04

And it is the same reactor that's used in the '869 patent. It's a 2-liter Applikon mentioned in the '869 patent. That is a -- that is an Applikon bioreactor. I went back and even looked. They never called it a fermentor. They were very clear of calling it a bioreactor because 09:33:07 09:33:11 09:33:17 09:33:21 09:33:25 09:33:27

that would be looked down upon in selling a product. "We don't sell fermentors. We sell bioreactors to the biotech industry." 09:33:30 09:33:32 09:33:35

Q Are you finished? 09:33:38

A (No verbal response.) 09:33:38

MR. MC CLOUD: I'll move to strike that answer as nonresponsive, and I'll ask my question again. 09:33:42 09:33:43 09:33:44

Q So you understand, based on the context of this sentence, that the use of the term "fermentation" is referring to a process for making antibodies? 09:33:45 09:33:47 09:33:50 09:33:51

MR. GUTMAN: Objection; asked and answered, lacks foundation. 09:33:53 09:33:55

A Because I'm skilled in the art and I have to make an interpretation despite it being indefinite, I am saying that this statement, which I keep saying is correct -- I stand by it. 09:33:59 09:34:03 09:34:06 09:34:10

Q I'm sorry. Which statement are you referring to? The statement in your declaration? 09:34:13 09:34:15

A Correct. 09:34:18

Q I'm asking about the sentence that we just read in the Dwivedi chapter. So I'll ask my question again for the third time. 09:34:19 09:34:21 09:34:25

You understand, based on the context of 09:34:26

this sentence, that they are referring to a fermentation process for the process of making antibodies? 09:34:29 09:34:30 09:34:33

MR. GUTMAN: Objection; asked and answered, lacks foundation, vague. 09:34:34 09:34:35

A I have to infer it because the word "fermentor" immediately sends up a flag on it. Why are they mentioning that? That's sloppy. 09:34:38 09:34:42 09:34:47

So in the whole context of the article from what -- I can't -- I might have read this a long time ago. But as I look it over, they're constantly referring to making a product that's a drug. 09:34:50 09:34:52 09:34:54 09:34:58 09:35:01

So that would imply that, yes, you're correct; it's making a human drug and not making beer or wine. So I'll -- I'll agree with that. 09:35:03 09:35:08 09:35:11

Q Thank you. 09:35:13

Could you turn to page 225, please. 09:35:20

Let me know when you get there. 09:35:27

A Yes. 09:35:29

Q Okay. And this is a chapter titled "Aeration, Mixing, and Hydrodynamics in Bioreactors." 09:35:34 09:35:37 09:35:40

A Yes. 09:35:40

Q Do you see that? 09:35:42

Amgen 2011 Annual Report and Financial Summary

AMGEN®

Pioneering science delivers vital medicines™

In many countries, the influence of regional and hospital payers also contributes to whether patients have access to certain products. For example, a product may be successfully listed on a national formulary, but may also be subject to further evaluations or competitive bidding by payers at a regional or hospital level. The impact of multiple layers of assessment can result in delay or failure to secure access and/or net price pressure.

Payers in some countries are using and others are beginning to experiment with alternative payment mechanisms (e.g., payment caps, risk sharing) as a means to maintain access to innovative therapies while increasing their budget certainty. Requirements for such payment mechanisms can impact Amgen's business through increased net price concessions and added administrative burden.

Fraud and Abuse Regulations Related to Reimbursement

As participants in government reimbursement programs, we are subject to various U.S. federal and state laws, as well as foreign laws, pertaining to healthcare "fraud and abuse," including anti-kickback laws and false claims laws. (See Government Regulation — Other.) Violations of fraud and abuse laws can result in stringent enforcement penalties up to and including complete exclusion from federal healthcare programs (including Medicare and Medicaid).

Manufacturing, Distribution and Raw Materials

Manufacturing

Biological products, which are produced in living systems, are inherently complex due to naturally-occurring molecular variations. Highly specialized knowledge and extensive process and product characterization are required to transform laboratory scale processes into reproducible commercial manufacturing processes. Our manufacturing operations consist of bulk manufacturing, formulation, fill and finish and distribution activities. Bulk manufacturing includes fermentation and/or cell culture, processes by which our proteins are produced, and also includes purification of the proteins to a high quality. The proteins are then formulated into a stable form. The fill process dispenses the formulated bulk protein into vials or syringes. Finally, in the finish process, our products are packaged for distribution.

We operate a number of commercial and/or clinical manufacturing facilities, and our primary facilities are located in the United States, Puerto Rico and the Netherlands. (See Item 2. Properties.) We also use and expect to continue to use third-party contract manufacturers to produce or assist in the production of certain of our large molecule marketed products as well as a number of our clinical product candidates. Manufacturing of Sensipar®/Mimpara®, our small molecule product, is currently performed by third-party contract manufacturers, except for certain finish activities performed by us in Puerto Rico.

The global supply of our products depends on actively managing the inventory produced at our facilities and by third-party contract manufacturers and the uninterrupted and efficient operation of these facilities. During the manufacturing scale-up process, and even after achieving sustainable commercial manufacturing, we may encounter difficulties or disruptions due to defects in raw materials or equipment, contamination or other factors that could impact product availability. (See Item 1A. Risk Factors — Manufacturing difficulties, disruptions or delays could limit supply of our products and limit our product sales and — We rely on third-party suppliers for certain of our raw materials, medical devices and components.)

Commercial Bulk Manufacturing

We operate commercial bulk manufacturing facilities in Puerto Rico and in several locations throughout the United States. (See Item 2. Properties.) We perform commercial bulk manufacturing for our proteins except Vectibix®, which is performed by a third-party contract manufacturer. We also supplement commercial bulk manufacturing for ENBREL, Prolia® and XGEVA® with a third-party contract manufacturer.

Commercial Formulation, Fill and Finish Manufacturing

We perform most of our commercial protein formulation, fill and finish manufacturing in our Puerto Rico facility. Formulation, fill and finish manufacturing for Nplate® and Vectibix® is performed by third-party

Confidential

Transcript of Stuart Watt

Date: September 23, 2019
Case: Genentech -v- Amgen (18-924-CFC)

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IN THE UNITED STATES DISTRICT COURT

FOR THE DISTRICT OF DELAWARE

- - - - - - - - - - - - - - - - - -

GENENTECH, INC., and CITY OF HOPE

Plaintiffs,

-vs-

AMGEN, INC.

Defendant.

Case No.: 1:18-CV-00924-CFC

C.A. No. (17-1407-CFC) (Consolidated)

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CONFIDENTIAL

Video-Recorded Deposition of STUART WATT

VOLUME I

Westlake Village, California

Monday, September 23, 2019

9:04 a.m.

Job No.: 263891

Pages: 1 - 302

Reported by: Tricia Rosate, RDR, RMR, CRR

CSR No. 10891

Video-Recorded Deposition OF STUART WATT,
held at:

Four Seasons Hotel
Tasting Room
Two Dole Drive
Westlake Village, California 91362
(818) 575-3000

Pursuant to Notice, before Tricia Rosate, RDR, RMR, CRR, CCRR, Certified Shorthand Reporter No. 10891, in and for the State of California.

There may be other parameters as well that I'm forgetting or not familiar with.

BY MR. FLETCHER:

Q Your understanding of the phrase "fermentation" in this context is that it refers to the cells growing or producing protein; is that right?

A Generally so, yes. Again, I think there's a dispute between the parties as to what "fermentation" -- specifically "following fermentation" -- means in the context of these claims.

Q You would --

A But as with respect to protein production processes in using host cells, fermentation historically had more to do with probably yeast or E. coli, bacterial production systems. But I guess the use of that term probably bled over into mammalian cell, as well.

And, generally, it means -- it can mean the growth of cells and the exhibition of the cellular machinery, cellular function in this kind of growth media, which is usually marked by the rate of protein production.

Q And that's your understanding of the

ordinary meaning of "fermentation" in the context of mammalian recombinant cell culture.

MR. GUTMAN: Objection. Form.

THE WITNESS: Yes, generally.

BY MR. FLETCHER:

Q Are you aware of any documents memorializing the fact reflected here, that these cells are still undergoing fermentation?

A I -- I don't remember --

I'm -- I'm not certain I ever saw any specific documents. Certainly, I was told that that's what's happening during the cool-down phase. That's what I know.

Q If you could please turn to page 9 of Dr. Loots' opinion. Heading "B" on page 9 states "The Relevant Law Regarding Invalidity."

Do you see that?

A I do.

Q Did you review this section of Dr. Loots' opinion when you reviewed it?

A Yes.

Q Were you aware of any errors in this section?

A I don't remember anything that cites to case law generally, the factors as referenced in the case