BJ www.biochemj.org

BJ Cell

Biochem. J. (2011) **435**, 499–508 (Printed in Great Britain) doi:10.1042/BJ20101303

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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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In vitro cultured mammalian cells respond to mild hypothermia (27–33 °C) by attenuating cellular processes and slowing and arresting the cell cycle. The slowing of the cell cycle at the upper range (31–33 °C) and its complete arrest at the lower range (27–28 °C) of mild hypothermia is effected by the activation of p53 and subsequent expression of p21. However, the mechanism by which cold is perceived in mammalian cells with the subsequent activation of p53 has remained undetermined. In the present paper, we report that the exposure of Chinese-hamster ovary-K1 cells to mildly hypothermic conditions activates the ATR (ataxia telangiectasia mutated- and Rad3-related kinase)–p53–p21 signalling pathway and is thus a key pathway involved in p53 activation upon mild hypothermia. In addition, we show that although $p38^{MAPK}$ (p38 mitogen-activated protein kinase) is also involved in activation of p53 upon mild hypothermia, this is probably the result of activation of $p38^{MAPK}$ by ATR. Furthermore, we show that cold-induced changes in cell membrane lipid composition are correlated with the activation of the ATR–p53–p21 pathway. Therefore we provide the first mechanistic detail of cell sensing and signalling upon mild hypothermia in mammalian cells leading to p53 and p21 activation, which is known to lead to cell cycle arrest.

Key words: ataxia telangiectasia mutated- and Rad3-related kinase (ATR), Chinese-hamster ovary cell (CHO cell), cold shock, hypothermia, lipidomics, metabolomics, p53.

INTRODUCTION

Under mildly hypothermic conditions (31–33 °C), mammalian cells proliferate slowly [1] and generally attenuate the processes of transcription and mRNA translation [2] (although protein folding may actually improve [3]), and the cell cycle proceeds at a much reduced rate [4]. However, below 30 °C, cells become arrested, predominantly in G_1 phase [5,6], normally the stage in the cell cycle when protein synthesis rates are optimal. Indeed, because of this, other strategies for inducing cell cycle arrest in late proliferative stage cultures of mammalian cells in an industrial sense have been investigated, including generation of cell lines with inducible expression of the general cyclin inhibitor p21 [7] and the addition of solvents, such as DMSO, to the growth medium, which also induces p21 expression [8]. However, exposure to mildly hypothermic conditions remains the most economic and most effective way of extending the productive life of cultured mammalian cells for large-scale recombinant protein production [9].

The slowing of the cell cycle at the upper range of mild hypothermia (31–33 °C) and its complete arrest at the lower range of mild hypothermia (27–28 °C) is regulated by the expression of p21 [10]. There are numerous examples (reviewed in [11]) of p21-induced cell cycle arrest protecting damaged or stressed cells from apoptosis, thus providing a time window within which the damage may be repaired or stress conditions removed. This is certainly the case for mildly cold-stressed cells, since they recover rapidly and fully on returning to 37 °C [12]. It is also well established that p21 induction in mildly hypothermic cells is subsequent to an increase in the stability and hence amounts of the tumour suppressor protein p53 [10,13] and to changes in the p53 isoform array [12], although the post-translational modification(s) generating these observed changes in p53 isoform pattern remain to be identified. Indeed, p53-deficient mammalian cells do not show cell cycle arrest at mildly hypothermic temperatures, confirming the key role of p53 in regulating this process upon cold shock [10,13]. However, the mechanism(s) by which p53 phosphorylation and amounts are unregulated upon mammalian cells being placed under hypothermic conditions, or how these conditions are sensed, are currently unknown.

In addition to cell cycle arrest and the general attenuation of transcription and translation, changes to the cell membrane composition are also observed when both prokaryotic [14] and eukaryotic [15] cells are exposed to hypothermic conditions. Essentially, cells respond to reduced temperature by increasing the polyunsaturated fatty acid content of membrane phospholipids, thereby maintaining the fluidity under hypothermic conditions, so-called homeoviscous adaptation [16]. At 37 °C, an increase in polyunsaturated fatty acid content of membrane phosphatidylcholines, induced by exposure to the Ca^{2+}-dependent phospholipase A_2 inhibitor BEL (bromoenol lactone), has been reported to arrest mammalian cells in G_1-phase by activation of the p53–p21 pathway [17]. This was subsequently shown to be mediated by phosphorylation of p53 at Ser^{15} by a member of

Abbreviations used: ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related kinase; BEL, bromoenol lactone; CHO, Chinese-hamster ovary; CIRP, cold-inducible RNA-binding protein; DMEM, Dulbecco's modified Eagle's medium; DNA-PK, DNA-dependent protein kinase; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; Mdm2, murine double minute 2; $p38^{MAPK}$, p38 mitogen-activated protein kinase; PC, principal component; PC-DFA, principal component-discriminant function analysis; PIKK, phosphoinositide 3-kinase-related kinase; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; siRNA, small interfering RNA; UTR, untranslated region.

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the PIKK (phosphoinositide 3-kinase-related kinase) family, ATR (ataxia telangiectasia mutated- and Rad3-related kinase) [17], a signalling pathway more usually associated with cell cycle arrest in response to compromised DNA replication [18]. Since there was no evidence of DNA damage in these cells with altered membrane composition, it was concluded that an increase in the ratio of polyunsaturated to saturated fatty acids in phosphatidylcholines in cell membranes independently activates the ATR–p53–p21 pathway [17]. Furthermore, the expression of the cold-shock protein CIRP [cold-inducible RNA-binding protein, also known as hnRNP (heterogeneous nuclear ribonucleoprotein) A18] is induced at mildly hypothermic temperatures in mammalian cells [19] and binds to the 3′-UTRs (untranslated regions) of certain transcripts, increasing their translation [20]. CIRP protein binds to the 3′-UTR of *ATR* mRNA, and overexpression of CIRP results in increased ATR protein levels [20].

In view of these reports and our own previous observations that changes in p53 isoform pattern are observed upon mildly hypothermic conditions in mammalian cells as are the detection of lipid droplets at lower temperature [12], we set out to investigate (i) whether p53 activation upon mild hypothermia was at least in part mediated through the ATR kinase signalling pathway, and (ii) whether mild hypothermia resulted in changes in lipid composition consistent with those previously reported to activate ATR (an increase in the ratio of polyunsaturated to saturated fatty acids), thus linking hypothermia-induced changes in membrane composition to hypothermia-induced cell cycle arrest. We show that p53 phosphorylation and activation in the commercially relevant CHO (Chinese-hamster ovary)-K1 cell line is mediated by the ATR–p53–p21 pathway and ATR signalling is thus a key pathway involved in p53 activation upon mild hypothermia, and, furthermore, that cold-induced changes in cell membrane lipid composition are associated with this. We therefore provide the first mechanistic detail of cell sensing and signalling upon mild hypothermia in CHO cells leading to p53 and p21 activation, which are known to subsequently result in cell cycle arrest.

EXPERIMENTAL

Cells and cell maintenance

CHO-K1 cells (originally sourced from the European Collection of Cell Cultures) were maintained in DMEM (Dulbecco's modified Eagle's medium)/F12 (Invitrogen), supplemented with 10 % (v/v) dialysed heat-inactivated FBS (fetal bovine serum) (PAA, catalogue no. A15–507), glutamine, glutamate, aspartate, nucleosides and non-essential amino acids (Invitrogen), at 37 °C in a 5 % CO_2 atmosphere as described previously [12]. HeLa (Ohio) cells (sourced from the A.T.C.C.) were maintained in DMEM supplemented with 10 % (v/v) FBS (PAA, catalogue no. A15–151), 2 mM glutamine and non-essential amino acids. ^{35}S-labelled amino acid incorporation into proteins was assessed as described in [12]. Exposure to mildly sub-physiological temperatures was undertaken in routine culture medium in appropriately regulated ($\pm$0.1 °C) incubators. Exposure to 15 μM BEL (Sigma) was for 6 h at 37 °C in normal growth medium. Caffeine (Sigma) was used at a final concentration of 2.5 mM, wortmannin (Sigma) at a final concentration of 20 μM, the p38 kinase inhibitor SP203580 (Calbiochem) at a final concentration of 10 μM, the ATM (ataxia telangiectasia mutated) inhibitor KU0055933 at a final concentration of 10 μM and the DNA-PK (DNA-dependent protein kinase) inhibitor NU7441 at a final concentration of 1 μM. Cells were exposed to these inhibitors for 30 min at 37 °C prior to transfer, without removal of the inhibitor, to mildly hypothermic conditions. For RNAi (RNA interference) knockdown, cells were transfected with validated siRNAs (small interfering RNAs) for Hs ATR (*Homo sapiens* ATR), *Hs_ATR_11* (Qiagen) and *Hs_ATR_12* (Qiagen), using HiPerfect reagent (Qiagen) as described in the manufacturer's instructions and a final siRNA concentration of 5 nM. When combining RNAi knockdown of ATR with inhibitor experiments, CHO-K1 cells were first exposed to ATR siRNA for 48 h at 37 °C, then 10 μM SP203580 was added for a further 30 min prior to transfer, in the continued presence of siRNA and SP203580, to 32 °C or 27 °C for 10 h.

Extraction of RNA, protein and lipids from cells

Total RNA was extracted from intact cells using the commercially available Qiagen RNeasy kit as per the manufacturer's instructions. Cell lysates for protein analysis were prepared by lysing PBS-washed 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 μg/ml leupeptin, 2 μg/ml pepstatin and 0.2 mM PMSF) and protein phosphatase inhibitors (50 mM NaF and 1 mM activated Na_3VO_4). For each lipid extraction, 5×10^6 CHO-K1 cells (60 % confluent) were washed with 10 ml of 0.8 % NaCl at the appropriate temperature and then scraped into 600 μl of solid-CO_2-chilled methanol, followed by extraction of lipids by vortex-mixing for 15 s with 600 μl of solid-CO_2-chilled chloroform and then freezing in liquid N_2 for 1 min before thawing on ice. This freeze–thaw procedure was repeated twice more. The chloroform extract was then washed twice by adding 900 μl of ice-cold water, vortex-mixing for 30 s followed by centrifugation at 16000 ***g*** for 15 min and removal of the aqueous layer. Samples were stored at -80 °C prior to transportation (as solutions) on solid CO_2 to Manchester for lipid analysis.

qRT-PCR (quantitative real-time PCR) analysis of mRNA levels

Relative quantification of mRNA levels was undertaken by qRT-PCR using the Bio-Rad Laboratories iScript™ one-step kit as described in the manufacturer's instructions with the following primers: human ATR, Quantitect primer assay Hs_ATR_1_SG (Qiagen); CHO ATR, forward, 5′-GTTAATCCATGGTCGAGC-3′, reverse, 5′-TTGTCATAGTACTTGGCAAGG-3′; human actin, forward, 5′-CCGAGGACTTTGATTGCAC-3′, reverse, 5′-AGTGGGGTGGCTTTTAGGAT-3′; and CHO actin, forward, 5′-AGCTGAGAGGGAAATTGTGCG-3′, reverse, 5′-GCAACGGAACCGCTCATT-3′. Reactions were carried out using a Mastercycler® ep Realplex thermocycler (Eppendorf) programmed for a reverse transcription incubation at 50 °C for 10 min, followed by a 95 °C hold for 5 min and subsequent 40 cycles of 10 s at 95 °C and 20 s at 55 °C.

SDS/PAGE and immunoblot analysis

For SDS/PAGE analysis, 10 % separation gels were prepared as described by Laemmli [21], loading 20 μg of protein lysate per lane. SDS/PAGE-resolved polypeptides were transferred on to a nitrocellulose membrane, which was then blocked with 5 % (w/v) non-fat dried skimmed milk powder in 0.2 % Tween 20/TBS (Tris-buffered saline). Primary antibodies were sourced as follows: anti-ATR, Santa Cruz Biotechnology (N19); anti-p53, Dako (clone DO-7); anti-p53 phosphorylated at Ser^{15}, Cell Signaling Technology (#9284); anti-p21, Santa Cruz Biotechnology (C19); and anti-β-actin, Sigma (clone AC15). Horseradish peroxidase-conjugated secondary antibodies were detected by enhanced chemiluminescence using Hyperfilm ECL (GE Healthcare). Linearity of the antibody response over the

concentration range of the target protein had been established previously [12].

Immunofluorescence microscopy

PBS-washed CHO-K1 cells grown on 13 mm glass coverslips were fixed in 3 % (w/v) paraformaldehyde in PBS for 15 min at 37 °C or 27 °C, then permeabilized with 0.5 % Triton X-100 in PBS for 10 min at room temperature (21 °C). After blocking in 0.1 % Tween 20/PBS containing 3 % (w/v) BSA, coverslips were incubated with anti-ATR antibody (1:50 dilution) overnight at 4 °C. Further processing and detection were as described in [12].

MS analysis of lipids

Profiling of the lipid fraction of cell extracts was performed using DIMS (direct infusion MS [22]). Chloroform extracts (400 μl) were diluted in 600 μl of methanol. Samples were infused into an electrospray Thermo Fisher Scientific LTQ-Orbitrap XL mass spectrometer operating in negative-ion mode at a flow rate of 5 $\mu l \cdot min^{-1}$ for 1 min. Accurate mass data were acquired in the Orbitrap mass analyser operating at a mass resolution of 100 000 (at m/z 400) and with a scan time of 1.2 s. All mass spectra were averaged to provide a single mass spectrum for each sample that was passed forward for further data processing and analysis. All mass peaks were binned to the nearest nominal mass (defined as mass bins).

Data analysis of lipid profiles

Multivariate PC-DFA (principal component-discriminant function analysis) using cross-validation was performed to inspect the clustering of sample classes as detailed in [23]. All data were normalized to a sum of 1. A PCA (principal component analysis) model was then constructed [24] with three of six samples per class; the first ten PCs (principal components) were extracted and these accounted for 99.8 % of the total explained variance. Next, these PCs were used by the DFA algorithm [25] with the *a priori* knowledge of the six different treatments. In order to validate this PC-DFA model, it was tested by cross-validation by the projection of the three remaining samples, a process that allows cluster integrity to be assessed; that is to say, if the projected samples co-cluster with samples used to construct the PC-DFA model, then the groupings observed and their relationships are valid. All multivariate analyses were performed in MatLab (MathWorks). To define statistically significant differences, Kruskal–Wallis analysis of variance was performed. For those mass bins of statistical significance, further analysis was performed to determine the accurate lipid mass contributing to this statistical difference. Putative identification of lipids (as the deprotonated ion, sodiated or potassiated negatively charged adducts) was performed by matching to the accurate mass of lipids in the Lipid Maps database (http://www.lipidmaps.org/) with a mass accuracy less than 2 p.p.m.

RESULTS

p53 is phosphorylated at Ser[15] when CHO-K1 cells are exposed to mildly hypothermic conditions

When CHO-K1 cells were transferred from 37 °C to 32 °C the subsequent growth rate was greatly reduced, and cells transferred to 27 °C ceased to proliferate (Figure 1A). Under both of these mildly hypothermic conditions, expression of p21 was

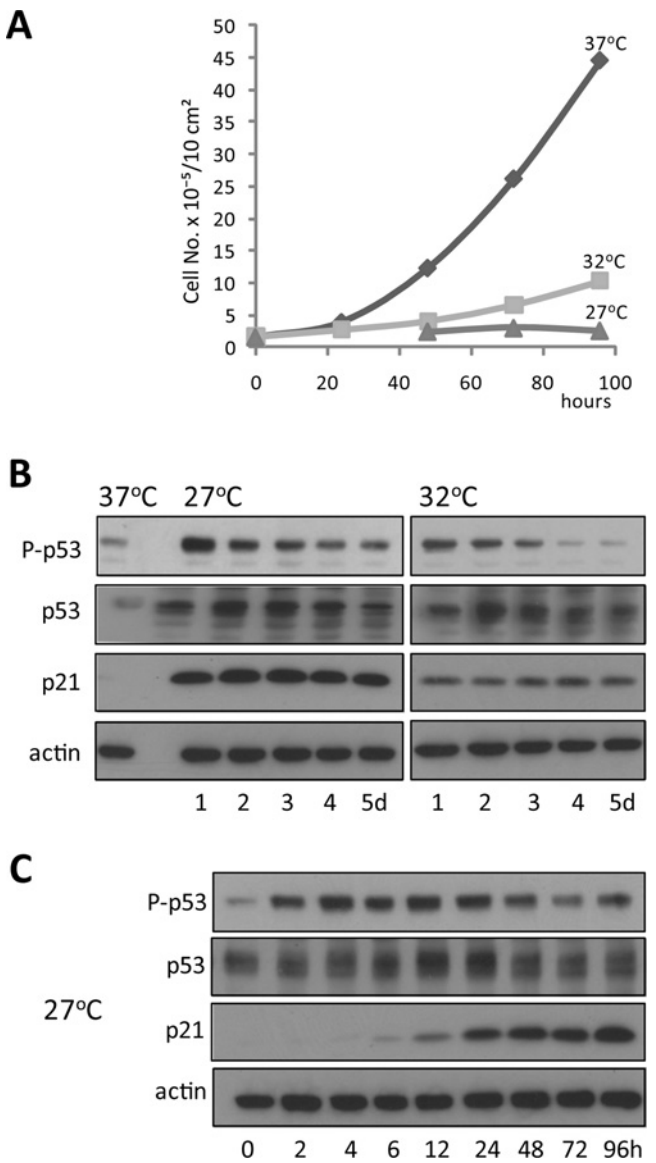

Figure 1 Mild hypothermia reduces cell proliferation and induces phosphorylation of p53 at Ser[15] and subsequent p21 expression

(**A**) Growth curves of CHO-K1 cells maintained at the indicated temperatures. (**B**) Immunoblot detection of p53 phosphorylated at Ser[15], total p53 protein and p21 in lysates of CHO-K1 cells maintained at the indicated temperatures for the indicated number of days. (**C**) Immunoblot detection of p53 phosphorylated at Ser[15], total p53 protein and p21 in lysates of CHO-K1 cells maintained at 27 °C for the indicated number of hours. In (**B** and **C**), immunoblot detection of β-actin served as an indicator of lysate protein loading.

induced and maintained throughout a 5 day period, whereas phosphorylation of p53 at Ser[15] initially increased dramatically, but then decreased towards the end of the 5 day period (Figure 1B). Notably, the levels of both p53 phosphorylation and p21 expression were greater at 27 °C compared with 32 °C in line with the proliferation status of the cells at these two temperatures, and at 32 °C cell proliferation was observed towards the end of the 5 day period when Ser[15] phosphorylation levels once again decreased (Figure 1B). A more detailed examination of the early period following the temperature shift to 27 °C (Figure 1C) clearly showed that the phosphorylation of p53 at Ser[15] preceded a modest increase in p53 levels that, in turn, preceded the induction of p21. This

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

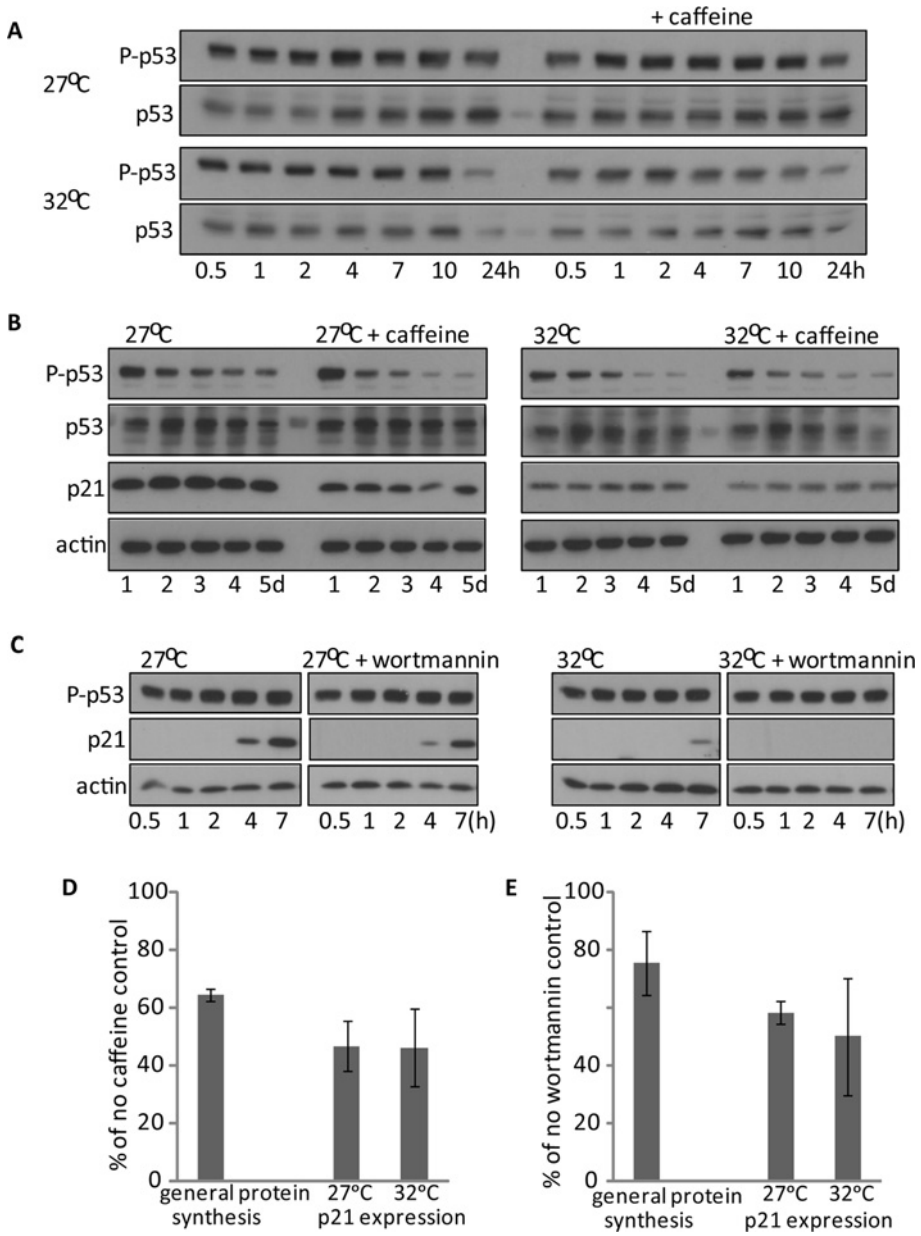

Figure 2 Caffeine inhibits both phosphorylation of p53 at Ser[15] and p21 induction associated with mild hypothermia, but wortmannin does not

(**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

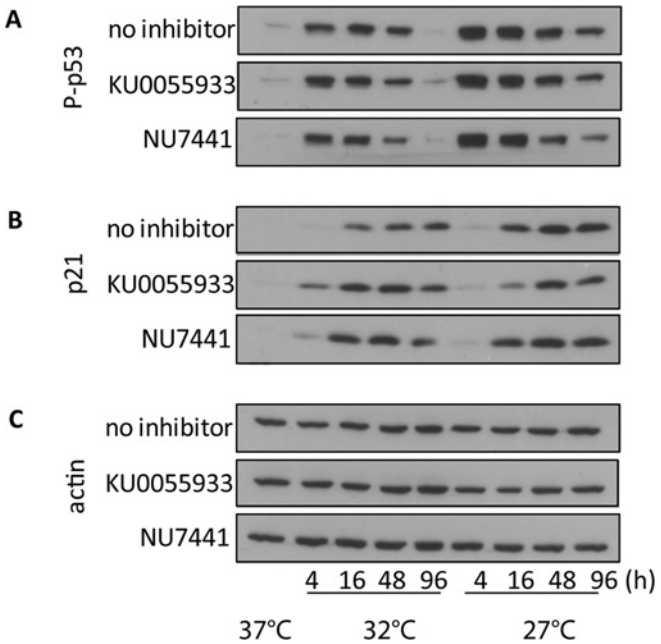

Figure 3 Specific inhibitors of DNA-PK and ATM do not abrogate the cold-induced phosphorylation of p53 and induction of p21

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.

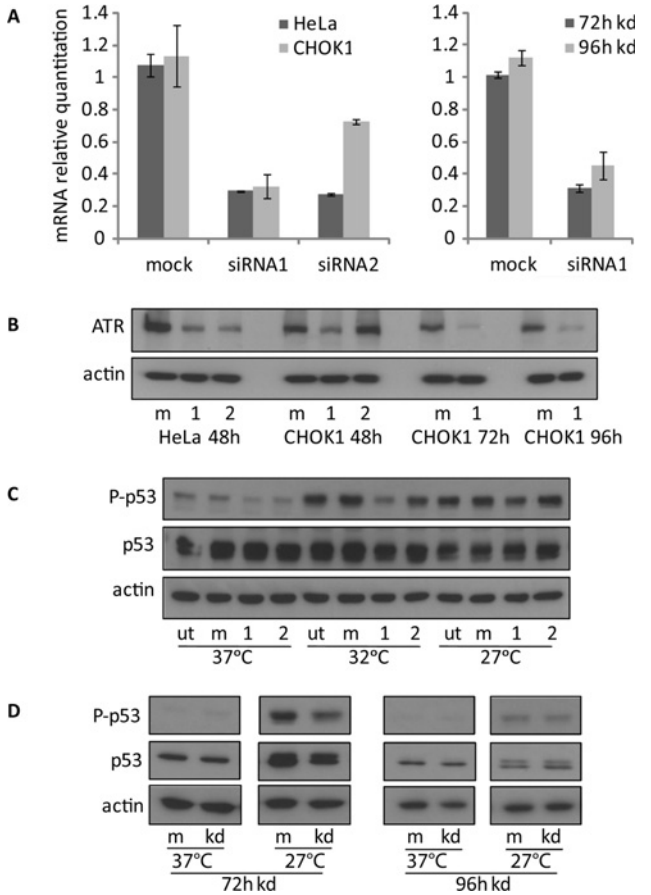

Figure 4 siRNA knockdown of ATR mRNA inhibits hypothermia-induced phosphorylation of p53 at Ser[15]

(**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

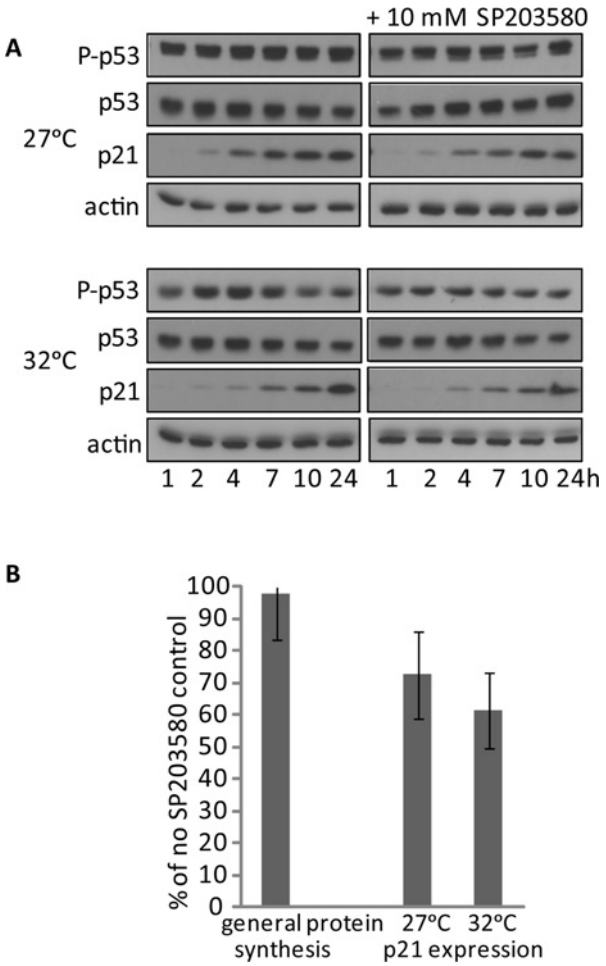

Figure 5 The p38^{MAPK} inhibitor SP203580 attenuates hypothermia-associated phosphorylation of p53 at Ser^{15} and p21 induction

(**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.

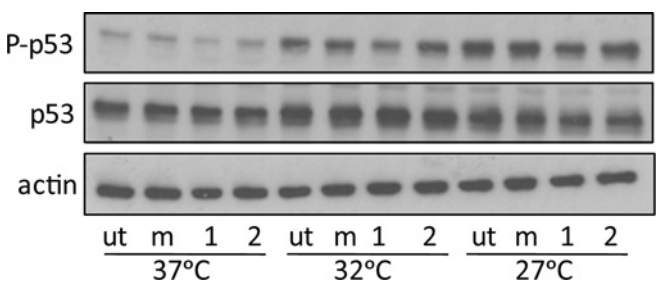

Figure 6 Inhibition of hypothermia-induced phosphorylation of p53 at Ser^{15} by ATR knockdown is not increased by additional inhibition of p38^{MAPK}

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.

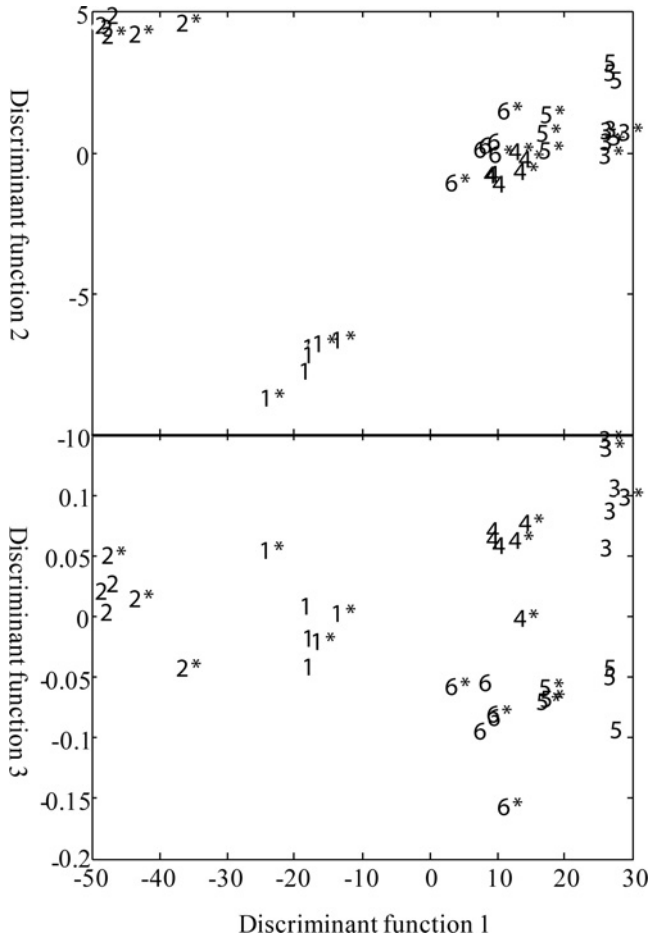

Figure 7 Exposure of CHO-K1 cells to mild hypothermia is associated with changes in the cellular lipid profile

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.

FUNDING

This work was in part funded by the Biotechnology and Biological Sciences Research Council (BBSRC), U.K. [grant numbers BB/F018908/1 (Kent; to C.M.S.) and BB/F018738/1 (Nottingham and Leicester; to A.W.)]. The contribution from the Manchester Centre for Integrative Systems Biology was funded in particular by the BBSRC and the Engineering and Physical Sciences Research Council (EPSRC) [grant number BB/C008219/1 (to R.G. and W.D.)].

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Received 17 August 2010/19 January 2011; accepted 2 February 2011
Published as BJ Immediate Publication 2 February 2011, doi:10.1042/BJ20101303

Biochem. J. (2011) **435**, 499–508 (Printed in Great Britain) doi:10.1042/BJ20101303

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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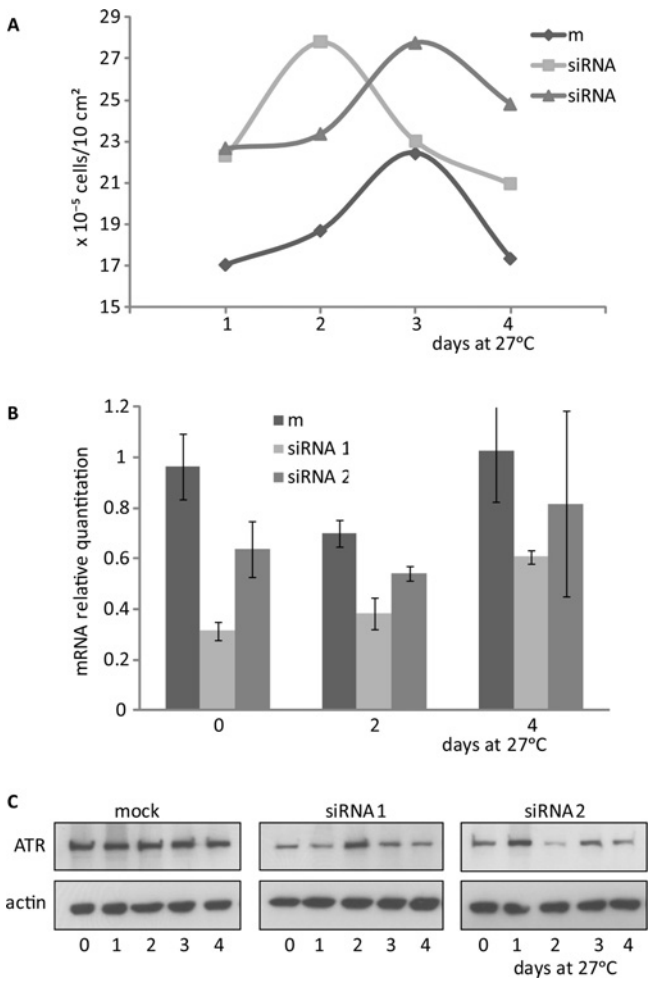

Figure S1 Effect of prolonged siRNA knockdown of ATR on cell proliferation at 27 °C

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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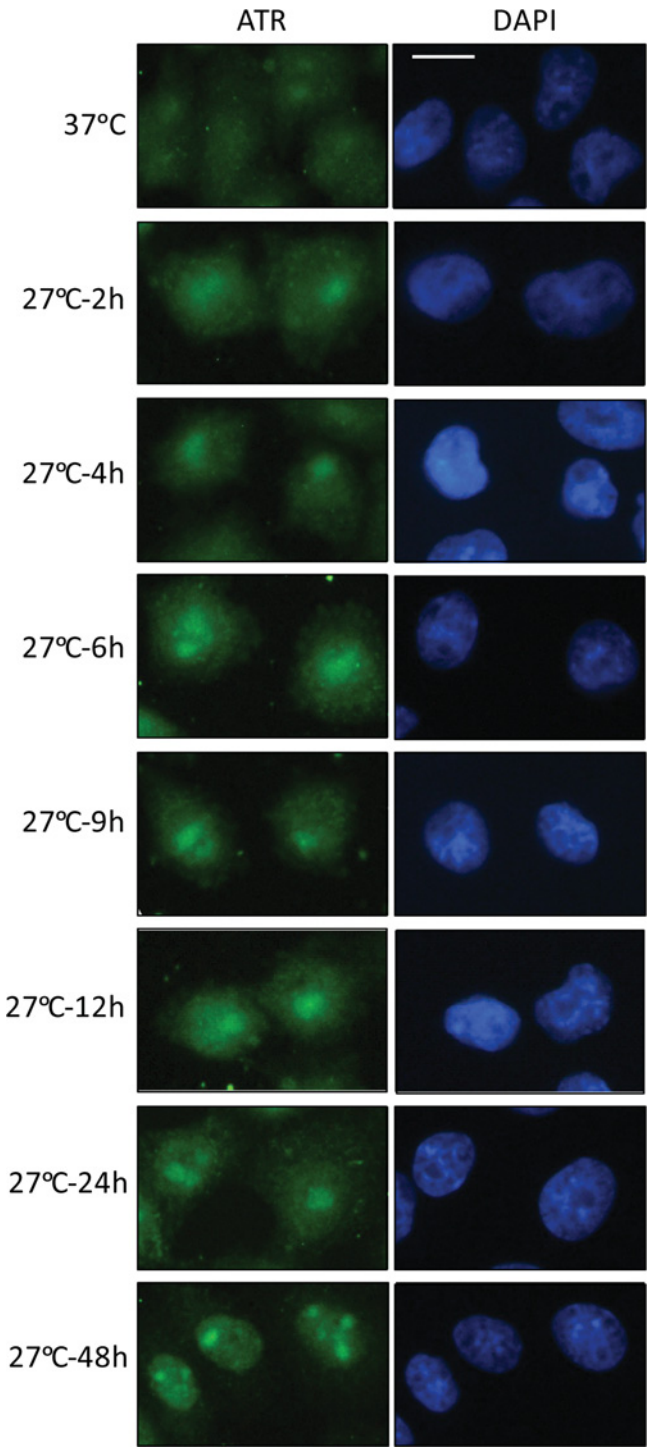

Figure S2 The intracellular localization of ATR changes during the early stages of hypothermia at 27°C

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

Received 17 August 2010/19 January 2011; accepted 2 February 2011
Published as BJ Immediate Publication 2 February 2011, doi:10.1042/BJ20101303