Yeast Transcription Termination Factor Rtt103 Functions in DNA Damage Response

Indukuri Srividya, Sirupangi Tirupataiah, Krishnaveni Mishra*
Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, India

Abstract
YKu70/YKu80 is a heterodimer that is essential for repair of DNA double strand breaks through non-homologous end joining pathway in the yeast Saccharomyces cerevisiae. Yku70/80 proteins are associated with telomeres and are important for maintaining the integrity of telomeres. These proteins protect telomeres from recombination events, nuclease attacks, support the formation of heterochromatin at telomeres and anchor telomeres to the nuclear periphery. To identify components in molecular networks involved in the multiple functions of Yku70/80 complex, we performed a genetic screen for suppressors of yku70 deletion. One of the suppressors identified was RTT103, which encodes a protein implicated in transcription termination. We show that rtt103Δ are sensitive to multiple forms of genome insults and that RTT103 is essential for recovery from DNA double strand breaks in the chromosome. We further show that Rtt103 associates with sites of DNA breaks and hence is likely to play a direct role in response to DNA damage.

Introduction
Genomes are constantly subjected to multiple forms of damage and if left unattended, can lead to mutations and chromosomal aberrations that result in cell death and diseases like cancer. Consequently, cells have evolved several mechanisms to detect and repair damage to the genome. Damage to DNA induces a DNA damage response (DDR) that essentially comprises of three arms. The first of these is the cell-cycle checkpoint that arrests the cell cycle in order to prevent damaged DNA from being replicated or transmitted; second, the DNA repair pathways that repair the break and lastly an apoptotic pathway leading to death of the cell if the damage is irreversible. DNA damage is generally induced by exposure to UV irradiation, genotoxic drugs, ionising radiation or other metabolites that generate reactive oxygen species. Depending on the type of lesion and the stage of cell cycle, different repair mechanisms may be activated. These pathways of repair and checkpoint mediated cell cycle arrest are conserved across eukaryotes, reviewed in [1,2].

A particularly dangerous form of damage is the DNA double-strand break (DSB). These may arise due to, among other factors, exposure of DNA to ionising radiations or genotoxic drugs. Cells repair this form of DNA damage either through non-homologous end joining (NHEJ) or through homology-mediated repair. In brief, during homology-mediated repair, such DNA lesions are recognized by the MRX complex (Mre11-Rad50-Xrs2) in budding yeast, S. cerevisiae. This in turn recruits Tel1, a DNA-dependent protein kinase that phosphorylates multiple substrates including histone H2A, which recruits repair proteins. Tel1 also phosphorylates Rad53, a protein kinase that activates Dun1, which in turn leads to induction of transcription of DNA damage inducible genes. Rad53 also induces cell cycle arrest through phosphorylation of other substrates like Cdc5 and Cdc20. The single stranded DNA generated at the breaks recruits a different set of proteins including Rpa1, that recruit another DNA-dependent protein kinase Mec1 which again induces the phosphorylation of Rad53 kinase via Rad9 kinase, leading to cell cycle arrest and transcriptional up regulation of the DDR genes (reviewed in [1]).

Although a lot is known about the different pathways that operate in repair of DNA lesions and the various checkpoint proteins that together contribute to cell cycle arrest and repair of damage, it is increasingly apparent that there are large gaps in our knowledge about the large number of cellular processes are actually required for recovery from damage. Multiple screens have been done to identify components that affect genome stability; each new screen has revealed new proteins and pathways that are involved in the response to DNA damage. Approaches involving the genome-wide measurement of transcriptional responses to DNA damage by UV or MMS show changes in transcriptional status of over 25% of the genome [3,4,5]. In competitive fitness assays using the whole genome knock-out strains of yeast, several pathways including those involved in ubiquitination, gene silencing, and transport across the mitochondrial membrane were identified [6]. Similarly in protein localization-based screens that look at the key damage sensors like H2Ax in mammals [7] or Rad52 in yeast [8], pathways involving nuclear transport, RNA processing, protein modification and chromosomal structural proteins were discovered. How all these responses contribute to recovery from DNA damage needs to be elucidated. An important outcome of these studies is the realization that even though there are a few core damage response genes and repair pathways that do
appear in most screens, a large number of new and previously unidentifed genes and molecular networks are discovered with each new screen.

In this work, we discovered that a transcription termination factor RTT103 (Regulator of Ty1 transposition) is critical for maintaining genome integrity. This work was initiated to derive insights into the function of Yku70/80 heterodimer in genome integrity. Yku70/80 is a DNA binding heterodimer consisting of Yku70 and Yku80 proteins, that are conserved from yeast to mammals [9]. They play a major role in repair of DNA breaks by NHEJ. Additionally, Yku70/80 are important for many telomeric functions, including loading of telomerase to telomeres, protecting telomeres from nucleolytic digestions, establishing stable silent chromatin at telomeres and also in anchoring telomeres to the nuclear periphery [10,11,12,13,14,15]. yku70/80 mutants are temperature sensitive and die at 37°C with enlarged budded cells that contain more than G2 DNA content [16,17].

In order to isolate interacting partners that contribute to the multiple roles of Yku proteins, we carried out a multicopy suppressor screen for temperature sensitivity of yku70Δ. In this process, we isolated RTTI03 as a partial suppressor of temperature sensitivity. We show that yku70Δ mutants are severely defective in repairing DNA breaks although they do not affect signalling of DNA damage or repair of non-chromosomal substrates by NHEJ. Further, we show that Rtt103 protein physically associates with the sites of DNA breaks and hence is likely to play a direct role in the repair of DNA breaks. This unanticipated role for Rtt103 in DNA repair adds it to the list of RNA processing factors like CSTF [18] and Sen1 [19] that have been recently shown to have roles in genome stability.

Materials and Methods

Yeast strains and plasmids

All strains used in this study were isogenic with W303 background (RAD5+) unless otherwise indicated. The genotypes of strains used are described in Table 1. Manipulations in the yeast strains were carried out by PCR based homologous recombination using vectors and methods described [20]. Gene manipulations were confirmed by Southern blots. All mutations used are gene replacements unless specified otherwise. Multicopy RTTI03 plasmid (CKM233) was constructed by digesting the KM93 plasmid with Hpal and NsiI, and ligating to YEpplac181 vector digested with PsI and Smal. Single copy RTTI03 was constructed by digesting the CKM233 plasmid with Kpnl and SphI and ligation to YCplac22 digested with Kpnl and SphI. pCH10 plasmid which contains RAD53-3myc in YCplac111 was from Marco Foiani [21].

Western blot analysis

To examine phosphorylation of Rad53, cells carrying RAD53+ were treated with 0.02% MMS for 2 hrs or left untreated and total protein extracted using TCA [21]. Briefly yeast extracts were prepared by glass bead beating in 20% trichloroacetic acid (TCA), washing the glass beads in 5% TCA and combining the wash with the lysate. The protein suspension was pelleted, re suspended in 1x Laemmli loading buffer (pH 8.0), boiled for 2 min, pelleted, and the supernatant was used for Western blots. Protein was run on 8% SDS-PAGE gels and transferred to PVDF membrane. Anti- myc staining was done with 9E10 (BabCo) antibody. Same blots were probed with Sir2p antibody (Santa Cruz) for loading control.

DNA Damage Assays

Plasmid rejoining assay. pRS313 DNA (100 µg) was digested with EcoRI to completion. This linearised DNA was then used to transform yeast by the lithium acetate method. Parallel transformations with the same competent cells were performed with an equivalent amount of uncut plasmid to enable normalisation for minor differences in transformation efficiencies between strains and between experiments. Following transformation, cells were plated and colonies arising on selective media (SC-HIS) after 3–4 days were counted. We typically obtained 1600 to 2200 transformants with the supercoiled plasmid in all strains; the linearized plasmid recovery was in the range of 1100 to 1500 in wildtype and rtt103Δ, while in yka70Δ we obtained around 40 colonies.

MMS assay. Yeast strains were grown overnight to mid-log phase in YPD medium. 10-fold serial dilutions of the strains were done and 5 µl of the sample was spotted on YPD plates and YPD containing different concentrations of MMS. The plates were incubated for 2–3 days at 30°C.

I-SceI endonuclease assay. For I-SceI endonuclease assay, KRY304 strain was used which has two I-Sce1 sites inserted in opposite orientations on each side of the URA3 gene on chromosome V. The sequence encoding the nuclease was inserted at a different locus and placed under the control of a galactose-inducible promoter. Strains were grown overnight and spread on SC-GLU and SC-GAL plates. Colonies were counted after incubation at 30°C for 4 days.

Chromosome hyper-recombination assays. The strains used for this study have ADE2 and URA3 genes flanked by lnu2-k repeats [22]. Recombination between the lnu2-k repeats results in loss of ADE2 and URA3. The number of recombinants was calculated by counting the number of colonies on SC+ FOA plates, which indicate the loss of URA3; recombination was confirmed by the appearance of red colonies showing loss of ADE2. Three independent segregants were used for quantification of recombination.

Chromatin Immunoprecipitation. ChIP experiments were done by following the method described [23]. Yeast strain KRY448 was grown in 50 ml of SC broth till OD600 reached 0.5–1.0. These cultures were cross-linked with formaldehyde and cross links were quenched with glycine. Cells were pelleted, washed with ice cold TBS buffer, and lysed with ice cold lysis buffer containing protease inhibitor. Lysates were prepared with glass beads and were then sonicated in Biorupter Sonifier at power setting of 15 sec pulse on and 2 min off to shear the chromatin to average length of 500 bp . The supernatant was pre cleared by adding 30 µl bed volume of Protein A Sepharose beads (Amersham Biosciences) and incubated for 1 hr at 4°C with constant rotation. Samples were centrifuged and 50 µl of sample was taken as input DNA. 1 µl of primary antibody against myc epitope of Rtt103p (Abcam) was added to the sample and incubated for overnight at 4°C with constant rotation. 30 µl of Protein A Sepharose beads were added to the chromatin antibody mix, and incubated for 2 hr at 4°C with constant rotation. Chromatin immunoprecipitate was eluted first with 1% SDS in TE and then with 150 µl of 0.67% SDS in TE buffer by incubating at 65°C for 10 min. DNA from bound and unbound chromatin (input sample) was purified by phenol:chloroform extraction and ethanol precipitation. DNA sample from ChIP experiments were analysed by Real-Time PCR using SYBER green master mix according to manufacturer’s instructions on an Applied Biosystems 7500 HT fast Real-Time PCR system. Primers for real-time PCR were designed by taking sequences at different regions near the URA3 gene flanked by I-Sce1 sites on chromosome V. Primer sequence information can be provided upon request. Relative quantification of immunoprecipitated DNA was done based on comparative Ct value method using sequence detection software.
Northern blots. Northern blots were done as described in [24]. RNA was extracted using Ambion RNA isolation kit. For Northern blots, 10 μg of RNA was loaded onto 1% formaldehyde agarose gels, separated by electrophoresis, and then transferred to N+ membranes. Blots were blocked for 1 hr at 55°C and then incubated with 32P-labeled DNA probes. Hybridizations were performed for 16 hr at 55°C. In order to characterize the temperature sensitivity of yku70Δ, we performed a multicity suppressor screen to identify processes that are compromised at 35°C. yku70Δ (KRY172) were transformed with a genomic library in a high copy number vector (gift from K. Nasmyth) and plated at 35°C and incubated for 2 days. Any large colony that appeared in two days was isolated and rechecked for improved growth at 35°C and 37°C. The plasmids were isolated and retransformed into yku70Δ and tested again for growth at elevated temperatures.

Two plasmids that reproducibly improved growth were found to contain the full length YKU70 gene. This plasmid (KM93) that partially suppressed the yku70Δ phenotype (Figure 1a). As shown in Figure 1a, yku70Δ is lethal at 37°C and partially defective for growth at 35°C. Upon over-expression of KM93 or just RTT103 gene, yku70Δ showed strong suppression of yku70 phenotype (not shown). The second plasmid (KM95), which partially suppressed the yku70Δ temperature sensitivity phenotype contained the complete sequence of the RTT103 gene. RTT103 was subcloned in YEplac181 vector and tested to see if it suppressed yku70 phenotype (Figure 1a). As shown in Figure 1a, yku70Δ was tested to see if it suppressed yku70 phenotype (Figure 1a). As shown in Figure 1a, yku70Δ was isolated and rechecked for improved growth at 35°C and 37°C. The plasmids were isolated and retransformed into yku70Δ and tested again for growth at elevated temperatures.

Multiple copies of RTT103 suppress the temperature and DNA damage sensitivity of yku70 mutants

yku70Δ/Δ mutants are sensitive to temperature; they grow slowly at 35°C and die at 37°C. In order to characterize the temperature

Table 1. List of the yeast strains used in this study.

| Name      | Genotype                  | Source/Reference          |
|-----------|---------------------------|---------------------------|
| KRY2      | MATa leu2-3,112 his3-11,15 ura3-1 ade2-21 trp1-1 can1-100 | Rodney Rothstein          |
| KRY3      | MA a leu2-3,112 his3-11,15 ura3-1 ade2-21 trp1-1 can1-100 | Rodney Rothstein          |
| KRY105    | MATa ade2-1 ade2-21 trp1-1 can1-100 | This Study               |
| KRY171    | KRY105 except yku70::KanMx | This Study               |
| KRY172    | KRY193 except MATa yku70::KanMx | This Study               |
| KRY193    | MATa ade2-1 URA3 TEL L | This Study               |
| KRY230    | KRY105 except MATa rtt103::KanMx | This Study               |
| KRY290    | KRY105 except MATa rtt103::KanMx | This Study               |
| KRY285    | KRY193 except MATa rtt103::KanMx | This Study               |
| KRY286    | KRY193 except rtt103::KanMx yku70::LEU2 | This Study               |
| KRY290    | KRY105 except MATa rtt103::KanMx yku70::LEU2 | This Study               |
| KRY371    | MATa Δ lys2::pGAL-ISCEI ISce1::URA3::ISce1 | This study               |
| KRY375    | KRY371 except rtt103::KanMx | This Study               |
| KRY376    | KRY371 except yku70::LEU2 | This Study               |
| KRY379a   | KRY371 rtt103::KanMx yku70::LEU2 | This Study               |
| KRY448    | KRY371 except RTT103 13Myc | This Study               |
| KRY482    | W303 rtt103 diploid | This Study               |
| KRY473    | W303 MATa rad1::LEU2 | This Study               |
| KRY631    | MATa RAI1 his3-1 leu2-10 ura3-300 (BY4739) | Arel Johnson            |
| KRY632    | KRY631 except rai1::KanMX | Arel Johnson            |
| KRY633    | MATa ura3-52 leu2-11 trp1-163 (FY23) | Arel Johnson            |
| KRY634    | KRY633 except MATa rai1-1 ts | Arel Johnson            |
| KRY615    | Mat a ade2-1 trp1, his3, ura3 can1-100 leu2-k::ADE2 URA3::leu2-k rad5-535 | Andre Aguilera          |
| KRY650    | MATa ade2-1 trp1, his3, ura3 can1-100 leu2-k::ADE2 URA3::leu2-k rtt103::KanMx rad5-535 | This study               |
| KRY652    | MATa ade2-1 trp1, his3, ura3 can1-100 leu2-k::ADE2 URA3::leu2-k rtt103::KanMx rad5-535 | This study               |
| KRY622    | MATa pGAL::HIS3::LEU2 rad5-535 | David Shore             |
| KRY624    | MATa pGAL::HIS3::LEU2 rai1::KanMx rad5-535 | This study               |
| KRY646    | MATa pGAL::HIS3::LEU2 rtt103::KanMx rad5-535 | This study               |
| KRY443    | KRY105 except mec1::TRP1, sml1::HIS3 | This study               |
| KRY445    | KRY105 except MATa rtt103::KanMx | This study               |

doi:10.1371/journal.pone.0031288.t001

Microscopy. For sporulation experiments, cultures were fixed with ethanol and stained with DAPI. Immunofluorescence experiments were performed as described in [25]. For localization of Rtt103, antibodies to myc epitope and Nsp1 were from Abcam. AlexaFluor conjugated anti mouse and anti rabbit antibodies were from Molecular Probes. Olympus inverted microscope IX61 was used to visualize and capture images.
temperature sensitivity was modest, we quantified the phenotype by plating cells at different temperatures. As shown in Figure 1b, we found that elevated dosage of \textit{RTT103} improved growth of \textit{yku70}D by 7 to 8-fold. However, \textit{RTT103} could not suppress lethality at 37°C. Therefore, we conclude that an elevated dose of \textit{RTT103} partially suppresses the temperature sensitivity of \textit{yku70} mutants.

Since \textit{YKU70} is involved in several processes including DNA repair, telomere metabolism and gene silencing we tested if multiple copies of \textit{RTT103} also suppressed any of these phenotypes. We found that while neither gene silencing nor telomere length defects of \textit{yku70} mutants were affected by \textit{RTT103} (data not shown), the DNA repair phenotype was partially suppressed by over-expression of \textit{RTT103} (Figure 2).

\textit{yku70/80} mutants are sensitive to MMS, an alkylating agent, which produces DSB during repair. Therefore, we plated wild type and \textit{yku70}D transformed with either empty vector or \textit{RTT103} on plates containing MMS. As shown in Figure 2a and Figure 2b, \textit{yku70}D mutants are over 50-fold more sensitive to MMS than wild type (row 4). Upon elevated dosage of \textit{RTT103}, there is a marked improvement in survival, and these cells are only 7-fold more sensitive than wild type, suggesting that \textit{RTT103} on multicopy plasmid partially suppresses the \textit{yku70} temperature sensitivity and MMS sensitivity. \textit{RTT103} on single copy plasmid could not suppress these phenotypes indicating that suppression requires multiple copies of \textit{RTT103} (data not shown).

\textit{rtt103} deletion enhances \textit{yku70} sensitivity to temperature and are sensitive to MMS

\textit{RTT103} was initially isolated in a screen for mutants that enhance the transposition of Ty1 elements [26]. It was later shown to participate in transcription termination [27]. Additionally, \textit{rtt103}D were also reported to increase the number of Rad52 foci [8]. Since elevated \textit{RTT103} dosage could suppress \textit{yku70} mutant sensitivity to DNA damage, we investigated if \textit{RTT103} is required for DNA repair. To this end we generated \textit{rtt103}D by replacing the complete \textit{RTT103} ORF with \textit{KanMx} (KRY285). The \textit{rtt103}D were tested for sensitivity to temperature. As shown in Figure 3a and as expected, growth of \textit{yku70}D was arrested at 37°C and impaired at 35°C. \textit{rtt103}D were not sensitive to increased temperatures; growth was comparable to that of wild type strains. However, the \textit{yku70rtt103} double deletions (rows 7, 8) showed increased sensitivity to temperature, as reflected by poor growth at 35°C when compared to \textit{yku70}D. Thus, \textit{rtt103}D exacerbates the temperature sensitivity of \textit{yku70}D.

We next checked the effect of \textit{rtt103}D on DNA repair. First, we tested sensitivity of the cells to MMS. Serially diluted cultures of wild type, \textit{yku70}D, \textit{rtt103}D and \textit{yku70rtt103} were plated on normal plates and incubated at 30°C, 35°C and 37°C for 2–3 days. (1b) Quantification of temperature sensitivity. The temperature sensitivity of \textit{yku70} was quantified by plating out appropriate dilutions of 3 independent cultures at the appropriate temperatures. Sensitivity of WT was set to 1. \textit{yku70} is approximately 40-fold sensitive and upon overexpression of \textit{RTT103}, the sensitivity to temperature is reduced by approximately 7 to 8 fold. Error bars indicate SD.

doi:10.1371/journal.pone.0031288.g001
YPD or YPD containing MMS. As shown in Figure 3b, rtt103Δ were sensitive to MMS, as much as or more than yku70Δ (compare rows 3, 4 to rows 5, 6). The double deletions were even more sensitive to MMS than either single mutant (row 7,8). These phenotypes were quantified by plating out three independent cultures: yku70Δ single mutants were 55-fold more sensitive than wild type, the rtt103Δ were 100-fold more sensitive, and the double mutants were over 700-fold more sensitive. These data indicate that rtt103 enhances the yku70 defective phenotypes in a synergistic manner.

rtt103Δ are unable to recover from DNA double strand breaks

As MMS generates multiple DNA breaks all over the genome, and could potentially affect several genome functions, we tested the role of RTT103 when single or two DSBs were introduced specifically. For this, we used a strain where two sites recognized by I-SceI endonuclease have been introduced bracketing URAS gene. This strain also contains an insertion of galactose inducible I-SceI endonuclease in the genome [28]. We generated yka70Δ and rtt103Δ deletions in this strain and tested the efficiency of colony formation on galactose plates. When I-SceI endonuclease is induced by galactose, DSBs are generated on either side of URAS gene. Wild type cells can repair the breaks and form colonies (Figure 4a). Strains that cannot repair the DNA break are expected to be growth arrested and unable to form colonies on galactose plates. We plated wild type, yka70Δ, rtt103Δ and yka70Δrtt103Δ deletions carrying the I-SceI sites and endonuclease on plates containing galactose. Numbers of full sized colonies that appeared after 3 days were counted. As shown in Figure 4a, yka70Δ, rtt103Δ and yka70Δrtt103Δ deletions were all severely affected and very few colonies could be recovered. However, wild type cells were able to form colonies as they could repair the breaks at the expected frequency [28]. We noticed that even though rtt103Δ deletions did not form many full-sized colonies, there were numerous tiny colonies on the plate. This suggests that rtt103Δ, unlike yka70Δ, underwent a few divisions before they succumbed.

RTT103 is not required for plasmid end joining or repair of UV induced damage

As the specific DSB assay indicated that RTT103 was required to repair these breaks in vivo, we directly tested if end joining was affected in rtt103Δ by a plasmid rejoining assay. This assay reports the efficiency of end-joining by NHEJ. When linearized CEN plasmids are transformed into yeast, they are recircularized by end joining (most efficient) or are integrated into the genome (less efficient). However, when end-joining is defective, as seen in yka70Δ/80 mutants or DNA ligaseIV mutants [29], very few transformants are recovered. Equal amounts of plasmids linearized with EcoRI were transformed into wild type, yka70Δ, rtt103Δ and

Figure 2. RTT103 partially suppresses the MMS sensitivity of yku70. WT (KRY193) and yku70 (KRY 172) mutants were transformed with empty vector, KM93 and RTT103. 5 μl of 10-fold serial dilutions of yeast cultures were plated on SC-LEU plates containing MMS and incubated at 30°C for 2–3 days. (2b) Quantification of MMS sensitivity. The MMS sensitivity of yku70 was quantified by plating appropriate dilutions on plates with and without MMS. Sensitivity of WT was set to 1. yku70 is approximately 60 fold sensitive and upon overexpression of RTT103 the sensitivity to MMS is reduced by approximately 8 fold. Quantification was done for three independent cultures and error bars show SD. doi:10.1371/journal.pone.0031288.g002
yku70 and rtt103 deletions, and the numbers of transformant colonies were counted. The data are represented as fraction of linear plasmid recovered relative to the supercoiled plasmid. As expected, a high number of transformants were obtained in wild type cells while very few could be recovered in yku70Δ (Figure 4b).

The number of transformants in rtt103Δ were comparable to wild type, suggesting that RTT103 is not required for end-joining of plasmids.

We then tested if rtt103Δ were sensitive to damage by UV irradiation, by comparing them to rad1Δ that is extremely sensitive to UV radiation and yku70Δ that is not sensitive to UV. We found that rtt103Δ were not sensitive to UV radiation (Figure 4c) and were quite indistinguishable from wild type. These results indicate that RTT103 is required for repair of chromosomal breaks although could be dispensable for UV damage.

**rtt103Δ are defective in sporulation**

As all these experiments indicated that RTT103 is essential for efficient repair of DNA damage, we asked if repair of naturally occurring DSBs by Spo11: many of these initiate recombination events and others are repaired without recombination. We asked if RTT103 has any effect on the repair of these breaks. For this, we generated diploid, rtt103Δ/rtt103Δ cells and plated on potassium acetate plates to induce sporulation. rtt103Δ/rtt103Δ diploids were severely defective in sporulation (0.5%; 4 out of 800 cells scored), although the wild type cells cells sporulated efficiently (35%) on the same plates. To confirm this, we stained the cells with DAPI. As shown in Figure 4d, we could clearly see 4 nuclei in many wild type cells but in rtt103Δ there were none. However, rtt103Δ carrying a copy of RTT103 on a plasmid could sporulate as efficiently as the wild type cells. We noted that a previous genome-wide screen for meiosis and sporulation reported that rtt103Δ sporulated normally [30]. However, we observed that rtt103Δ/rtt103Δ diploids obtained from BY4741 and BY4742 also were severely defective in sporulation (no spores at all in 4000 cells scored). It is possible that rtt103Δ escaped detection in the previous genome-wide study. These data indicate that RTT103 is essential for successful meiosis, probably because it is essential to repair the DSBs induced during this process.

**DNA damage signalling is intact in rtt103Δ**

A key difference between the plasmid end-joining assay and the chromosome break assays is the nature of response to damage:

*yku70&rtt103* double mutants are MMS sensitive and enhance the temperature sensitivity and MMS sensitivity of *yku70* mutation. 5 ul of ten-fold serial dilutions of wild type (KRY193), yku70 (KRY172), rtt103 (KRY285) and yku70&rtt103 (KRY286) double mutants were spotted on YPD plates and incubated at 30°C, 35°C and 37°C (3a) or on plates containing MMS (3b) for 2 to 3 days. Two independent cultures for each mutant are shown here. rtt103Δ are sensitive to MMS and enhance both the temperature sensitivity and MMS sensitivity of yku70Δ. (3c) Quantification of MMS sensitivity. The MMS sensitivity of yku70, rtt103, rtt103 yku70 was quantified as described in Figure 2. The sensitivity to MMS for yku70, rtt103 and rtt103 yku70 were approximately 55, 100 and 750 fold respectively. Values plotted are from three independent cultures and error bars show SD.
**Figure 4. rtt103Δ are sensitive to various kinds of DNA damage.** (4a) rtt103Δ are sensitive to Sce-I endonuclease. Strains of WT (KRY304), yku70 (KRY376), rtt103 (KRY375) and rtt103 yku70 (KRY379) with two Sce-I sites on either side of the URA3 gene were induced with galactose to produce DSBs. The relative survival on galactose versus glucose was calculated from three independent cultures for each strain and error bars show SD. (4b) WT (KRY105), yku70 (KRY171), rtt103 (KRY230) and rtt103 yku70 (KRY290) strains were transformed with supercoiled or linearized pRS313. The transformants were plated on SC-HIS plates in duplicates and incubated at 30°C for 2–3 days. The value plotted is the percentage of linear plasmid recovered relative to supercoiled plasmid for each strain from three independent transformation experiments. (4c) Wild type (KRY105), yku70 (KRY171), rad1 (KRY473) strains were grown to mid log phase, 10-fold serially diluted and spotted on YPD plates. They were then exposed to UV radiation and incubated at 30°C for 2 days. (4d) rtt103Δ homozygous strains are severely defective in sporulation (i) and (ii) WT and
When there is a chromosome break, cells arrest their division cycle and signal the presence of the break to the repair proteins. This signal transduction cascade culminates in the recruitment of proteins that execute the repair, and when the repair is complete, the signal is turned off. But in case of plasmids, as the break lies on an extra-chromosomal element and not on the chromosome, there is no cell cycle arrest or activation of the signalling cascade. Because rtt103Δ are sensitive to plasmids but not to chromosomal breaks, we reasoned that the end-joining process per se is not affected, but the signal transduction cascade could be affected in the rtt103Δ. In order to test this, we checked the phosphorylation of Rad53, the final effecter kinase in the pathway. Rad53 phosphorylation activates the phosphorylation of Dun1 protein by Rad53 kinase; Dun1 activation leads to transcriptional upregulation of a set of repair specific genes. Rad53 is phosphorylated in response to DNA damage primarily by Mecl, and this is facilitated through interactions between Rad9 and Rad53.

Wild type, yka70Δ and rtt103Δ were transformed with plasmid encoding RAD53-9xMYC [21] and DNA damage introduced with MMS treatment for 2 hours. Total protein was extracted and Western blots were performed using anti-myc antibody. As shown in Figure 5, the untreated cells show a sharp band at ~110 kDa. Upon treatment with MMS, we see an upward shift in the Rad53 band, which appears diffuse. This indicates that Rad53 gets phosphorylated upon DNA damage. The shift in molecular weight is same in all three strains, showing that the damage signalling cascade is active in rtt103Δ. This phosphorylation was also seen after I-SceI induced damage (data not shown).

We performed two more controls to strengthen this data. First, the same blots were re-probed with antibodies to Sir2 protein to establish that the diffuse bands were not due to abnormal separation of proteins. As shown in the lower panel of Figure 5, Sir2 appeared as a sharp band. Second, we confirmed that the phosphorylation of Rad53 is dependent on DNA-damage signalling by testing the same in a mutant defective in signalling, mec1 (lanes 8 to 11). No phosphorylation of Rad53 could be detected in mec1 and rtt103mec1 double mutants as MEC1 is required for phosphorylation of Rad53, supporting the hypothesis that the phosphorylation of Rad53 seen in rtt103Δ is due to the activation of the signalling cascade.

These experiments showed that the signalling pathway for the damaged DNA is intact in rtt103Δ. The downstream effect of this signalling is the transcriptional upregulation of the DNA damage signature genes, namely, DUN1, RAD54, RNR2, and RAD51. Their expression is substantially induced in response to both MMS and ionizing radiation [31]. To test if rtt103Δ had any defects in the induction of transcription, Northern blots were done to assess RNA levels of RNR2, RAD51, DUN1 and PLM2 genes in wild type and rtt103Δ under normal condition and after DNA damage (Figure S1). We found, as expected, that these genes were induced upon DNA damage and that there was no difference in the levels of RNA induced in wild type and rtt103Δ. The result with these representative genes indicates that the defect in rtt103Δ is unlikely to affect the expression levels of all DDR genes. However, it is possible that expression of other untested genes are affected by rtt103Δ, since previous genome-wide transcriptional studies have reported transcriptional induction of several genes. These data indicate that the DNA damage is sensed and the core downstream response is activated in a normal manner in rtt103Δ.

Transcription termination factors show differential response to DNA repair

Rtt103p copurifies with Rat1 and Rai1 proteins and is synthetically lethal with rai1 [27]. Rat1, Rai1, and Rtt103 proteins were found to crosslink very strongly at the S’ ends of the genes predicting their involvement in transcription termination. Since Rat1, Rai1, and Rtt103 proteins act in a complex during transcription termination, we wanted to test if defects in RAT1 and RAI1 genes show any DNA damage sensitivity when exposed to DNA damaging agents. As shown in Figure 6a, growth of rtt103Δ was inhibited by MMS, whereas, growth of rat1Δ and rai1Δ was comparable to the corresponding wild type strains. To further investigate if these factors were required when specific DSBs were introduced, we generated rai1Δ and rtt103Δ in a strain carrying galactose inducible HO endonuclease that generates a single break in the MAT locus. As shown in Figure 6b, although rtt103Δ are very sensitive to endonuclease induction, rai1 were not. These results indicate that transcription termination per se is not responsible for sensitivity of rtt103Δ to DNA breaks and suggest a unique, or at least a more prominent, role for RTT103 in maintaining genome stability.

Figure 5. rtt103Δ show normal Rad53 phosphorylation. WT, yka70, rtt103, mec1 and rtt103mec1 null strains containing RAD53-Myc was treated with MMS for 2 h and anti-Myc western blots were performed. The slower moving fuzzy band indicates phosphorylation of Rad53. Fuzzy bands are visible in all lanes treated with MMS and contain wild type MEC1. mec1 mutants (lanes 9, 11) do not produce phosphorylated Rad53 upon MMS treatment. Wild type sample was loaded in two lanes of the gel for better comparison of the position of the fuzzy band. Same blot was probed with Sir2 antibody to show that separation of proteins was normal and identical in all lanes.

doi:10.1371/journal.pone.0031288.g005
rrt103Δ do not enhance chromosomal recombination events

Connections between transcription and genome instability have been well established by studying mutants in the multiple processes that ultimately lead to the accumulation of mature RNA in the cytoplasm [32]. For example, mutations in the components of the THO complex, consisting of Tho2, Hpr1, Mft1 and Thp1, lead to transcription associated hyper-recombination phenotype [33]. Recently, this has been shown for a transcription termination factor in yeast and humans, Sen1 [19,34]. Sen1 prevents transcription-associated recombination by restricting the occurrence of RNA:DNA hybrids that might occur during transcription. A hallmark of THO complex mutants and sen1-1 is the increased chromosomal instability that results from promoting recombination between direct repeats. We tested if rtt103Δ also showed similar hyper-recombination phenotypes in the assay described previously [22]. rtt103Δ, yku70Δ and hpr1Δ deletions were generated in a strain carrying leu2-k::URA3-ADE2:: leu2-k direct repeat system (Figure 7a) and recombination was tested in wild type and mutants (Figure 7b). As expected, wild type cells had low levels of recombination and hpr1Δ had more than 1000-fold increase in recombination. However, rtt103Δ had recombination frequencies indistinguishable from wild type cells. These results show that rtt103Δ do not stimulate hyper-recombination between repeats. As the sen1-1 mutant showed increased hyper-recombination, we conclude that Rtt103 is unlikely to function in the same pathway as Sen1 to prevent genome instability. Additionally, sen1-1 shows synthetic genetic interactions with genes involved in the homologous repair pathway and none with genes involved in NHEJ.

However, rtt103Δ appears to have the opposite mode of action, by showing synthetic phenotype of increased sensitivity to DNA damage with yku70Δ as described in the earlier sections. We also tested the rtt103Δrad52Δ double deletions for damage sensitivity and found that they did not show increased sensitivity (Figure S2). Taken together these data indicate that although both rtt103Δ and sen1Δ affect genome stability, their mechanisms might be distinct.

Rtt103 associates with sites of DNA damage

The results shown above indicated that Rtt103 is critical for DNA repair but did not follow the same pathways as Sen1, the other transcription termination factor shown to affect genome stability. In order to gain some clues to its possible mechanisms, we tested if Rtt103 associates with the sites of damage. Rtt103 was first tagged with 13 MYC. It produced an approximately 75 kDa protein and localized to the nucleus. Cells expressing this myc-tagged Rtt103 showed wild type levels of sensitivity to MMS indicating that the myc-tagged protein was functional (Figures S3 and S4). We then introduced this tagged protein in the I-SceI strain, and performed ChIP experiments to test the binding of RTT103 to DSBs (shown schematically in Figure 8a). I-SceI enzyme was induced for 3 hours and then proteins were cross-linked with formaldehyde and immunoprecipitated using anti-myc antibodies. We used Southern blots to confirm that the 3 hour induction produced endonuclease digests as expected (Figure S5). PCR primers were designed to amplify the site of damage, and 0.2 kb, 0.5 kb, 1 kb, 2 kb, and 3 kb away from the site of scission. SPS2 on chromosome IV and a telomere-proximal site on chromosome VI were chosen as negative controls. Additionally, SPS2 on chromosome IV and a telomere-proximal site on chromosome VI were chosen as negative controls. Additionally,
3’ ends of two genes, \textit{PMA1} and \textit{ADH1}, where Rtt103 was shown to be enriched were also tested. As shown in Figure 8b, except for 3’ ends of \textit{PMA1} and \textit{ADH1}, where Rtt103 was expected to be enriched, there was no significant association of Rtt103 with any of the other regions tested when the endonuclease was not induced (blue bars). However, upon induction, the association of Rtt103 went up more than 5-fold only in the vicinity of the cut site (red bars). \textit{SPS2}, telomere regions, regions further away from the cut site, \textit{PMA1} and \textit{ADH1} did not show any increased association upon endonuclease induction, suggesting that Rtt103 protein was binding specifically to regions of damage. We also carried out ChIP experiments at the same time for Yku80myc and confirmed that association of Yku80 with damaged sites was as expected (Fig. S6). These data strongly indicate that Rtt103 functions through association at the site of damaged DNA.

**Discussion**

Telomeres are specialized ends of the chromosomes consisting of tandem repetitive sequences and proteins that bind to these sequences. Telomeres protect chromosome ends from fusion and prevent the natural chromosome ends from being recognized as DSBs by the DNA repair apparatus. Proteins involved in DSB repair, Yku70/80p, are localized to the telomeres and are critical for protection of chromosome ends. In an effort to understand the mechanisms through which this heterodimer protects chromosome ends, we isolated suppressors of \textit{yku70Δ}. In the process we discovered an unanticipated role for transcription termination factor, Rtt103, in maintaining genome integrity.

Rtt103 is an abundant nuclear protein, however no clear function had been demonstrated previously. Our work for the first time connects \textit{RTT103} to recovery from DSBs. We show that \textit{RTT103} is essential for survival when genome integrity is compromised. Rtt103 also associates with sites of DNA damage and is likely to play a direct role in maintenance of genome integrity. Rtt103 contains a carboxyl-terminal domain interacting domain (CID) or RPR. This domain interacts with the conserved C-terminal domain of RNA polymerase II and proteins are recruited via this interaction to the actively transcribed chromatin [35]. \textit{RTT103} is not essential; but it is synthetically lethal with several RNA 3’-end processing factors including \textit{RAI1}, \textit{CTK1} and \textit{REF2}. Based on the interactions and the preferential association of Rtt103 with 3’-ends of genes, it is proposed that Rtt103 facilitates termination of transcription along with Rat1 and Rai1 proteins. \textit{rtt103Δ} do not show obvious defects in termination [27].

Maintenance of genome integrity is essential for survival of cells and multiple pathways contribute to this process. Recently, evidence has accumulated to indicate the critical role of 3’-end processing in responding to damage [18]. A general response to UV treatment is the reduction of poly A+ mRNA [36] and 3’-end processing is also affected. CstF-50 is a component of CstF (cleavage stimulation factor), which is an essential polyadenylation
factor and interacts with the CTD domain of RNAP-II to promote RNA processing. Upon UV-induced damage, it also interacts with BARD1 (BRCA1 associated RING domain protein) and through its association with BARD1 and BRCA1, promotes the inhibition of 3'–end processing. Thus CstF may coordinate the RNA processing activity with DDR. This work also shows that CstF-50, RNAP-II, BARD1, and BRCA1 associated with the sites of repaired DNA [18]. Sen1 helicase has recently been shown to be important in maintaining genome integrity [19]. SEN1 prevents transcription-associated genome instability by preventing R-loop formation. R-loops are thought to form during transcription between the denatured DNA and the nascent transcript. These loops induce recombination leading to genome instability. The transcription termination mutant rat1-1 did not show transcription-dependent recombination, suggesting that defect in transcription termination per se is unlikely to be the cause for such recombination defects. This is similar to our observation that rat1-1 and ratl are not as sensitive to MMS as rt103. Another intriguing link between RNA processing and genome stability is indicated by the consistent co-purification of KU70/80 and DNAPK during isolation of the human pre-RNA processing complex [37].

Our data support a more direct role for RTT103 in responding to DNA breaks. Firstly, we see sensitivity of rt103Δ to MMS and DNA DSBs, but not to UV radiation. As transcriptional responses are seen for all the three types of genome insults described here, a general transcriptional defect is likely to affect all types of repair. However, it still remains formally possible that transcripts specifically involved in these responses are affected in rt103Δ. This possibility could be addressed by studying genome-wide transcriptional response to different DNA damaging agents in rt103Δ. Secondly, we do not see sensitivity to DNA damage in rat1-1 and ratl-1 mutants that have even stronger termination defects. Thirdly, Rtt103 does not show the same types of genome instability or epistatic relationships with DNA repair pathways as those associated with mutations in sen1 helicase and other RNA processing mutants which promote genome stability through prevention of R loops. Lastly, our studies clearly show over 5-fold enrichment of Rtt103 protein at the sites of DNA damage upon induction of damage. This strongly suggests that Rtt103 protein functions at the sites of damage. There is some indirect evidence from various genome-wide studies to suggest that RTT103 might be involved in damage response and other chromosomal functions since Rtt103 interacts genetically with a plethora of genes involved in chromatin or genome stability [38]. For example, rt103Δ show increased Rad52 foci, a mark of increased spontaneous damage [8]. These could be due to spontaneous DSBs in the unperturbed cells. rt103 is synthetically sick with dne2, a mutant that is sensitive to multiple forms of DNA damage [39]. rt103 was also found to produce
growth defects in synthetic combinations with condensins [40] and rtt103 is synthetically sick with or2-1 and or5-1 [41]. In all these networks neither rad1 nor rad1 appear. Given that Rtf1 is essential and may have been missed in these screens, if termination defects were the primary reason for picking up rtt103, rad1 is likely to have appeared in a few of these screens. This suggests that RTT103 may have an additional role in protecting genome integrity.

As Rtt103 has been isolated with transcription termination complexes and our studies show that it is essential for DNA damage response, we suggest a hitherto unidentified role for this machinery in genome stability. It is possible that termination complexes become associated with DNA damage sites to disengage RNA polymerase from the damaged sites in order to prevent synthesis of aberrant transcripts. Isolation of interacting complexes of Rtt103 in the presence of damaged DNA might indicate mechanistic basis for this function. Direct testing of other proteins in the termination complex for binding to the damaged sites will also be informative. In sum our results imply an essential role for RTT103 in promoting genome stability and further investigations will be required to understand the mechanistic basis of this contribution.

Supporting Information

Figure S1 Expression of several DNA damage responsive genes is similar in WT and rtt103Δ. RNA was isolated from WT and rtt103Δ and northern blots were done to check the expression of RNR2, RAD51, DUN1 and PLM2, rtt103A show similar pattern of upregulation of RNR2 and DUN1 as seen in WT cells. Upper panel shows the northern blots probed with the indicated probes while the lower panel shows the corresponding agarose gel stained with ethidium bromide as loading control. (TIF)

Figure S2 Epistasis analysis of rtt103 with rad52 and yku70. WT and strains carrying deletions of rtt103, rad52, rtt103Δrad52 and rtt103Δyku70 were tested for sensitivity to MMS. rtt103Δ does not affect the sensitivity of rad52 to DNA damage induced by MMS. rtt103Δ exacerbates the sensitivity of yku70 to MMS. (TIF)

Figure S3 Rtt103 is nuclear localized. The Rtt103 Myc strain was stained with anti-rabbit myc antibody. Rtt103p is localized in the nucleus. The nucleus is marked by both DAPI staining and antibody to a nuclear pore complex protein, Nsp1. (TIF)

References

1. Harrison JC, Haber JE (2006) Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet 40: 209–235.
2. Wool RD, Mitchell M, Lindahl T (2005) Human DNA repair genes, 2005. Mutat Res 577: 275–283.
3. Burrell GW, Gaever G, Chu AM, Davis RW, Brown JM (2001) A genome-wide screen in Saccharomyces cerevisiae for genes affecting UV radiation sensitivity. Proc Natl Acad Sci U S A 98: 12668–12673.
4. Jelinsky SA, Ester P, Church GM, Samson LD (2000) Regulatory networks revealed by transcriptional profiling of damaged Saccharomyces cerevisiae cells: Rpf1 links base excision repair with proteasome. Mol Cell Biol 20: 0157–0167.
5. Fry RG, DeMott MS, Coggrove JP, Begley TJ, Samson LD, et al. (2006) The DNA-damage signature in Saccharomyces cerevisiae is associated with single-strand breaks in DNA. BMC Genomics 7: 313.
6. Hansway D, Chiu JR, Xia G, Oshiro G, Wiznoler EA, et al. (2009) Previously uncharacterized genes in the UV- and MMS-induced DNA damage response in yeast. Proc Natl Acad Sci U S A 99: 10605–10610.
7. Paulsen RD, Soni IV, Wollman R, Hahn AT, Yee MC, et al. (2009) A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. Mol Cell 35: 228–239.
8. Aclaro D, Lisby M, Rothstein R (2007) Genome-wide analysis of Rad52 foci reveals diverse mechanisms impacting recombination. PLoS Genet 3: e228.
17. Mages GJ, Feldmann HM, Winnacker EL (1996) Involvement of the Saccharomyces cerevisiae HDF1 gene in DNA double-strand break repair and recombination. J Biol Chem 271: 7910–7915.

18. Mirkin N, Fonseca D, Mohammed S, Cevher MA, Manley JL, et al. (2006) The 3′ processing factor CstF functions in the DNA repair response. Nucleic Acids Res 36: 1792–1804.

19. Mischke HE, Gomez-Gonzalez B, Grzegorczyk P, Rondon AG, Wei W, et al. (2011) Yeast Sen1 helicase protects the genome from transcription-associated instability. Mol Cell 41: 21–32.

20. Longtine MS, McKenzie A, 3rd, Demarini DJ, Shah NG, Wach A, et al. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14: 953–961.

21. Pellicioli A, Lucca C, Liberi G, Martini F, Lopes M, et al. (1999) Activation of Rad33 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. EMBO J 18: 6561–6572.

22. Santos-Rosa H, Aguilera A (1995) Isolation and genetic analysis of extragenic suppressors of the hyper-deletion phenotype of the Saccharomyces cerevisiae hpr1 delta mutation. Genetics 139: 57–66.

23. Shi Y, Di Giannantonio DC, Taylor D, Sarkeshik A, Rice WJ, et al. (2009) Molecular architecture of the human pre-mRNA 3′ processing complex. Mol Cell 33: 365–376.

24. Cherry JM, Adler C, Ball C, Chervitz SA, Dwight SS, et al. (1998) SGD: Saccharomyces Genome Database. Nucleic Acids Res 26: 73–79.

25. Budd ME, Tong A, Kosman P, Peng X, Boone C, et al. (2005) A network of multi-tasking proteins at the DNA replication fork preserves genome stability. PLoS Genet 1: e61.

26. Lunde BM, Reichow SL, Kim M, Suh H, Leeper TC, et al. (2010) Cooperative interaction of transcription termination factors with the RNA polymerase II C-terminal domain. Nat Struct Mol Biol 17: 1195–1201.

27. Ljungman M, Zhang F, Chen F, Raina AJ, McKay BC (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 18: 583–592.

28. Marcand S, Pardo B, Gratas A, Calahum S, Callahan I (2008) Multiple pathways inhibit NHEJ at telomeres. Genes Dev 22: 1153–1158.

29. Milne GT, Jia S, Shannon KB, Weaver DT (1996) Mutations in two Ku homologs define a DNA end-joining repair pathway in Saccharomyces cerevisiae. Mol Cell Biol 16: 4189–4198.

30. Enyenihi AH, Saunders WS (2003) Large-scale functional genomic analysis of sporulation and meiosis in Saccharomyces cerevisiae. Genetics 163: 47–54.

31. Gasch AP, Huang M, Metzner S, Botstein D, Elledge SJ, et al. (2001) Genomic expression responses to DNA-damaging agents and the regulatory role of the yeast ATR homolog Meclp. Mol Biol Cell 12: 2987–3003.

32. Aquilera A (2002) The connection between transcription and genomic instability. EMBO J 21: 195–201.

33. Aguilera A (2002) The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability. EMBO J 21: 3526–3535.

34. Ljungman M, Zhang F, Chen F, Raina AJ, McKay BC (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 18: 583–592.

35. Shi Y, Di Giannantonio DC, Taylor D, Sarkeshik A, Rice WJ, et al. (2009) Molecular architecture of the human pre-mRNA 3′ processing complex. Mol Cell 33: 365–376.

36. Cherry JM, Adler C, Ball C, Chervitz SA, Dwight SS, et al. (1998) SGD: Saccharomyces Genome Database. Nucleic Acids Res 26: 73–79.

37. Lunde BM, Reichow SL, Kim M, Suh H, Leeper TC, et al. (2010) Cooperative interaction of transcription termination factors with the RNA polymerase II C-terminal domain. Nat Struct Mol Biol 17: 1195–1201.

38. Ljungman M, Zhang F, Chen F, Raina AJ, McKay BC (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 18: 583–592.

39. Shi Y, Di Giannantonio DC, Taylor D, Sarkeshik A, Rice WJ, et al. (2009) Molecular architecture of the human pre-mRNA 3′ processing complex. Mol Cell 33: 365–376.

40. Ljungman M, Zhang F, Chen F, Raina AJ, McKay BC (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 18: 583–592.

41. Ljungman M, Zhang F, Chen F, Raina AJ, McKay BC (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 18: 583–592.

42. Ljungman M, Zhang F, Chen F, Raina AJ, McKay BC (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 18: 583–592.

43. Ljungman M, Zhang F, Chen F, Raina AJ, McKay BC (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 18: 583–592.