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RESEARCH ARTICLE

A systems-level approach to understanding transcriptional regulation by p53 during mammalian hibernation

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ABSTRACT

Presumably to conserve energy, many mammals enter into hibernation during the winter. Homeostatic processes such as transcription and translation are virtually arrested. To further elucidate transcriptional regulation during hibernation, we studied the transcription factor p53. Here, we demonstrate that changes in liver mRNA and protein concentrations of known regulators of p53 are consistent with activation. p53 mRNA and protein concentrations are unrelated. Importantly, p53 protein concentration is increased ~2-fold during the interbout arousal that punctuates bouts of torpor. As a result, both the interbout arousal and the torpid state are characterized by high levels of nuclear-localized p53. Chromatin immunoprecipitation assays indicate that p53 binds DNA during the winter. Furthermore, p53 recruits RNA polymerase II, as indicated by nuclear run-on data. However, and consistent with previous data indicating an arrest of transcriptional elongation during torpor, p53 'activity' does not result in expected changes in target gene transcripts. These data demonstrate the importance of using a systems level-approach in understanding a complex phenotype such as mammalian hibernation. Relying on interpretations of data that are based on steady-state regulation in other systems may be misleading in the context of non-steady-state conditions such as torpor.

KEY WORDS: Torpor, Nuclear run-on, Metabolic depression

INTRODUCTION

During the winter, hibernating mammals such as ground squirrels oscillate between bouts of metabolic depression (torpor), wherein core body temperature may be below −2°C, and euthermic periods (interbout arousals), wherein body temperature approaches euthermic values (~36°C) (van Breukelen and Martin, 2002; Carey et al., 2003). Oxygen consumption during torpor may be as low as 1/100th of the active rate (Carey et al., 2003). Hibernation represents a unique metabolic constraint. Hibernators must balance a need to conserve energy with maintenance of cellular structure. Many energetically expensive physiological processes normally vital to homeostasis are dramatically reduced during torpor. Processes such as transcription and translation are not sustainable given the severe metabolic depression. Earlier, we demonstrated marked depressions of both hepatic transcription and translation (van Breukelen and Martin, 2001; van Breukelen and Martin, 2002). Nuclear run-on data from hibernating golden-mantled ground squirrels demonstrate a moderate inhibition of transcriptional initiation (~50%), but indicate that elongation of transcripts is essentially arrested during torpor (van Breukelen and Martin, 2002). Translational regulation during hibernation is more elaborate. Polysome analyses reveal that the bulk of initiation is depressed as animals enter into torpor (van Breukelen and Martin, 2001). 4E-binding protein 1, a potent regulator of the cap binding protein, eIF4E, is absent in summer, present in winter, and differentially phosphorylated between torpor and the interbout arousal. These data suggested a role for cap-independent mechanisms in promoting differential translation (van Breukelen et al., 2004). Transcripts bearing internal ribosome entry sites preferentially accumulate on ribosomes during the torpor bout (Pan and van Breukelen, 2011).

Some investigators noted that transcription factors such as p53 and NF-κB move in and out of the nucleus during the torpor cycle (Fleck and Carey, 2005; Allan and Storey, 2012). An earlier study indicated that nuclear p53 protein concentration was significantly reduced by 4-fold in torpid ground squirrels (Fleck and Carey, 2005). A seemingly plausible interpretation was that such movement must have functional significance. In other words, it was hypothesized that differential transcription was evident in hibernators. Given our previous work demonstrating a virtual arrest of transcription, we address this issue in the present study of golden-mantled ground squirrels [*Spermophilus lateralis* (Say 1823)] by exploiting a systems-level approach towards understanding whether the transcription factor p53 is functional during mammalian hibernation. p53 protein is a well-known transcription factor that plays a key role in the response to a variety of cellular stresses, such as DNA damage, hypothermia, hypoxia and metabolic stress (Hirao et al., 2000; Zhang et al., 2010; Koumenis et al., 2001; Puzio-Kuter, 2011). The half-life [~20 min under normal conditions (Maltzman and Czyzyk, 1984)] and activity of p53 is extremely well regulated by a tremendous number of processes; estimates are that at least 160 known proteins contribute to the regulation of p53 (Toledo and Wahl, 2006).

RESULTS

mRNA or protein concentrations of five known p53 regulators are consistent with activation of p53 during steady-state conditions

Many researchers use mRNA or protein concentrations of known regulators of p53 to indicate p53 activity in steady-state conditions (e.g. Ofir-Rosenfeld et al., 2008; Kim et al., 2007). We chose five well-described regulators – p53BP2, p53INP1, Mdm2, Mdm4 and RPL26 – all of which were consistent with an activation of p53 during winter. p53BP2 works as an activator of p53 and its mRNA concentrations were significantly increased by ~2.6-fold during winter (P=0.003; Fig. 1A). Protein concentrations of RPL26, another p53 activator, were significantly increased nearly 2-fold during winter (P=0.005; Fig. 2C). In contrast, Mdm2 works as an inhibitor of p53 and its protein concentrations were significantly reduced by 30–40% during winter (P=0.005; Fig. 2A). mRNA concentrations of

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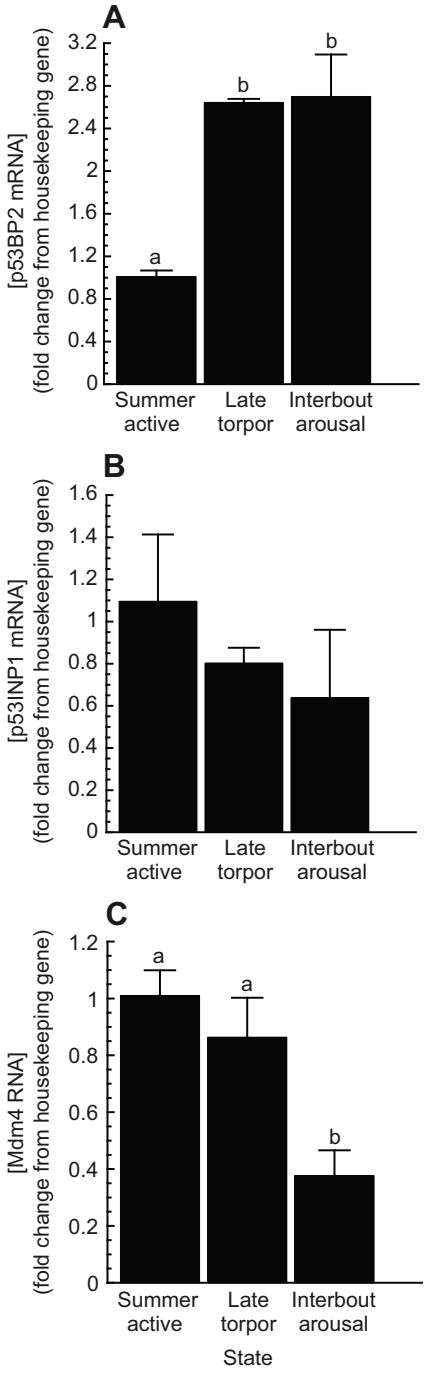

Fig. 1. mRNA concentration of known p53 regulators in liver as a function of torpor state. (A) p53BP2, (B) p53INP1 and (C) Mdm4. Relative expression values are shown as fold change ± s.e.m. for triplicates in reference to the housekeeping genes GAPDH or actin. Treatment groups with different letters are significantly different (P<0.05, ANOVA with Tukey *post hoc* analysis).

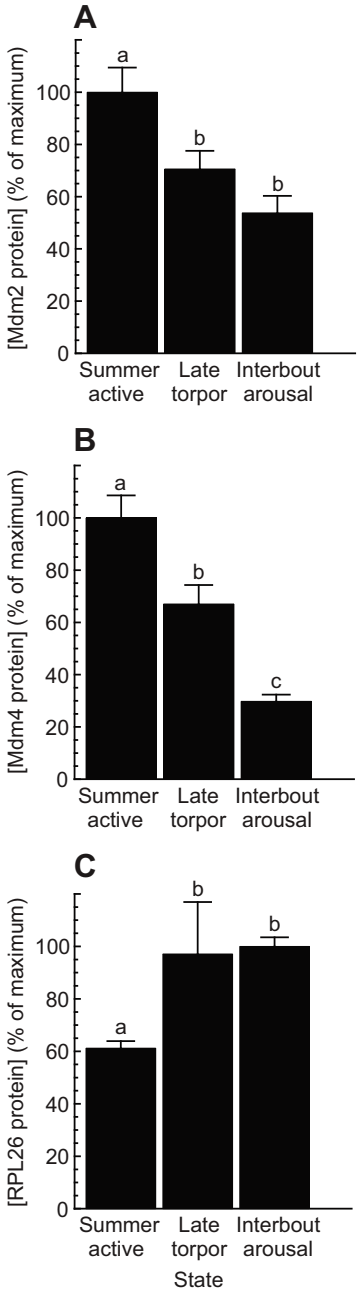

Fig. 2. Protein concentration of known p53 regulators in liver as a function of torpor state. (A) Mdm2, (B) Mdm4 and (C) RPL26. Protein concentrations were normalized to actin protein concentration; n=3 animals for each torpor state. Treatment groups with different letters are significantly different (P<0.05, ANOVA with Tukey *post hoc* analysis). Western blot images are available as supplementary material Fig. S2.

Mdm4, another p53 inhibitor, remained unchanged in late torpor (LT), but were significantly reduced by ~60% during interbout arousal (IBA) (P=0.014; Fig. 1C). Protein concentrations of Mdm4 were significantly reduced by 30–70% during winter (P=0.007; Fig. 2B). p53INP1 can either inhibit or promote p53 activity. mRNA concentrations of p53INP1 remained unchanged as a function of torpor state (P=0.23; Fig. 1B). These results are summarized in supplementary material Fig. S1.

Protein and mRNA concentrations of p53 are not related

qRT-PCR data indicate that p53 mRNA concentrations were reduced by ~60% in LT, but restored during IBA, as compared with summer active (SA) values (P=0.008; Fig. 3A). In contrast to mRNA concentration, western blot analyses indicate that p53 protein abundance was unchanged between LT and SA (P=0.13; Fig. 3B,C). These data underscore a lack of correlation between protein and mRNA concentrations. Such a result is not very surprising because the correlation between protein and mRNA concentrations may be less than 0.4 in a variety of organisms including mammals (Gygi et al., 1999; Forner et al., 2006; Gry et

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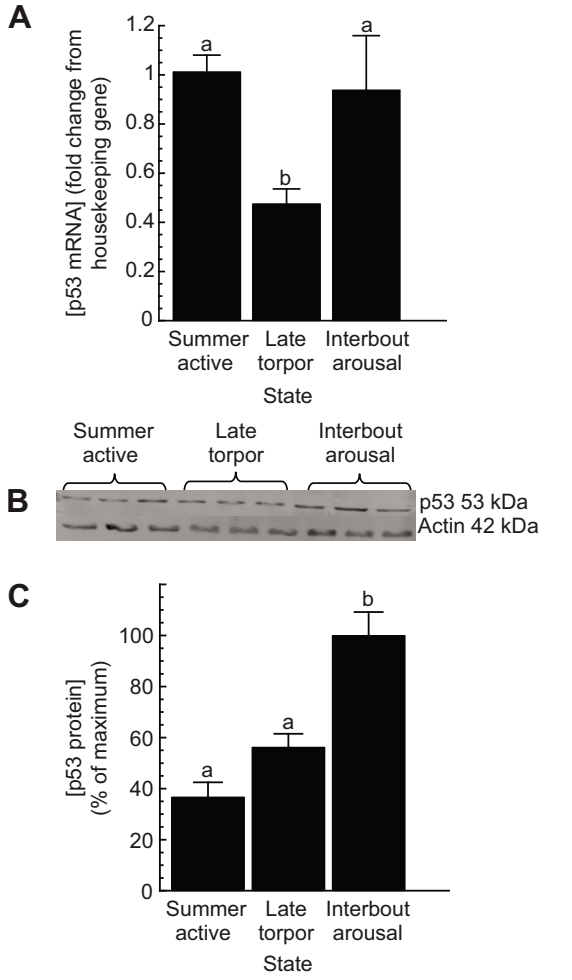

Fig. 3. mRNA and protein expression levels of p53 in liver as a function of torpor state. (A) mRNA concentrations of p53 were measured by qRT-PCR. Relative expression values are shown as fold change ± s.e.m. for triplicates in reference to the housekeeping gene GAPDH. (B) A western blot using antibodies against p53 protein was used to obtain (C) quantitative changes in p53 protein concentration as a function of torpor state. p53 protein concentration was normalized to actin protein concentration, n=3 animals for each torpor state. Treatment groups with different letters are significantly different (P<0.05, ANOVA with Tukey *post hoc* analysis).

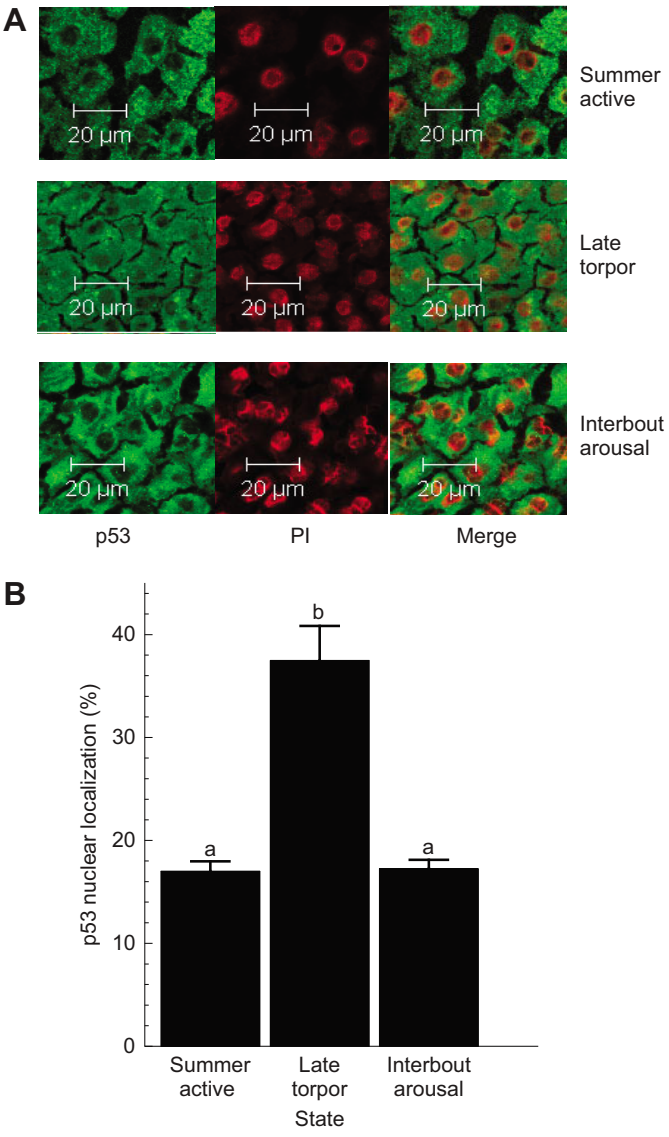

Fig. 4. Immunohistochemical analysis of subcellular localization of p53 protein. (A) Representative confocal images are shown as single and overlaid images. Green staining represents p53 protein. Red staining represents DNA in the nucleus. (B) Quantification of image analyses from A. Percent localization was determined by comparing green signal from nucleus with total green signal. One hundred clearly identifiable cells were used for each animal (n=3 animals for each torpor state). Treatment groups with different letters are significantly different (P<0.05, ANOVA with Tukey *post hoc* analysis). PI, propidium iodide.

al., 2009). Importantly, p53 protein was significantly elevated by ~2-fold during IBA (P=0.0008; Fig. 3B,C).

p53 protein is localized to the nucleus during winter

Transcription factors must be localized to the nucleus to work. Using immunohistochemical analysis followed by quantitative confocal microscopy, the subcellular localization of p53 protein as a function of torpor state was determined. Representative images of immunolocalization of p53 protein are shown in Fig. 4A. Analyses revealed an ~2-fold increase in the percentage of p53 protein found in the nucleus of LT animals (P=0.0048; Fig. 4B). We note the percentage of p53 protein found in the nucleus of IBA animals is less than that found in LT animals (P=0.0048; Fig. 4B). However, the total amount of p53 protein was increased by ~2-fold in IBA animals (P=0.0008; Fig. 3B,C). Thus, the amount of nuclear p53 protein in IBA animals is still ~82% of that found in LT animals, and significantly higher than that found in SA animals (30% of the LT value).

p53 binds to DNA during winter

Transcription factors must bind DNA to function. Chromatin immunoprecipitation (ChIP) assays were used to determine the degree to which p53 protein bound DNA. Data indicate ~40% more association of p53 with DNA during winter (P=0.0005; Fig. 5A). One p53 target gene, *mdm2*, was amplified from both ChIP DNA and Input DNA. The ChIP DNA was diluted ~50-fold to allow comparison with input DNA, suggesting that the binding of p53 was specific (Fig. 5B).

Nuclear run-on assays suggest moderate recruitment of RNA polymerase II during winter

A major result of transcription factor activity is the alteration of recruitment of RNA polymerase II and subsequent transcription of

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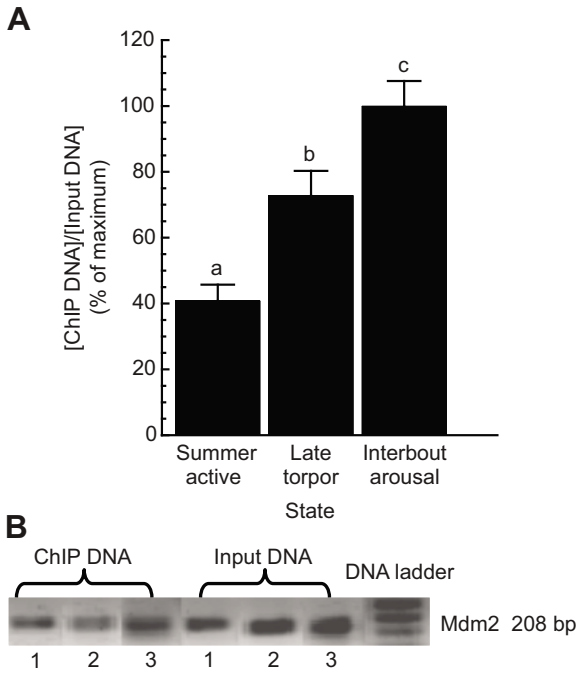

Fig. 5. Chromatin immunoprecipitation (ChIP) analysis of the association of p53 protein and DNA as a function of torpor state. (A) Results are expressed as a ratio of the concentration of immunoprecipitated DNA (ChIP DNA) to the concentration of input DNA. Treatment groups (n=3 animals for each torpor state) with different letters are significantly different (P<0.05, ANOVA with Tukey *post hoc* analysis). (B) PCR confirmation of the presence of known p53 target gene Mdm2 in ChIP and input DNA. ChIP DNA was diluted 50-fold prior to use to allow for comparison with input DNA. Lanes were cut and reassembled in this figure to allow for comparison.

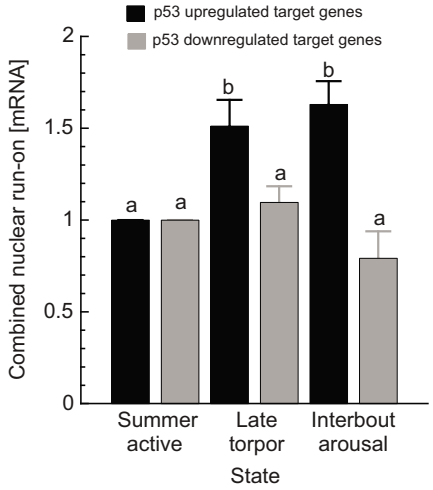

Fig. 6. Summary data for qRT-PCR-based nuclear run-on assay to determine whether p53 recruited RNA polymerase II to known target genes. Details of target genes are given in Table 1. Treatment groups with different letters are significantly different (P<0.05, ANOVA with Tukey *post hoc* analysis).

target genes. In a nuclear run-on assay, isolated nuclei are afforded the opportunity to elongate pre-initiated transcripts. When nuclei are allowed to elongate under similar conditions, the rate of transcription is proportional to the recruitment of RNA polymerase II (van Breukelen and Martin, 2002). It should be noted that estimates of new transcriptional initiation events *in vitro* by isolated nuclei are very low (1–2%). Thus, the nuclear run-on assay allows for an assessment of the amount of transcriptional initiation that had occurred at the moment of isolation. Known upregulated and downregulated p53 target genes were quantified using qRT-PCR. Linearity of the assay was ensured by restricting elongation to 5 min (van Breukelen and Martin, 2002). Further, only newly synthesized mRNA was quantified by subtracting the initial amount of mRNA in each sample. qRT-PCR-based nuclear run-on assays for known p53 target genes indicate that expression of known upregulated genes were increased by ~1.5-fold during winter as compared with SA values (P=0.0076; Fig. 6, Table 1). However, run-on activity for known downregulated genes was not statistically reduced in winter as compared with SA values (P=0.5; Fig. 6).

Target gene cellular mRNA concentrations are not consistent with p53 activity during winter

The gold standard in determining efficacy of a transcription factor is whether cellular mRNA concentrations are altered. If p53 were an effective transcriptional regulator, one would expect that mRNA concentrations of upregulated genes would be increased. qRT-PCR indicates that cellular mRNA concentrations of 'upregulated' target genes were significantly reduced during torpor, with the exception of *GADD45A*, whose mRNA concentration was significantly increased by ~1.5-fold in LT (P=0.0035; Fig. 7). mRNA concentrations of 'downregulated' genes were similarly reduced during winter from SA values, except for *CRYZ* in LT (P=0.0068; Fig. 8). These results suggest that p53 is ineffective in modulating upregulated and downregulated target genes, implying little role of p53 in directing differential transcription during torpor.

DISCUSSION

Hibernation is manifested by global decreases in transcription and translation in accordance with metabolic demands. Depending on ambient temperature, oxygen consumption during hibernation may be reduced to as low as 1/100th of active rates (Wang and Lee, 2000). When one considers that transcription accounts for as much as 10% of standard metabolic rate in mammalian cells (Rolfe and Brown, 1997), it follows that transcription must be severely depressed during torpor. Incorporation of radiolabeled precursors into RNA is severely reduced during torpor depending on tissue type (Osborne et al., 2004; Bocharova et al., 1992). Because organs such as the heart and brain must function during torpor, it seems likely that these tissues would experience higher transcriptional activities than tissues such as muscle. Indeed, such differences have been noted (Osborne et al., 2004). Use of nuclear run-on assays from liver allowed for mechanistic insight into how this transcriptional depression is effected; overall, transcriptional initiation is reduced by ~50% during torpor, but the cold temperatures typical of hibernation would essentially arrest elongation (van Breukelen and Martin, 2002). Data from nuclear run-on assays in hibernating hamsters are consistent with a significant role for depression of elongation as well (Osborne et al., 2004; Berriel Diaz et al., 2004).

One might expect that employment of a hibernation phenotype, with the associated reduction in metabolism, might be accompanied by dramatic changes in gene expression. Tempering that expectation is how such changes could be accomplished given the reductions in transcription. Perhaps differential mRNA degradation and/or transcriptional efforts during phases such as the entrance stage of hibernation, before there are large changes in body temperature,

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Table 1. Effect of hibernation state on transcriptional recruitment by p53

Category	Gene	Summer active	Late torpor	Interbout arousal
Upregulated target genes	*GADD45A*	1.00±0.08	1.51±0.20	1.65±0.38
	BAX	1.00±0.14	0.91±0.17	1.75±0.33
	PIG3	1.00±0.23	1.98±0.50	1.87±0.48
	IGFBP3	1.00±0.09	1.73±0.29	1.43±0.20
	maspin	1.00±0.03	1.75±0.21	2.17±0.23
	DDB2	1.00±0.25	1.11±0.30	1.18±0.32
	DRAM	1.00±0.08	1.59±0.23	1.36±0.18
Downregulated target genes	*CRYZ*	1.00±0.14	1.13±0.17	0.64±0.13
	CDC25C	1.00±0.15	1.04±0.18	0.52±0.09
	SLC38A2	1.00±0.11	1.32±0.25	1.19±0.20
	ODC1	1.00±0.04	0.90±0.08	0.82±0.11
Housekeeping gene	*actin*	1.00±0.03	0.97±0.06	0.98±0.09

Values are relative fold changes in mRNA concentration from nuclear run-on assays for p53 target genes. Values after 5 min of transcriptional elongation in isolated nuclei were compared against values prior to elongation (time 0). Known upregulated p53 target genes include growth arrest and DNA damage inducible gene (*GADD45A*); Bcl-2 associated X gene (*BAX*); p53 inducible gene 3 (*PIG3*); insulin-like growth factor-binding protein 3 (*IGFBP3*); serpin peptidase inhibitor, clade B ovalbumin, member 5 (*maspin*); DNA damage-binding protein 2 (*DDB2*); and DNA-damage regulated autophagy modulator 1 (*DRAM*). Known downregulated p53 target genes include crystallin, zeta (quinone reductase; *CRYZ*); cell division cycle 25 homolog C (*CDC25C*); sodium-coupled neutral amino acid transporter 2 (*SLC38A2*); and ornithine decarboxylase 1 (*ODC1*). *actin* was used as a non-target gene control. Values are represented as means ± s.e.m.; *n*=3 animals per group. Summer active values were arbitrarily set to 1.

could yield significant changes in transcript abundance. There have been numerous investigations of differential gene expression during torpor (Srere et al., 1992; Epperson et al., 2004; Schwartz et al., 2013; Morin and Storey, 2005; Yan et al., 2006; Yan et al., 2008). Although some mRNAs such as α_2-macroglobulin (Srere et al., 1992) and thyroxine-binding globulin (Epperson et al., 2004) have been observed to change by 5- to 15-fold during torpor, most mRNA abundances are unchanged. The relative paucity of changes observed in the above-mentioned studies suggests few substantial changes to gene expression. Furthermore, total mRNA concentrations are surprisingly constant throughout the hibernation cycle, suggesting that there is little net mRNA degradation (Frerichs et al., 1998; O'Hara et al., 1999).

There are reports of transcription factor movements, including p53, into and out of the nucleus during torpor (Fleck and Carey, 2005; Allan and Storey, 2012; Carey et al., 2000). A presumption is that the movement would predict transcription factor function during hibernation. Could some transcription factors work during hibernation and might this then lead to differential gene expression? Given the role of p53 in mediating stress in cells, we hypothesized that p53 may be an exemplary transcription factor to study in the context of hibernation. p53 has a role in regulating oxygen consumption and apoptosis as well as responses to hypoxia and nutrient deprivation (Vousden and Lane, 2007; Maddocks and Vousden, 2011; Puzio-Kuter, 2011). These processes are important during hibernation, e.g. apoptosis must be regulated during hibernation (van Breukelen et al., 2010).

At least 160 proteins have been reported to regulate p53 activity (Toledo and Wahl, 2006). During steady-state conditions, changes in mRNA or protein abundance of p53 regulatory proteins have

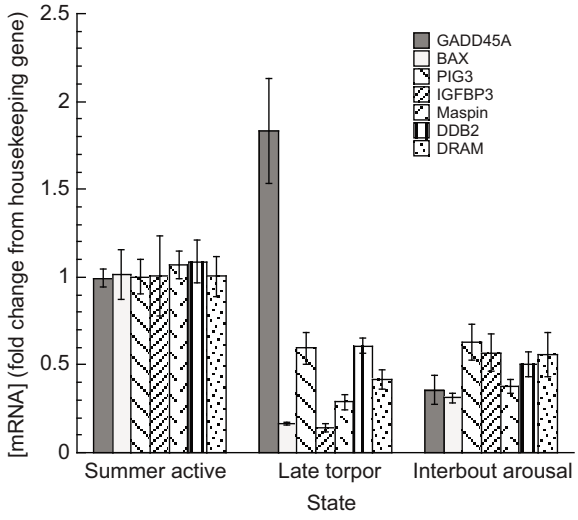

Fig. 7. Cellular concentrations of mRNAs for known upregulated p53 target genes. The mRNA concentrations of all but GADD45 were significantly reduced in winter. GADD45 mRNA abundance was increased by ~1.5-fold in LT. Values represent mean ± s.e.m. of qRT-PCR results as in Fig. 3, except actin was used for normalization (*n*=3 animals for each torpor state).

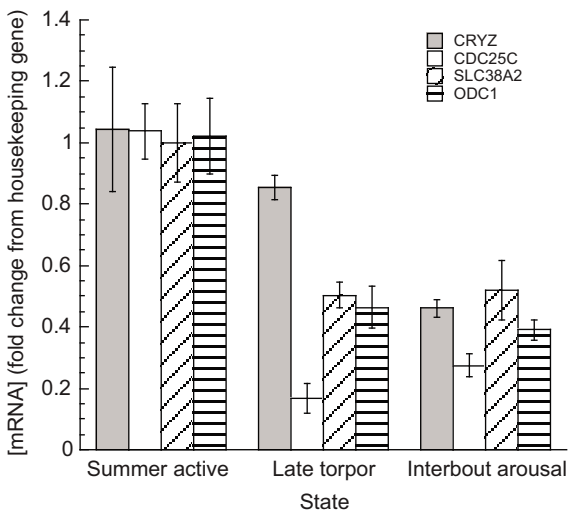

Fig. 8. Cellular concentrations of mRNAs for known downregulated p53 target genes. The mRNA concentrations of all but CRYZ (not reduced in LT) were significantly reduced in winter. Values represent mean ± s.e.m. of qRT-PCR results as in Fig. 3, except actin was used for normalization (*n*=3 animals for each torpor state).

been associated with changes in p53 transcription factor function (Ofir-Rosenfeld et al., 2008; Kim et al., 2007; Lambert et al., 1998). Changes in mRNA or protein concentration of five known regulators of p53 are seemingly consistent with an activation of p53 as a transcription factor during winter in hibernators (supplementary material Fig. S1). It is important to note these data do not actually address activity per se. To understand whether p53 works as a bona fide transcription factor during hibernation, we addressed its ability to effect changes in transcript abundance.

As a result of changes in either p53 protein concentration (IBA; Fig. 3) or localization (LT; Fig. 4), p53 accumulates in the hepatocyte nucleus of winter animals. These data are inconsistent with a previous report that indicated that nuclear p53 protein concentrations were 4-fold less in hibernating ground squirrel as compared with SA animals (Fleck and Carey, 2005). This earlier study only examined enriched nuclear fractions and no attempt was made to investigate cytosolic or total p53 protein concentrations. In the present study, quantitative immunohistochemistry and western blot analyses were performed to study subcellular localization of p53.

An important consideration of p53 transcription factor function is whether p53 binds DNA. Analysis of a ChIP assay indicates an approximately 40% increase in binding of DNA by p53 in winter squirrels (Fig. 5A). These data are rather moderate in comparison to other ChIP results. In human cancer models, p53 activation results in an up to ~20- to 30-fold increase in p53 binding (Lambert et al., 1998; Gu and Roeder, 1997; Luo et al., 2004). When one considers that availability of p53 protein in the nucleus was approximately ~2- to 2.5-fold higher in winter squirrels than in summer squirrels (Fig. 3C, Fig. 4B), a 40% increased binding of DNA by p53 in winter seems rather low, suggesting inefficient recruitment. Furthermore, a previous report indicated that p53 that was localized to the nucleus of cancer cells was transcriptionally inactive despite its binding to DNA (Wolff et al., 2001). Thus, the degree to which p53 may be directing differential transcription in hibernation could be significantly less than even the 40% suggested by the increased DNA binding revealed by the ChIP assay.

A nuclear run-on assay was employed to determine if RNA polymerase II was being differentially recruited by p53. Run-on assays of genes known to be upregulated by p53 indicate RNA polymerase II recruitment was increased ~1.5-fold during winter as compared with SA values (Fig. 6, Table 1). Data from other studies suggest that this degree of recruitment may be expected. Similar 1.5- to 3.5-fold changes in nuclear run-on activity are expected following transcription factor activation (Vyhlidal et al., 2002). However, a 1.5-fold increase in RNA polymerase II recruitment based on a nuclear run-on assay does not necessarily result in a 1.5-fold increase in cellular mRNA concentration. Because there is negligible new transcriptional initiation, nuclear run-on assays do not address a temporal aspect of transcription, e.g. p53 activity could result in multiple recruitments of RNA polymerase II across time and thus significantly higher transcript concentrations (Weaver, 2011). Importantly, run-on activity for known downregulated genes was not reduced in winter as compared with SA values (Fig. 6). That p53 may have resulted in enhanced recruitment of RNA polymerase II of upregulated but not downregulated genes is inconsistent with a well-coordinated role for p53 in regulating transcription. We contend that p53 has, at most, a rather limited role in directing differential transcription because only upregulated and not downregulated target genes appeared to have been affected and because the recruitment of RNA polymerase II for upregulated target genes was modest.

The ultimate result of transcription factor function should be the alteration of cellular mRNA concentrations. Transcript concentrations for genes known to be upregulated and downregulated by p53 were generally much lower during winter than during summer (Figs 7, 8). These data clearly suggest ineffective regulation of transcription by p53 during torpor. One could argue that perhaps p53 function during hibernation defended mRNA concentrations and that those concentrations would be even lower without an effective p53. However, the fact that both upregulated and downregulated genes were similarly affected does not support such an assertion. p53 activation may result in as high as 8- to 64-fold changes in mRNA concentrations of target genes (Zhao et al., 2000).

Combined with earlier data suggesting a virtual cessation of transcriptional elongation in the liver during hibernation (van Breukelen and Martin, 2002), a more parsimonious explanation for the data presented here might be one of serendipitous recruitment. Transcription initiation levels during torpor are only moderately depressed to ~50% of that of active animals (van Breukelen and Martin, 2002). The limited initiation of RNA polymerase II to DNA might be expected to result in a relatively large amount of free polymerase. A relative abundance of p53 protein in the nucleus and its associated increased binding to DNA during winter might then result in serendipitous recruitment of RNA polymerase II to p53 targets. In other words, while overall transcription initiation is moderately reduced, initiation at p53 target sites (for upregulated genes) is serendipitously increased. However, because transcriptional elongation is essentially arrested, little realized transcription results.

Complicating this interpretation are the data for IBA animals. In IBA animals, one would have expected that even serendipitously enhanced binding of p53 to DNA and the enhanced recruitment of RNA polymerase II would have resulted in changes in cellular transcript abundance. In this steady-state condition of normothermy, we speculate that alternative regulation might be in place to regulate affected mRNA concentrations, e.g. through enhanced specific mRNA degradation. For instance, if increased mRNA synthesis were accompanied by increased mRNA degradation, there may be no net change in mRNA concentration. Given the tremendous regulation on p53 metabolism, such events may not be out of the question. However, additional studies would be required to address this speculation.

An appropriate question is if p53 is not serving a role as a transcription factor, then what role does it play in hibernation? p53's cellular roles are as diverse as they are important. However, one must question whether the observed data may be the result of simply imprecise coordination of cellular processes (van Breukelen et al., 2008). SUMOylation of p53 has been found to be an important component of its localization to the nucleus (Stindt et al., 2011). Interestingly, SUMO conjugate concentrations increase significantly during torpor (Lee and Hallenbeck, 2006; Lee et al., 2007). Previous work from our laboratory suggests that the homologous process of ubiquitylation is poorly coordinated at lower temperatures (van Breukelen et al., 2008; Velickovska et al., 2005; Velickovska and van Breukelen, 2007). Increased SUMOylation of p53 might result in a nuclear localization and stabilization of p53. Even if unintended, p53 could still function, at least partially, in that context. One might then expect that hibernators, and perhaps other organisms, have evolved a mechanism to ensure that 'accidental' activation of p53 does not result in deleterious events. The data from Wolff and colleagues (Wolff et al., 2001), indicating p53 that bound DNA did not result in transcriptional activation in cancer cells, may indeed be the result of such a mechanism.

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Conclusions

Perhaps the most important lesson here is that cautious data interpretation is appropriate in the context of a depressed and non-steady metabolic state. Traditional interpretations would have suggested activation by p53 as evidenced by known regulators of p53 metabolism, p53 protein concentrations, p53 nuclear localization, p53 binding to DNA, and even p53 effects on initiation of transcription of known upregulated target genes. Utilization of a systems-level analysis of function reveals that p53 does not result in efficient and expected changes to mRNA concentrations of target genes. An incomplete systems-level approach would have resulted in a very different and misleading interpretation of the data.

MATERIALS AND METHODS

Animals and tissue collection

Golden-mantled ground squirrels (*Spermophilus* [*Callospermophilus*] *lateralis*) were captured in July or early August from Southern Nevada, California or Utah. Some squirrels were used immediately as a summer active (SA) control. The remaining squirrels were surgically implanted with temperature-sensitive radiotelemeters (model VM-FH disc; Mini Mitter, Sun River, OR, USA), which allow for precise measurement of body temperature throughout the hibernation season. Implanted squirrels were housed in an environmental chamber at 4°C and allowed to hibernate. The body temperature of torpid squirrels was ~5°C. All animals were killed by CO_2 asphyxiation except for the torpid animals. Torpid animals were killed by decapitation because of their low respiratory rates. Livers were obtained from ground squirrels representing various stages of a torpor bout: animals killed in summer used as a seasonal control (SA; n=3); animals that had completed ~80% of the predicted torpor bout [typically 7 days; late torpor (LT); n=3]; and animals that were euthermic between torpor bouts [interbout arousal (IBA); n=3]. Livers were removed, snap-frozen in liquid N_2, and stored at −80°C until use. All experiments were approved by the University of Nevada Las Vegas Institutional Animal Care and Use Committee.

Rationale for investigation of known regulators of p53 in other systems

p53 is tightly regulated by a tremendous array of motifs. We wanted to exploit techniques often utilized by other researchers to determine whether there was indirect evidence for p53 activation. Five known regulators of p53 were examined for change in either mRNA or protein abundance. mRNA concentrations of p53BP2, p53INP1 and Mdm4 were measured by qRT-PCR (primers are listed in supplementary material Table S1). The protein expression levels of Mdm2, Mdm4 and RPL26 were measured by western blot with specific antibodies against those proteins. p53BP2 binds to the DNA-binding domain of p53 and normally enhances its binding to specific consensus sequences on target genes (Iwabuchi et al., 1998). p53INP1 is thought to either enhance or decrease p53 activity depending on the interaction of homeodomain-interacting protein kinase 2 (HIPK2) and its role in ubiquitin-mediated proteolysis of Mdm2 (Tomasini et al., 2003; Wang et al., 2001). Mdm4 can bind p53 and inhibit p53 transactivation (Finch et al., 2002). Mdm2 is perhaps the best-known negative regulator of p53 as it appears to be the primary ubiquitin ligase responsible for regulating p53 via ubiquitin-mediated proteolysis (Kubbutat et al., 1997). RPL26 binds p53 mRNA and enhances p53 translation (Takagi et al., 2005).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from approximately 50 mg liver using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Quality of total RNA was verified by examining the migration of rRNA on a 0.8% agarose gel. RNA concentration was measured on a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Superscript III reverse transcriptase (Invitrogen) was used to generate single-stranded cDNA from 0.5 µg of total RNA with oligo dT and random primers, as per the manufacturer's instructions. qRT-PCR was run with PerfeCTa SYBR Green SuperMix Reaction Mixes (VWR, Radnor, PA, USA) using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). mRNA transcript abundances were determined using specific primers listed in supplementary material Tables S1 and S3. Specificity of all primers was confirmed using conventional PCR and sequencing of products. All samples were run in triplicate. All qRT-PCR reactions were run as follows: 2 min at 95°C, 15 s at 95°C, 30 s at 52°C, and 30 at 72°C (36 cycles) with a mixture containing 1 µl of cDNA template, 12.5 µl qPCR Supermix and 200 nmol l^{-1} of each primer in a total volume of 25 µl (VWR), as per the manufacturer's instructions. Data were collected during the 30 s–52°C extension step. Melt curves were performed using the following program: 1 min at 95°C, 1 min at 55°C, and 80 cycles of 10 s at 55°C with a step of 0.5°C every cycle. Melting curve analyses showed no primer–dimers or non-specific products. The efficiency of the primers was controlled as described previously (Pan and van Breukelen, 2011). Concentrations were compared using actin mRNA as a reference.

Protein sample preparation

Approximately 100 mg of frozen liver samples were pulverized in liquid nitrogen and then homogenized in 300 µl ice-cold lysis buffer A or B using a glass Teflon homogenizer (DuPont, Wilmington, DE, USA). Buffer A contained 50 mmol l^{-1} Tris-HCl, pH 7.5, 150 mmol l^{-1} KCl, 1 mmol l^{-1} dithiothreitol (DTT), 1 mmol l^{-1} EDTA, 50 mmol l^{-1} glycerolphosphate, 1 mmol l^{-1} EGTA, 50 mmol l^{-1} NaF, 10 mmol l^{-1} sodium pyrophosphate, 0.1 mmol l^{-1} orthovanadate and 50 nmol l^{-1} okadaic acid. Samples were centrifuged at 10,000 ***g*** for 10 min at 4°C to remove insoluble cellular debris. Buffer B contained 50 mmol l^{-1} Tris HCl, pH 8.3, 20% glycerol, 2% SDS and 0.4 mol l^{-1} β-mercaptoethanol. Samples were centrifuged at 10,000 ***g*** for 30 min at 4°C to remove insoluble cellular debris. The supernatant was collected, aliquoted and stored at −80°C until further analysis. Sample protein concentrations were determined by a modified Lowry assay.

Western blotting

p53 blotting

A total of 80 µg total protein samples extracted with lysis buffer A were heat-denatured at 100°C for 5 min. Samples were cooled on ice for 5 min and then electrophoresed on 12% (30:0.5 acrylamide:bisacrylamide) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to hydrophobic polyvinylidene difluoride membrane. Nonspecific protein-binding sites were blocked overnight at 4°C in 10 mmol l^{-1} Tris-HCl, pH 8, and 150 mmol l^{-1} NaCl (Tris-buffered saline; TBS) blocking solution containing 0.5% Tween-20 (TTBS) and 3% dry milk. After one 5 min wash in TBS, two successive 5 min washes in TTBS, and a final wash in TBS, membranes were incubated overnight at 4°C with goat anti-p53 polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalog no. sc-1313) at a dilution of 1:200 in TTBS with 3% milk and goat anti-actin polyclonal HRP-conjugated antibody at a dilution of 1:5000 in TTBS with 1% milk (Santa Cruz Biotechnology, catalog no. sc-1615; as a control). Washes were performed as described above. The membrane probed with p53 primary antibody was incubated with donkey anti-goat IgG-HRP secondary antibody at a dilution of 1:5000 (Santa Cruz Biotechnology, catalog no. sc-2020). Washes were performed as described above before detection was performed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a Typhoon 9410 Variable Mode Imager (GE Healthcare, Piscataway, NJ, USA).

Mdm4, Mdm2 and RPL26 blotting

The same protocol described above was used, except 50 µg total protein samples extracted with lysis buffer B were used with all three antibodies. Primary antibody incubations were performed with: a goat polyclonal antibody to Mdm4 diluted 1:200 (Santa Cruz Biotechnology, catalog no. SC-14738), followed by incubation with donkey anti-goat IgG-HRP secondary antibody at a dilution of 1:5000 (Santa Cruz Biotechnology, catalog no. SC-2020); a rabbit polyclonal antibody to phospho-Mdm2 (Ser 166) antibody diluted 1:1000 (Cell Signaling Technology, Beverly, MA, USA, no. 3521S), followed by anti-rabbit secondary antibody at a dilution of 1:5000 (Amersham Life Science, Arlington Heights, IL, USA, no. 356501); and a rabbit polyclonal antibody to RPL26 antibody diluted 1:1000 (Cell Signaling Technology, no. 2065S), followed by anti-rabbit secondary antibody at a dilution of 1:5000 (Amersham Life Science, no. 356501).

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Immunohistochemistry

Frozen livers were embedded in Tissue-Tek OCT compound (VWR Scientific) and equilibrated to −20°C for 20 min. Frozen sections were cut at a thickness of 7 µm with a Vibratome Ultrapro 5000 cryostat (Sigma-Aldrich, St Louis, MO, USA) and subsequently mounted on glass slides coated with freshly prepared 0.01% poly-L-lysine (Sigma-Aldrich). Sections were fixed with 1:1 methanol/acetone (v/v) solution for 10 min at −20°C and then rehydrated in PBS solution (140 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4, 8.1 mmol l^{-1} Na_2HPO_4, pH 7.4) for 10 min at room temperature. The fixed slides were immersed in blocking solution (3% milk in PBS) overnight at 4°C. After three 5 min washes in PBS, the sections were incubated with goat anti-p53 polyclonal IgG antibody (Santa Cruz Biotechnology, catalog no. sc-1313) diluted 1:200 in blocking solution overnight at 4°C. After three 5 min washes in PBS, the sections were reacted with Alexa Fluor 488 conjugated donkey anti-goat IgG antibody (Invitrogen, catalog no. A-11055) diluted 1:1000 in blocking solution for 1 h at room temperature. After another three 5 min washes in PBS, the sections were counterstained with propidium iodide (1 µg ml^{-1}; Enzo Life Sciences, Farmingdale, NY, USA) to label nuclei. Finally, tissue sections were visualized with laser scanning confocal fluorescent microscopy (Carl Zeiss, Jena, Germany). One hundred clearly identified cells were used for quantification for each animal.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the MAGnify chromatin-immunoprecipitation system (Invitrogen), as per the manufacturer's instructions. Briefly, 150 mg pulverized liver tissue were homogenized in PBS buffer and cross-linked in 1% formaldehyde for 10 min at room temperature. The crosslinking was quenched with addition of 2.5 mol l^{-1} glycine to a final concentration of 0.125 mol l^{-1}. The liver cells were lysed to release chromatin from the nuclei. Chromatin DNA was sheared to generate fragments of approximately 200 to 500 bp in length using a probe sonicator (Branson Sonifier 450, VWR Scientific; duty cycle: constant; output control: 3; output: 5; 30 s ON plus 2 min OFF, 16 cycles on ice; supplementary material Fig. S3). One hundred microliters of chromatin DNA was reserved as an input control (input DNA). Dynabeads Protein A/G were pre-coupled with 10 µg goat anti-p53 polyclonal IgG antibody (Santa Cruz Biotechnology, catalog no. sc-1313). Sheared chromatin DNA was incubated overnight with antibody-conjugated beads at 4°C with rotation. The binding was reversed by incubating for 15 min at 55°C. Immunoprecipitated chromatin DNA (ChIP DNA) was purified and quantified by PCR reactions with the corresponding primers listed in supplementary material Table S2 (Integrated DNA Technologies, San Diego, CA, USA). PCR reactions were performed using both ChIP and input DNA samples as templates. The PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

The concentrations of ChIP DNA were measured using Hoechst 33258 dye. Briefly, 5 µl aliquots were added to 40 µl assay buffer (10 mmol l^{-1} Tris-HCl, pH 7.0, 100 mmol l^{-1} NaCl, 10 mmol $^{-1}$ EDTA and 100 ng ml^{-1} Hoechst stain 33258), equilibrated to 30°C, and read using a spectrofluorometer (Shimadzu, Kyoto, Japan) with excitation/emission wavelengths of 350/455 nm. The fluorescence readings were compared against a standard curve (r^2=0.995) constructed from serial dilutions of herring sperm DNA (Sigma). The concentrations of input DNA were measured on a NanoDrop spectrophotometer (Thermo Scientific). To confirm that the ChIP assay pulled down target genes, mdm2 DNA was amplified from ChIP DNA and input DNA. ChIP DNA samples were diluted 50-fold before PCR amplification to allow for ready comparison with input DNA samples.

Nuclear preparation

Nuclear preparations were performed essentially as described previously (van Breukelen and Martin, 2002). Briefly, ~1 g of frozen liver tissues was gently homogenized on ice using a Dounce homogenizer in lysis buffer (10 ml 10 mmol l^{-1} Hepes, pH 7.6, 25 mmol l^{-1} KCl, 0.30 mmol l^{-1} spermine, 1 mmol l^{-1} spermidine, 1 mmol l^{-1} EDTA and 40% glycerol) with 10 strokes of pestle A (loose fitting) and two strokes of pestle B (tight fitting). After coarse filtering through a cloth towel, the homogenate was centrifuged at 800 ***g*** for 10 min at 4°C. The pellet was resuspended in 3 ml ice-cold resuspension buffer (10 mmol l^{-1} Hepes, pH 7.6, 25 mmol l^{-1} KCl, 0.15 mmol l^{-1} spermine, 0.5 mmol l^{-1} spermidine, 1 mmol l^{-1} EDTA and 10% glycerol), and carefully laid over a 0–35% gradient of iodoxanol (Optiprep; Nycomed Pharma, Oslo, Norway) prepared in the resuspension buffer. The gradient was centrifuged at 10,000 ***g*** for 20 min at 4°C. The pellet containing the nuclei appeared on the tube wall at the outer side of the rotor, which was collected and washed in 15 ml washing buffer (10 mmol l^{-1} Tris-HCl, pH 7.5, 10 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} NaCl). The resulting suspension was centrifuged at 800 ***g*** for 10 min at 4°C. The final pellet was resuspended with an equal volume of ice-cold storage buffer (50 mmol l^{-1} Tris-HCl, pH 8.3, 5 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} EDTA and 40% glycerol) and stored at −80°C until further use.

The DNA concentrations in the nuclei were measured using Hoechst 33258 dye. Five-microliter aliquots of isolated nuclei were incubated in 1 ml extraction buffer (1 mol l^{-1} NaCl, 1 mol l^{-1} NH_4OH and 0.2% Triton X-100) for 10 min at 37°C. A total of 12.5 µl of the resulting DNA solution was added to 750 µl assay buffer (10 mmol l^{-1} Tris-HCl, pH 7.0, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} EDTA and 100 ng ml^{-1} Hoechst stain 33258), equilibrated to 30°C, and read using a spectrofluorometer (Shimadzu, Kyoto, Japan) with excitation/emission wavelengths of 350/455 nm. The fluorescence readings were compared against a standard curve (r^2=0.996) constructed from serial dilutions of herring sperm DNA (Sigma-Aldrich).

qRT-PCR-based nuclear run-on assay

Nuclear run-on assays were titrated to ensure that the transcriptional activity was within the linear range, and the optimal conditions for nuclear run-on assays were determined to be 5 min at 25°C, as described previously (van Breukelen and Martin, 2002). All assays were performed in triplicate. Briefly, for the 0 min assay, one 20 µl aliquot of nuclei lysate was added to a 190.25 µl cold mixture of reaction buffer [47.8 mmol l^{-1} Tris-maleate, pH 7.6, 71.6 mmol l^{-1} KCl, 19.1 mmol l^{-1} $(NH_4)_2SO_4$, 2.3 mmol l^{-1} magnesium acetate, 1.9 mmol l^{-1} $MnCl_2$], 14.3 mmol l^{-1} DTT, 47.8 units ml^{-1} RNasin (Promega, Madison, WI, USA), NTP mix (310 µmol l^{-1} ATP, 310 µmol l^{-1} UTP, 620 µmol l^{-1} CTP, 620 µmol l^{-1} GTP), 157 µl TRIzol LS (Invitrogen) with 1 µl 20 mg ml^{-1} glycogen. For the 5 min assay, another 20 µl aliquot containing the same amount of nuclei was incubated at 25°C for 5 min in a 52.25 µl mixture containing the same amount of reaction buffer, DTT, RNasin and NTP mix. The reaction was stopped by adding 157 µl TRIzol LS with 1 µl 20 mg ml^{-1} glycogen.

Total RNAs were immediately extracted from the both solutions containing TRIzol reagents (Invitrogen) as per the manufacturer's protocol. Superscript III reverse transcriptase (Invitrogen) was used to generate single-stranded cDNA from 2 µl of total RNA with oligo dT and random primers, as per the manufacturer's instructions. qRT-PCR was run with PerfeCTa SYBR Green SuperMix Reaction Mixes (VWR) using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories). The primers used are listed in supplementary material Table S3 and actin was used as a reference gene. All qRT-PCR reactions were run as follows: 2 min at 95°C, 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C (36 cycles) with a mixture containing 1 µl of cDNA template, 12.5 µl qPCR Supermix and 200 nmol l^{-1} of each primer in a total volume of 25 µl (VWR), as per the manufacturer's instructions. Data were collected during the 30 s–60°C extension step. Melt curves were performed using the following program: 1 min at 95°C, 1 min at 55°C, and 80 cycles of 10 s at 55°C with a step of 0.5°C every cycle. Melting curve analyses showed no primer–dimers or non-specific products. The efficiency of the primers was controlled as described previously (Pan and van Breukelen, 2011).

Statistical analyses

The qRT-PCR data were analyzed to show the relative changes in gene expression levels based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and subjected to ANOVA followed by Tukey multiple comparisons tests. Relative band intensities observed by western blotting and immunohistochemical data determined with confocal microscopy were quantified densitometrically using Image Quanta software (Molecular Dynamics, Sunnyvale, CA, USA) and subjected to ANOVA followed by Tukey multiple comparisons tests. ChIP data were analyzed by ANOVA.

The nuclear run-on data were analyzed according to a previously described method using LinReg PCR (11.0) software (Ruijter et al., 2009). Results with *P*-values <0.05 were considered statistically significant. R software was used for all data analyses (R Development Core Team, 2008).

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Competing interests
The authors declare no competing financial interests.

Author contributions
P.P. contributed to conception, design and execution of the experiments. M.D.T. contributed to execution of the experiments. F.v.B. contributed to conception and design of experiments. P.P. and F.v.B. were responsible for manuscript preparation.

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