Human Molecular Genetics, 2005, Vol. 14, No. 12 ***1699–1708***
doi:10.1093/hmg/ddi181
Advance Access published on May 11, 2005

Cancer chemoprevention by the antioxidant tempol acts partially via the *p53* tumor suppressor

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Received March 18, 2005; Revised and Accepted May 3, 2005

We previously demonstrated that the nitroxide antioxidant tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) increased latency to tumorigenesis and doubled (100%) the lifespan of *Atm*-deficient mice, a mouse model of ataxia telangiectasia, which displays accelerated oxidative damage and stress. Tempol treatment of cancer-prone *p53*-deficient mice resulted in a small but significant (25%) increase in lifespan by prolonging latency to tumorigenesis, demonstrating that existing oxidative stress and damage are not necessary for the chemopreventative effects of tempol. However, the relatively small effect on latency in *p53*-deficient mice and the finding that tempol-mediated resistance to oxidative insult was p53-dependent suggested a more direct role of p53 in the chemopreventative effects of tempol. Surprisingly, tempol treatment specifically increased serine 18 phosphorylation of p53 (but not γ-H2AX) and p21 expression in primary thymocytes *in vitro* in a p53-dependent fashion. Inhibition of phosphoinositide 3-kinase (PI3K) family members suggested that SMG-1 was responsible for the tempol-mediated enhancement of p53 serine 18 phosphorylation. These data suggest that the chemopreventative effect of tempol is not solely due to the reduction of oxidative stress and damage but may also be related to redox-mediated signaling functions that include p53 pathway activation.

INTRODUCTION

Although cancer research has led to successful treatment and early detection of most cancers, the mortality rate from this disease has not declined in the last 30 years (1). The American Cancer Society estimates that in 2002 over half-a-million Americans died of cancer (Weir, D.H., 2002, Oncologist at the US Centers for Disease Control and Prevention. *Oncolink*. http://www.oncolink.org). Although genetic factors have been shown to play a significant role in cancer development, it has also been estimated that 50% of cancer deaths are associated with environmental agents (1). It is possible that specific life choices can affect the ability of an organism to deal with the damage that cancerous environmental agents cause. Supporting this, there is a strong correlation found between a diet high in fruit and vegetables with a lower incidence of cancer, suggesting that some factor(s) in these foods aid in cancer prevention (2). The high levels of antioxidants in these foods suggest a role for these compounds in chemoprevention. A number of human studies have investigated the role of antioxidants in chemoprevention, however, definitive evidence for their specific role in cancer prevention has remained elusive (2–4).

Our previous work provides support for a chemopreventative effect of antioxidants, because the nitroxide antioxidant and superoxide dismutase (SOD) mimetic tempol increased tumor-free survival in both *Atm*-deficient and C3H mice (5,6). We found that increased latency to tumorigenesis in *Atm*-deficient mice was associated with reduced oxidative stress and damage in cancer-prone tissues, suggesting that

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the chemopreventative effects of tempol resulted from the reduction of oxidative stress and damage (5). However, we recently found that genetically increased oxidative stress and damage by reduction of *Sod1* and *Sod2* in *Atm*-deficient mice do not increase tumorigenesis (Erker *et al.*, manuscript in preparation). This suggests that elevated levels of oxidative stress and damage in *Atm*-deficient mice (7–10) is not the exclusive cause of cancer and that the chemopreventative effect of tempol results from reduction of this oxidative stress and damage, as well as through other undefined mechanisms.

In order to further test the relationship between reduced oxidative stress and chemoprevention, we investigated the effect of nitroxide antioxidants in a cancer-prone mouse model that does not display oxidative stress, the *p53*-deficient mouse (11,12). *p53*-deficient mice are a model of the human cancer-prone syndrome, Li–Fraumeni syndrome, and succumb to a spectrum of tumors, primarily lymphomas, by about 6 months of age (11). To date, there is no evidence that *p53*-deficient mice have elevated levels of oxidative stress. p53 is a direct target of ataxia telangiectasia mutated (ATM) in response to DNA double strand breaks (DSBs) caused by ionizing radiation (IR) or chemical carcinogens. ATM directly phosphorylates serine 15 in human p53 or serine 18 in mouse p53 (reviewed in 13,14). This phosphorylation signal synergizes with ATM-dependent phosphorylation of MDM2 and CHK2 (13–16) to increase p53 protein levels through stabilization. Once activated, p53 acts as a transcription factor binding to DNA and regulating the transcription of genes that play a role in cell cycle control and apoptosis such as *p21/WAF1/Cip1* (*p21*) and *Bax* (13,14,17,18). Transcriptional activation of p21 mediates, in part, a p53-dependent G1 arrest (19) by binding to cyclin/CDK complexes (20). Thus, p53 plays a crucial role in coordinately regulating the distinct pathways that control responses to DNA damage (21).

ATM is a member of the PI(3)K-related kinases (PIKK) along with ATR, DNAPK and the newest member SMG-1. All PIKK members are serine–threonine kinases containing a phosphotransferase homology domain of PI(3)Ks. All four aforementioned PIKKs are involved in responding to and repairing DNA DSBs. Each member displays a characteristic response to damaging agents; ATM, DNAPK and SMG-1 are activated within minutes following treatment with IR, whereas ATR responds to the same stimuli hours later. ATR also responds to other types of DNA damage and is an essential gene (22).

All PIKK members, except DNAPK, are implicated in phosphorylation of p53 at serine 15 in response to DSBs (13,14,21,23–27). Phosphorylation at this site causes a cell cycle delay, allowing time for DNA repair or if DNA damage is extensive, activates apoptosis (17,18). This phosphorylation site has also been implicated in response to oxidative stress in mice and cancer cell lines (28–30), suggesting that it may respond to modulation of reactive oxygen species (ROS) levels. Both ATM and SMG-1 have similar kinetics, maximally phosphorylating p53 (serine 15) 1 h after IR (13,23). However, they display differential sensitivity to DSBs with SMG-1 phosphorylating p53 (serine 15) at lower IR doses than ATM (23). The importance of PIKKs in genome surveillance and cancer prevention is underscored by the cancer-prone *Atm*- and *p53*-deficient mice, which succumb to tumors between 2 and 6 months of age (11,12,31).

As *p53*-deficient mice do not display oxidative damage and stress, and p53 is a direct target of ATM family members, we tested the ability of tempol to prevent cancer development in the *p53*-deficient mice by administering tempol continuously via the diet. Tempol treatment significantly increased the lifespan of *p53*-deficient mice without any reduction in oxidative stress and damage or increased resistance to an oxidative insult. These results suggested that the chemopreventative effect of tempol does not require pre-existing oxidative stress and damage. In addition, the increased latency to tumorigenesis was greater in $Atm^{-/-}$ (100%) than in $p53^{-/-}$ (25%) mice, suggesting that some aspects of the chemopreventative effects were p53-dependent. Consistent with this, we found that tempol enhanced phosphorylation of p53 and induced p21 protein expression after IR in the absence of *Atm*, suggesting an important effect of tempol in cellular signaling and activation of the p53 pathway.

RESULTS

Tempol increases latency to tumorigenesis in $p53^{-/-}$ mice

We treated $p53^{-/-}$ mice with tempol to determine whether it plays a more general role in chemoprevention than simply reversing existing oxidative damage and stress present in *Atm*-deficient mice. Tempol was administered chronically via the diet, after weaning, in four separate cohorts of $p53^{-/-}$ mice. Mean tumor-free survival of tempol-treated *p53*-deficient mice was significantly increased ($P = 0.0066$) from 21.4 to 25 weeks, when all four studies were combined and analyzed (Fig. 1). There was no change in weight in wild-type or $p53^{-/-}$ mice fed with tempol or placebo chow (data not shown). However, the increase in tumor-free survival of the *p53*-deficient mice was smaller (~25%) than the doubled lifespan previously seen in tempol-treated *Atm*-deficient mice (5).

During the course of the survival study, mice that displayed obvious tumors or were otherwise sick were autopsied when death was imminent. As previously shown for *Atm*-deficient mice (5), there was no change in tumor spectrum (primarily lymphoma) between tempol and placebo-treated $p53^{-/-}$ (data not shown) mice (11). These data are consistent with the interpretation that tempol prolonged latency to tumorigenesis without affecting tumor type in both *Atm*- and *p53*-deficient mice.

Tempol treatment does not reduce body weight in *p53*-deficient mice

As weight reduction and caloric restriction also prevents cancer and increases lifespan in tumor-prone mice (32–35), it should be noted that caloric restriction cannot explain the chemopreventative effects seen in both $Atm^{-/-}$(5) and $p53^{-/-}$ mice (Fig. 1). First, although we found weight reduction in tempol-treated $Atm^{-/-}$ mice (5), there was no such weight reduction in the *p53*-deficient or wild-type mice treated with

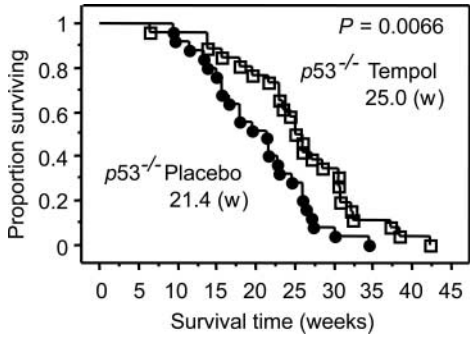

Figure 1. Tempol increases longevity of $p53^{-/-}$ mice and does not change oxidative stress or damage. Kaplan–Meier analysis for tumor-free survival of $p53^{-/-}$ mice fed food with (open squares) and without (closed circles) tempol at weaning.

tempol (data not shown). Secondly, food intake was the same for both tempol- and placebo-treated mice (data not shown). Finally, tumor spectrum has been shown to shift in response to caloric restriction (32–34), and we saw no such change with $Atm^{-/-}$ (5) or $p53^{-/-}$ (data not shown) tempol-treated mice. Besides, previous work demonstrated that C3H wild-type mice treated with tempol have a significant reduction in weight, whereas our wild-type 129 mice do not show any such tempol-mediated weight reduction (6). As the C3H mice from the previous study weighed twice our wild-type tempol-treated mice at 5 months of age (data not shown), these discrepancies may be a result of the mouse background (6). Regardless of the differential tempol-mediated effect on C3H and 129 wild-type mice, our data demonstrate that caloric restriction cannot explain the chemopreventative effects of tempol seen in both $Atm^{-/-}$ and $p53^{-/-}$ mice.

Tempol treatment does not further reduce oxidative damage and stress *in vivo* in $p53^{-/-}$ mice

The chemopreventative effects of tempol were previously associated with reduction of oxidative stress and damage in $Atm^{-/-}$ mice (5), suggesting that the chemopreventative effect of tempol either requires oxidative stress or reduces ROS levels below levels seen in untreated mice. Therefore, we determined whether oxidative stress and damage were decreased by tempol treatment in the *p53*-deficient mice. Consistent with previous work (5,6), the thymus of *Atm*-deficient mice displayed increased levels of oxidative stress and damage when compared with wild-type, measured by western blot of HO-1 protein (Fig. 2) and oxidized protein carbonyl groups (Fig. 2B and C). Tempol treatment lowered the elevated levels of oxidative stress (Fig. 2A) and damage in the $Atm^{-/-}$ thymus (Fig. 2B and C) (5). *p53*-deficient mice displayed similar levels of HO-1 and protein carbonyl groups as wild-type mice, and tempol treatment did not further decrease these normal levels of oxidative stress (Fig. 2A) or protein damage (Fig. 2B and C) in $p53^{-/-}$ thymi.

To directly investigate the effect of tempol on cellular oxidative status, we determined the effect of tempol treatment on intracellular ROS by measuring the conversion of non-fluorescent 2′,7′-dichlorodihydrofluorescein to fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) quantitatively by flow cytometry (36,37). DCF fluorescence was similar in thymocytes from $p53^{-/-}$ and wild-type mice and tempol slightly reduced DCF intensity in thymocytes from both wild-type and $p53^{-/-}$ mice (Fig. 2D). As mitochondria are the primary site of ROS induced damage, we determined whether mitochondrial membrane potential was similar in $p53^{-/-}$ and wild-type mice after tempol treatment (38). Consistent with a normal oxidative status, mitochondrial membrane potential was similar in wild-type and *p53*-deficient mice, and there was no detectable change in mitochondrial membrane potential with tempol treatment (Fig. 2E). These data are consistent with the interpretation that there is no increase in ROS in cells from $p53^{-/-}$ mice when compared with wild-type and that tempol reduces ROS to the same extent in both $p53^{-/-}$ and wild-type mice.

To test whether tempol might protect $p53^{-/-}$ cells from ROS, wild-type and $p53^{-/-}$ thymoctytes were treated with an extracellular oxidative insult, hypoxanthine and xanthine oxidase (HX/XOD), in the presence or absence of tempol, and cell viability was measured using an MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) viability assay (5,39). After 18 h in culture, no toxic effects of tempol were observed in wild-type thymoctytes at concentrations up to 1 mM (data not shown). Tempol treatment (1 mM) resulted in significant protection to wild-type cells subjected to extracellular HX/XOD oxidative stress after 4 h of treatment, with little toxic effect in the absence of oxidative stress (Fig. 2F). Surprisingly, tempol did not provide protection to thymocytes from $p53^{-/-}$ mice when exposed to oxidative insult by HX/XOD (Fig. 2F), over a range of doses and concentrations (data not shown). As we have shown previously that both wild-type and *Atm*-deficient cells are rescued from oxidative insult by tempol treatment (5), these results demonstrate that the rescue of cells from an oxidative insult of HX/XOD by tempol is p53 dependent.

p53 pathway targets are affected by tempol treatment

Tempol-mediated chemoprevention of *p53*-deficient mice was reduced (Fig. 1) when compared with that seen with *Atm*-deficient mice (5), and there was a tempol-mediated p53-dependent response to protect thymocytes from an oxidative insult (Fig. 2F). To further clarify possible p53 dependent and independent effects of tempol treatment, microarray analysis was performed on wild-type and $Atm^{-/-}$ primary thymocytes *in vitro*. We found that some p53 downstream targets were affected by 1 mM tempol treatment in both wild-type and $Atm^{-/-}$ thymocytes (data not shown). Previous work demonstrated that p53 was phospohorylated at serine 15 in response to oxidative stress in human leukemic cell lines (28–30), supporting a role for p53 in a redox-mediated pathway. We hypothesized that p53 could be a target of tempol treatment, reasoning that p53 phosphorylation may be enhanced by tempol.

We examined p53 (serine 18) phosphorylation after IR with or without tempol treatment. Six and a half hours after tempol treatment, phosphorylation of p53 at serine 18 was increased in both irradiated (Fig. 3A) and unirradiated (Fig. 3A, overexposure) primary thymocytes from wild-type and $Atm^{-/-}$ mice.

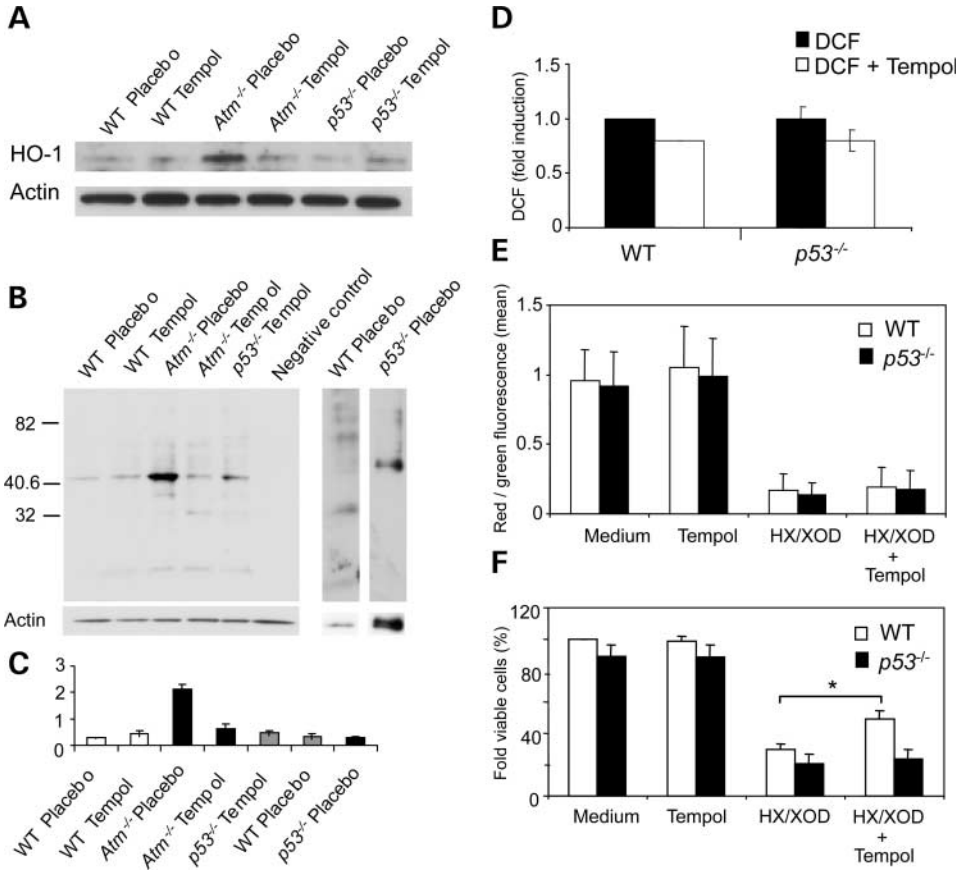

Figure 2. Tempol treatment does not further reduce oxidative damage and stress *in vivo* in $p53^{-/-}$ mice. (**A**) Levels of oxidative stress measured as HO-1 levels in the thymus of tempol and placebo-treated wild-type (WT), $Atm^{-/-}$ and $p53^{-/-}$ mice. (**B**) Oxidative protein damage measured as levels of oxidized protein carbonyls in the thymus of placebo and tempol-treated mice of the same genotypes as (A). (**C**) Quantitation of a single band across genotypes and treatments. (**D**) Levels of ROS measured by mean DCF fluorescence in primary thymocytes from wild-type (WT) and $p53^{-/-}$ mice treated with (white) and without (black) tempol, represented as fold change from wild-type control samples. (**E**) Analysis of $\Delta\Psi_m$ on primary thymocytes from wild-type (WT) (white) and $p53^{-/-}$ (black) mice by JC-1 flow cytometry. Graph represents quantitative analysis of the ratio of red (575 nm) and green fluorescence (525 nm) of four independent experiments for each genotype and treatment; wild-type (WT) in white and $p53^{-/-}$ in black. (**F**) MTT survival analysis of primary thymocytes from wild-type (WT) in white and $p53^{-/-}$ in black; mice that were untreated (medium), treated with tempol (tempol), treated with an extracellular oxidative insult (HX/XOD) and treated with an oxidative insult plus tempol (HX/XOD + tempol). Data are shown as mean ± SEM (*$P < 0.05$).

There was no significant effect on p53 phosphorylation at 30 min or 3 h post-IR (data not shown). No phosphorylated p53 (serine 18) was detected in lysates from $p53^{-/-}$ lymphoma cell lines (data not shown). Tempol treatment increased phosphorylation of p53 by ~4-fold in both wild-type and $Atm^{-/-}$ thymocytes after IR treatment (Fig. 3B). As expected, wild-type cells demonstrate a stronger phosphorylation of p53 in response to IR when compared with $Atm^{-/-}$ cells (Fig. 3A and B), because *Atm* is the primary PI(3)K involved in this phosphorylation event (13,21). The comparable increase of p53 phosphorylation in $Atm^{-/-}$ thymocytes demonstrates that another PI(3)K, besides ATM, may be involved in this response.

To determine whether p53 phosphorylation was biologically relevant, we measured p21 expression in response to tempol treatment and 2.5 Gy IR in primary thymocytes from both wild-type and $Atm^{-/-}$ mice (Fig. 3C). p21 expression was slightly but not significantly increased in irradiated wild-type thymocytes treated with tempol. However, there was a significant increase in p21 expression in $Atm^{-/-}$ tempol-treated thymocytes after IR (Fig. 3C and D; $P < 0.05$), demonstrating that tempol-mediated enhancement of p53 phosphorylation was functionally relevant.

In order to investigate whether tempol-mediated increase in p21 expression was p53 dependent, $p53^{-/-}$ thymocytes were treated with 1 mM tempol and 2.5 Gy IR. Six hours after tempol and IR treatment, p21 expression was unchanged in $p53^{-/-}$ thymocytes *in vitro* (Fig. 3C and D). This suggests that the observed tempol-mediated increase in p21 expression is p53 dependent.

To determine whether PI(3)K activity was generally enhanced by tempol treatment, we examined γ-H2AX phosphorylation. γ-H2AX is a direct target of ATM and DNAPK, two members of the PI(3)K family, via phosphorylation on serine 139 in response to DNA damage (40). Irradiated wild-type thymocytes treated with tempol *in vitro* did not demonstrate increased levels of γ-H2AX phosphorylation in response to tempol treatment (Fig. 3A and 3B, far right panels), and similar findings were also observed in $Atm^{-/-}$ thymocytes (data not shown).

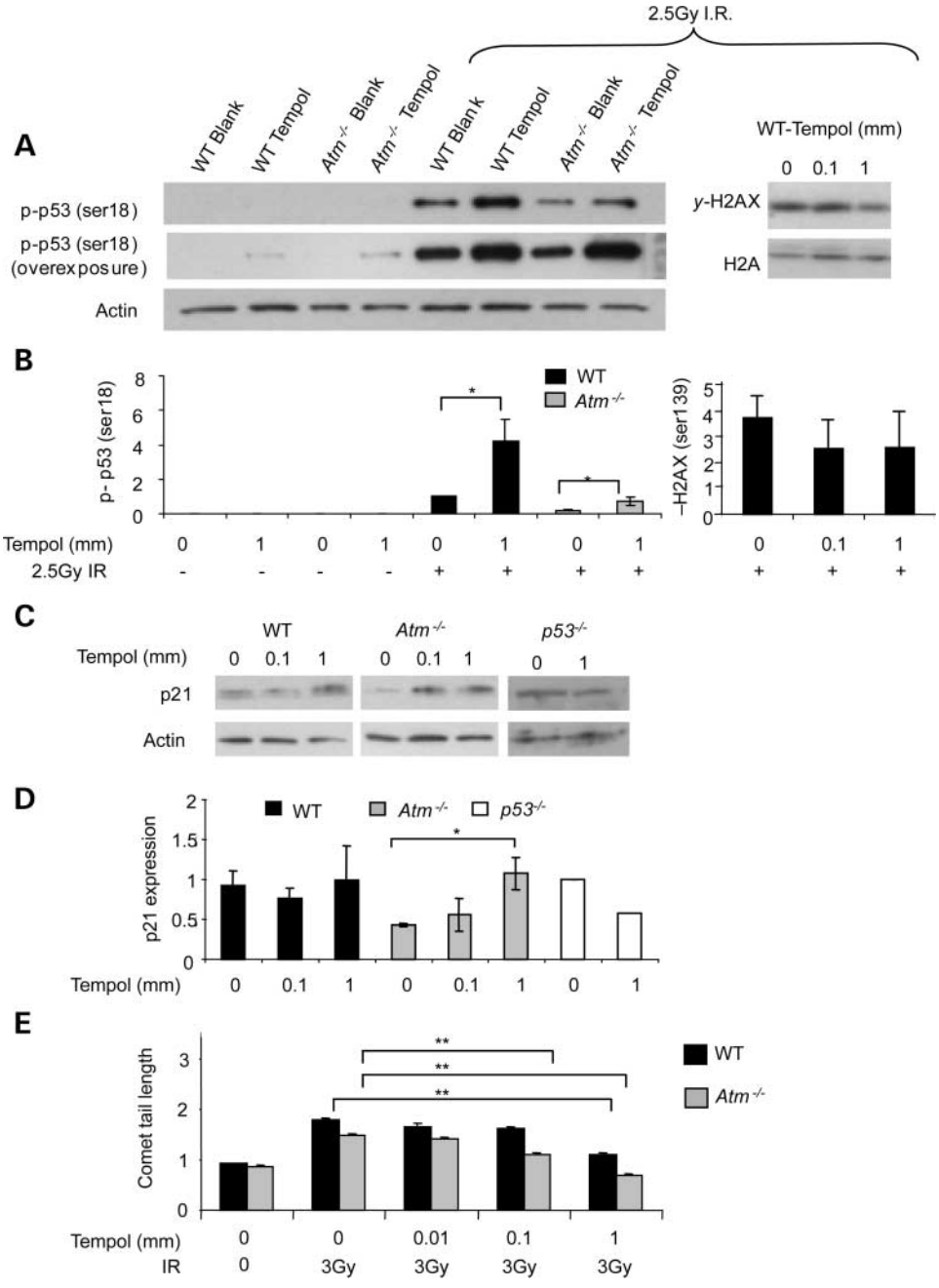

Figure 3. Tempol treatment increases phosphorylation of p53 at serine 18 and induces p21 expression *in vitro*. (**A**) Left panel: Western blot analysis of the levels of phosphorylation of p53 at serine 18 in both wild-type (WT) and $Atm^{-/-}$ primary thymocytes, with an actin loading control. Right panel: Representative western blot of γ-H2AX phosphorylation at serine 139 in primary thymocytes from wild-type mice with an H2A loading control. (**B**) Left panel: Quantitation of phosphorylation of p53 at serine 18 versus actin for three mice of each genotype. Right panel: Quantitation of γ-H2AX phosphorylation versus H2A loading control for three wild-type (WT) mice. (**C**–**D**) Measurement of p21 protein expression level, with an actin loading control. (C) Representative western blot of p21 expression in primary thymocytes from wild-type (WT), $Atm^{-/-}$ and $p53^{-/-}$ mice. (D) Quantitation of p21 expression versus actin loading control for three mice of each genotype, no SEM is shown for $p53^{-/-}$ because $n = 2$. (**E**) Tempol treatment reduces DNA damage in primary thymocytes after IR. DSBs were analyzed by the comet assay that specifically recognizes this form of DNA damage. Reading left to right: Primary thymocytes from wild-type (black) and $Atm^{-/-}$ (gray) mice treated with 3 Gy of IR with increasing concentrations of tempol. IR significantly increased comet tail length, demonstrating that this assay can detect DSBs ($^{*}P > 0.05$, $^{**}P < 0.01$).

This demonstrates that the tempol-mediated activation of p53 and p21 induction was not due to general increases in PI(3)K family member activity but resulted from specific activation of p53.

Tempol reduces DNA DSBs *in vitro*

As Atm and p53 are important mediators of DNA damage responses, and activation of p53 suggests an increase in

DSBs, we investigated the possibility that tempol may be increasing the amount of DNA DSBs in thymocytes *in vitro*, increasing phosphorylation of p53. We performed a comet assay that specifically examines DNA DSBs (41) because Atm and p53 mediate responses to this form of DNA damage (42). There were no significant differences in comet tail length between untreated wild-type and $Atm^{-/-}$ thymocytes (Fig. 3E). Primary thymocytes from either genotype treated with 3 Gy of IR displayed a significant increase in comet length (Fig. 3E), demonstrating that this assay can detect DSBs. There was a significant decrease in IR-induced DSBs when thymocytes from either wild-type or $Atm^{-/-}$ mice were pre-treated with 0.1 mM and 1 mM Tempol (Fig. 3E, $^{**}P < 0.01$). Thus, tempol treatment is not mediating p53 activation via increased DNA DSBs in both wild-type and *Atm*-deficient thymocytes and further suggests that tempol activates ROS-mediated signaling pathways to increase phosphorylation of p53 and activate this pathway.

Inhibition of SMG-1 reduces the tempol-mediated increase in phosphorylation of p53 at serine 18

Acute p53 phosphorylation at serine 18 in response to IR is SMG-1 and ATM-dependent process, but other PI(3)K family members (e.g. ATR) are also implicated in this phosphorylation event at later times and could be activated by tempol (13,21,23,25). Of the PI(3)K family member proteins, only ATM, SMG-1 and ATR phosphorylate p53 at serine 18 (13,21,23–27). At 6 h post-IR, we inhibited these PI(3)K members in tempol-treated irradiated primary thymocytes with two commonly used PI(3)K inhibitors, wortmannin and LY294002. Wortmannin has an IC50 of 2 μM for SMG-1 (23), 5 μM for ATM and DNAPK and 100 μM for ATR (43). The DNAPK-specific inhibitor LY294002, which does not affect the activities of ATM or ATR, has an IC50 of 100 μM for DNAPK (40,44,45). There was no effect of DMSO vehicle treatment alone on phosphorylation of p53 (data not shown). Both wild-type and $Atm^{-/-}$ thymocytes demonstrated a significant increase in phosphorylation of p53 at serine 18 in response to tempol treatment after IR (Fig. 3B). However, wild-type thymocytes had a stronger response than $Atm^{-/-}$ thymocytes (Fig. 4A). To better compare phosphorylation response across genotype and treatment, they were separated by genotype (Fig. 4B, wild-type and C, $Atm^{-/-}$). Treatment with 200 μM wortmannin blocked all PI(3)Ks (40), completely eliminating phosphorylation of p53 (Fig. 4B and C). ATM, SMG-1 and DNAPK (but not ATR) were inhibited in both wild-type and $Atm^{-/-}$ cells by 10 μM wortmannin treatment. This treatment resulted in reduced phosphorylation of p53 in both wild-type and *Atm*-deficient thymocytes, suggesting that ATR is not involved in this response (Fig. 4B and C). The DNAPK inhibitor, LY294002, did not affect the increased phosphorylation of p53 in response to tempol in wild-type cells (Fig. 4B), confirming that this PI(3)K is not involved in tempol enhanced p53 phosphorylation (14). However, inhibiting SMG-1 alone with 5 μM wortmannin in both wild-type and $Atm^{-/-}$ thymocytes reduced phosphorylation of p53 after tempol treatment (Fig. 4B and C). The slight increase in phosphorylation of p53 (serine 18) in wild-type cells treated with 5 μM wortmannin is likely due to active ATM in these cells because ATM is not inhibited at this concentration (Fig. 4B). Taken together, these data suggest that SMG-1 may be specifically activated in the tempol-mediated increase in p53 phosphorylation at serine 18.

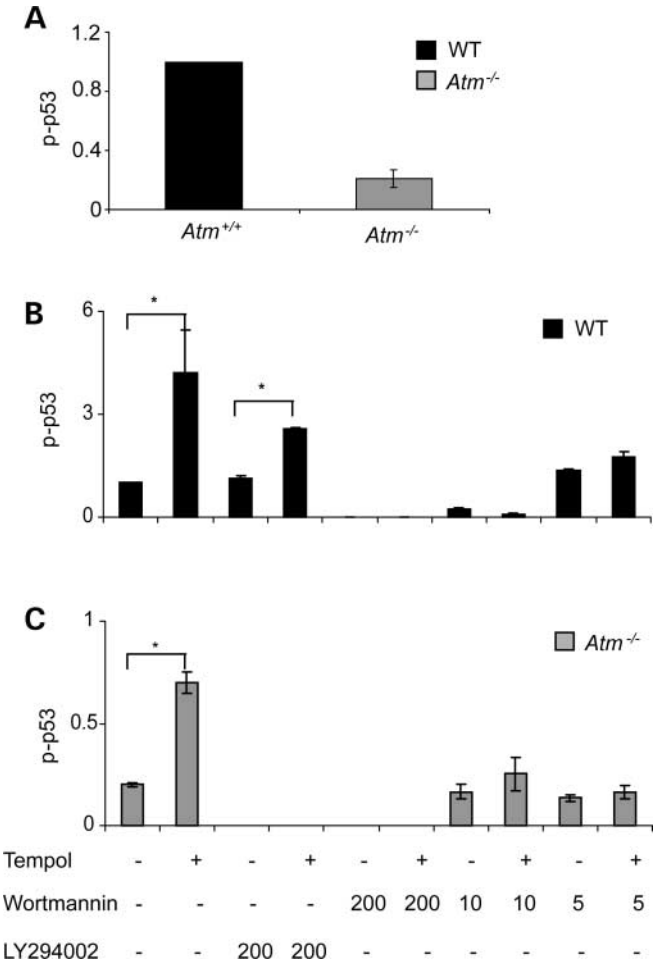

Figure 4. Measurement of p53 phosphorylation at serine 18 in primary thymocytes after treatment with PI(3)K inhibitors. (**A**) Quantitation of increased phospho-p53 (serine 18) 6 h after 2.5 Gy IR. Quantitation of phosphorylation of p53 (serine 18) after IR and tempol treatment, separated by genotype wild-type (**B**) and $Atm^{-/-}$ (**C**). Mouse primary thymocytes were treated with or without tempol in the presence of PI(3)K inhibitors, 6 h post-IR (2.5 Gy). The bottom of the graph shows the treatment. From top to bottom: tempol (1 mM treatment), wortmannin (μM units) and LY294002 (μM units). Starting from left to right: blank and tempol alone; 200 μM LY294002; 200 μM wortmannin, 10 μM wortmannin and 5 μM wortmannin ($^{*}P < 0.05$). ND, not determined.

DISCUSSION

Cancerous tissues have increased levels of ROS, oxidative stress and damage (46). ROS are important signaling molecules implicated in inducing cellular proliferation (47–51) and are also implicated in causing DNA damage, which could produce potential cancer causing DNA lesions (52,53). As such, ROS are thought to play a multifaceted role in tumor initiation, progression and maintenance (54,55).

There is evidence that decreasing intracellular ROS by treatment with antioxidants reduces cellular proliferation and cancer development (56,57). Our previous work provides support for a chemopreventative effect of antioxidants because the nitroxide antioxidant and SOD mimetic tempol increased tumor-free survival in both *Atm*-deficient (5), which display increased oxidative stress and damage (7–10), and C3H mice (6). We found that treatment of *Atm*-deficient mice with tempol increased latency to tumorigenesis and that this chemopreventative effect was associated with reduced oxidative damage and stress (5). Here, we investigated whether this effect was primarily due to reduction of oxidative stress and damage by feeding tempol to *p53*-deficient mice which do not display an oxidative stress phenotype but are cancer prone.

Tempol treatment significantly increased tumor-free survival of *p53*-deficient mice (Fig. 1), demonstrating a general chemopreventative effect of tempol on cancer-prone mice (5). However, the increase in tumor-free survival of the *p53*-deficient mice was smaller (~25%) than the doubled lifespan previously seen in tempol-treated *Atm*-deficient mice (5). Tempol treatment of $p53^{-/-}$ mice did not have any effect on oxidative stress and damage, presumably because these mice display no oxidative stress phenotype (Fig. 2B–F). The reduced effect on survival in $p53^{-/-}$ mice (Fig. 1), the lack of tempol-mediated rescue in response to an oxidative insult in $p53^{-/-}$ thymocytes (Fig. 2F) and the effect of tempol on p53 pathway targets as measured by microarray analysis (data not shown) suggest a tempol-mediated p53-dependent effect. Therefore, we considered the possibility that tempol was directly affecting p53 itself. Consistent with this hypothesis, tempol treatment increased p53 phosphorylation at serine 18 (Fig. 3A and B) and induced p21 protein expression (Fig. 3C and D). Activation of the p53 pathway by tempol was not due to increased DNA damage as measured by the comet assay (Fig. 3E). It is not surprising that altering intracellular ROS levels via antioxidant treatment results in increased phosphorylation of p53 at serine 18 because this site has been implicated in redox-mediated responses (23,30,58). Tempol specifically activated the p53 pathway because phosphorylation of another direct target of ATM, γ-H2AX, was not affected by tempol treatment (Fig. 3A and B). Treatment with PI(3)K inhibitors suggested that SMG-1 was at least in part responsible for the increased phosphorylation of p53 at serine 18 (Fig. 4). SMG-1 activation is consistent with the observation that γ-H2AX phosphorylation was unchanged after tempol treatment (Fig. 3A and B) because ATM and DNAPK are the sole PI(3)Ks responsible for the phosphorylation of this protein (40). We also demonstrated a 4-fold increase in phosphorylation of p53 (serine 18) after tempol treatment in both wild-type and $Atm^{-/-}$ thymocytes (Fig. 3A and B). Interestingly, the specific kinase activity of SMG-1 at this site is 3.5-fold higher than that of ATM *in vitro* (23).

SMG-1 is similar to ATM in amino acid sequence and function (23). It is widely expressed in human tissues and is located in both the cell nucleus and the cytoplasm (59,60). SMG-1 responds primarily to IR-induced DSBs but responds to a lesser extent to single strand DNA damage caused by UV-B light (23). *Smg-1*-deficient cells have not been produced, in the form of either transformed cell line or mouse embryonic fibroblasts, as *Smg-1*-deficient mice have not been produced. As RNAi for ATR, SMG-1 and ATM has been successfully used in human cell lines, we investigated this option (23). Unfortunately, we were unable to demonstrate an increase in p53 phosphorylation (serine 18) in response to tempol treatment in both U2OS and Jurkat tumor cell lines, as well as in $Atm^{-/-}$ lymphoma cell lines (data not shown), suggesting that the p53 pathway is functionally disrupted in these tumor cell lines. Consistent with this hypothesis, previous work has demonstrated that the p53 signaling pathway is not intact in a large number of leukemic cell lines (28,61).

Studies in our laboratory suggest that modulation of oxidative stress and damage in cancer-prone mice is not the only reason for the chemopreventative effects of tempol. When oxidative stress was increased genetically by mating *Atm*-deficient mice with mice deficient for the antioxidant enzyme *Sod1* or *Sod2*, there was no effect on latency to tumorigenesis (Erker *et al.*, manuscript in preparation). This suggests that the cause of cancer in $Atm^{-/-}$ mice was not solely due to elevated ROS levels and that reduction of oxidative stress and damage alone by antioxidants would not be sufficient to prevent tumorigenesis in these mice.

Taken together with the data presented in this article, it is reasonable to hypothesize that the chemopreventative effects of tempol are due, at least in part, to activation of the p53 pathway and modulation of redox-mediated signaling. Published results demonstrate that activation of the p53 pathway results in cell cycle checkpoint activation and increased apoptosis (13,14,62,63). We see no evidence of increased apoptosis in tempol-treated mice because thymocyte maturation is unaffected by tempol treatment. CD4+/CD8+ thymocyte populations are unchanged in all genotypes after tempol treatment for 1 month, and the number of apoptotic cells was reduced in tempol-treated $Atm^{-/-}$ thymi (data not shown). Increased phosphorylation of p53 at serine 18 and p21 induction may decrease cellular proliferation in $Atm^{-/-}$ mice (17,18). If reduced cellular proliferation is responsible for the chemopreventative effects of tempol, this delay may act either to artificially restore a defective cell cycle checkpoint or to generally prolong the cell cycle. We did not see any effect of tempol on proliferation (data not shown), probably due to the low number of proliferating thymocytes. Regardless, either cell cycle delay or reduction in proliferation would decrease cell and tumor growth, increasing latency to tumorigenesis. This decrease in growth may also slow the generation of chromosomal translocations that are required for tumorigenesis in $Atm^{-/-}$ mice (31,64).

Our findings demonstrate that tempol has significant chemopreventative effects on two cancer-prone mouse models, $Atm^{-/-}$ and $p53^{-/-}$ mice (Fig. 1) (5). We also provide evidence that tempol modulates redox-mediated cellular signaling partly through activation of the p53 pathway (Fig. 3). As the *Atm*- and *p53*-deficient mice are good models of many aspects of human AT and Li–Fraumeni syndrome, our results raise the possibility that tempol could be used in chemoprevention in humans with cancer-prone syndromes and may be especially effective if p53-dependent DSB repair and cell cycle checkpoint-deficient mechanisms are intact.

MATERIALS AND METHODS

Mice and tempol treatment

Atm-deficient mice (31) and *p53*-deficient mice (12), in 129S6 background, were used, and all animal procedures were performed according to protocols approved by both the NIH ACUC and the UCSD Animal Subjects Committee. In most cases littermates were used, and when these were not available, age-matched controls were used. Powdered tempol was mixed with bacon-flavored mouse chow (Bio-Serv, NJ, USA) at a concentration equivalent to 58 mM (10 mg/g of food) as previously described (5,6). Sixty-nine $p53^{-/-}$ mice were fed either tempol or placebo food in four different cohorts. Overall, 38 mice were fed tempol-treated food and 31 were fed placebo food (seven tempol-treated and six placebo fed mice in the first, 11 tempol-treated and 10 placebo-fed mice in the second, 10 tempol-treated and 10 placebo-fed mice in the third and 10 tempol-treated and five placebo-fed mice in the fourth cohort).

Western blot to detect oxidative stress and protein carbonyls

Thymi of age-matched mice (average of 2 months of age), fed placebo or tempol-treated food as mentioned previously, were used for analysis of HO-1 levels and carboxy-modified proteins by western blot analysis as previously described (5,65).

Intracellular ROS in primary thymocytes

Thymi were isolated from 1-month-old $p53^{+/+}$ and $p53^{-/-}$ mice, and six independent experiments were performed. 2′,7′-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes) fluorescence was performed as previously described (5).

Mitochondrial membrane potential ($\Delta\Psi_m$)

Control thymocytes and cells were treated as previously described (5). Data analysis was performed with Coulter Epics Elite software version 4.1 (Beckman Coulter Inc., Fullerton, CA, USA).

Cell viability assay

Mitochondrial respiration and cellular activity was measured in thymocytes via the MTT assay (39) as previously described (5).

Cell culture

Isolated thymocytes, *Atm*-deficient AT4 thymoma and *p53*-deficient lymphoma cells, were derived from primary tumors of thymic origin (31). These cells were cultured as previously described (5). All primary thymocytes and cell lines were grown at 37°C and 5% CO_2.

Microarray analysis

Thymocytes from 2-month-old wild-type and $Atm^{-/-}$ mice were isolated and treated with or without 1 mM tempol for 6 h ($n = 1$). RNA was isolated by the standard Trizol method (Invitrogen), resuspended in DEPC water and checked for integrity via agarose gel electrophoresis. The MG-U74AV2 chips were used (Affymetrix). Data was analyzed with dCHIP software (Dr Cheng Li, Harvard). p53-specific targets were specifically measured by comparing fold change in expression from wild-type untreated.

Western blot analysis

Cells were isolated from age-matched mice (average of 2 months of age) and treated with tempol 30 min prior to 2.5 Gy IR. Western blot analysis was done as previously described (5,66). Phospho-p53 (serine 18) (Cell Signaling Technology no. 9284S) and actin (Santa Cruz Biotech, Santa Cruz, CA, USA, sc-1616) were incubated at 1:500 in 3% milk TBS-T; p21 c-19 (Santa Cruz Biotech, sc-397) was incubated at 1:200 in 3% milk TBS-T and γ-H2AX (serine 139) (Upstate Biotechnology no. 07164) and H2A (Upstate Biotechnology no. 07146) were incubated at 1:500 in 5% milk TBS-T. Primary antibody incubation was followed by the treatment described earlier(5). Quantitation for all blots was done in the linear range for each antibody, with total signal divided by total protein signal (actin), to calculate fold expression or phosphorylation compared with wild-type or $Atm^{-/-}$ thymocytes not treated with tempol.

Wortmannin and LY294002 inhibition of PI3-kinases

Primary thymocytes isolated from age-matched mice were treated with wortmannin (Sigma catalog no. W1628) or LY294002 (Calbiochem catalog no. 440202) 1 h prior to tempol treatment. Both wortmannin and LY294002 are dissolved in DMSO, so 1 μl or less was used, and a DMSO control was also run in tandem with the other samples. Tempol was added 30 min prior to 2.5 Gy IR. Western blot analysis for phospho-p53, p21 and actin was performed as described previously.

Comet assay

To measure DNA DSBs, $Atm^{+/+}$ and $Atm^{-/-}$ thymocytes were treated with 10 μM tempol for 30 min in DMEM +5% FCS. Cells were then irradiated with a Shepard Model I Cs^{137} source at 3 Gy/min (UCSD Cancer Center) and incubated for 3.5 h. The non-denaturing comet assay was performed (53) and slides prepared according to published protocols (67), with 2.5×10^5 cells plated per slide. Slides were electrophoresed at 75 V for 20 min in 1× TBE, dipped in methanol, dried overnight and stained with 2 μg/ml ethidium bromide. Comets were visualized with a fluorescent microscope (Leica) and images were captured using a Spot2 cooled CCD camera. For each mouse, 100 comet tails/treatment were measured in Adobe Photoshop ($n = 400$ cells).

Statistical analysis

Survival time of mice was used to generate Kaplan–Meier survival curves that were compared using log-rank

(Mantel–Cox) test. A two factorial analysis of variance (ANOVA) (genotype × treatment) was used to analyze data from measuring intracellular ROS in thymocytes. Post-hoc comparisons were made using Scheffe's *F*-test for measuring intracellular ROS in thymocytes. For the mitochondrial membrane potential (JC-1), MTT assay, p53, p21 and γ-H2AX phosphorylation experiments, an unpaired Student's *t*-test was performed.

ACKNOWLEDGEMENTS

We would like to thank Judy Nordberg at the San Diego VA Hospital FACS Core for help and advice with flow cytometry. Dr Cheng Li of the Wing Wong Lab graciously provided the DNA-Chip Analyzer (dChip), Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard School of Public Health. We are grateful to Joe Aguilera of the UCSD Cancer Center Irradiation Core. We are also grateful to Amy Sullivan, Jennifer MacArthur and Scott E. Williams for technical help to Robert Abraham, Christoph Geisen and Courtney Havens, for their help and sharing of reagents, as well as to Jeff Long for his assistance with the statistical analysis in this paper.

Conflict of Interest statement. None declared.

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