Identification, validation, and targeting of the mutant p53-PARP-MCM chromatin axis in triple negative breast cancer

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Over 80% of triple negative breast cancers express mutant p53. Mutant p53 often gains oncogenic function suggesting that triple negative breast cancers may be driven by p53 protein type. To determine the chromatin targets of this gain-of-function mutant p53 we used inducible knockdown of endogenous gain-of-function mtp53 in MDA-MB-468 cells in conjunction with stable isotope labeling with amino acids in cell culture and subcellular fractionation. We sequenced over 70,000 total peptides for each corresponding reciprocal data set and were able to identify 3010 unique cytoplasmic fraction proteins and 3403 unique chromatin fraction proteins. The present proteomics experiment corroborated our previous experiment-based results that poly ADP-ribose polymerase has a positive association with mutant p53 on the chromatin. Here, for the first time we report that the heterohexomeric minichromosome maintenance complex that participates in DNA replication initiation ranked as a high mutant p53-chromatin associated pathway. Enrichment analysis identified the minichromosome maintenance members 2–7. To validate this mutant p53- poly ADP-ribose polymerase-minichromosome maintenance functional axis, we experimentally depleted R273H mutant p53 and found a large reduction of the amount of minichromosome maintenance complex proteins on the chromatin. Furthermore a mutant p53-minichromosome maintenance 2 direct interaction was detected. Overexpressed mutant p53, but not wild type p53, showed a protein-protein interaction with minichromosome maintenance 2 and minichromosome maintenance 4. To target the mutant p53- poly ADP-ribose polymerase-minichromosome maintenance axis we treated cells with the poly ADP-ribose polymerase inhibitor talazoparib and the alkylating agent temozolomide and detected synergistic activation of apoptosis only in the presence of mutant p53. Furthermore when minichromosome maintenance 2–7 activity was inhibited the synergistic activation of apoptosis was blocked. This mutant p53- poly ADP-ribose polymerase –minichromosome maintenance axis may be useful for theranostics.

INTRODUCTION

Missense mutations in the TP53 gene often result in mutant p53 (mtp53) protein with gain-of-function (GOF) properties that are associated with multiple types of cancers, including lung and breast cancer.1 Mutations in p53 are found in 80% of triple negative breast cancers (TNBC).2–4 A number of studies have been carried out to elucidate the mtp53-associated breast cancer transcriptome but the mtp53-targeted proteome is less well studied.5–8 Mtp53 has not been found to interact with DNA site-specifically but has been found to interact with cancer cell DNA in association with other cofactors. Importantly mtp53 modifies chromatin structure to up-regulate vascular endothelial growth factor receptor 29,10 and GOF mtp53 modifies major chromatin pathways by upregulating methyltransferase chromatin regulatory genes MLL1, MLL2, and the acetyltransferase MOZ.11,12 While changes in the transcriptome are a part of the mechanism of action of GOF mtp53, there are also transcription-independent mtp53 functions on chromatin that require further elucidation.

Very few studies have focused on the mtp53-associated proteome but new work strongly indicates that alternative experimental approaches are required to understand the complexity of the mtp53 pathway.7,13 A multiomics approach recently identified the proteasome machinery as a common target of missense mtp53.7 We are the only group to report on the influence of endogenous GOF mtp53 on the spatial segregation of the cancer cell proteome.6 The mtp53-associated cytosolic proteome targets include up-regulation of cytoplasmic poly ADP-ribose polymerase (PARP) when mtp53 is depleted14 and a decrease in the cytosolic mavelonate pathway enzymes (which is in agreement with previous transcriptome data).5 During validation of the spatially segregated proteins we discovered that down-regulation of mtp53 caused a chromatin-segregated decrease of PARP.6 We now report on the chromatin-segregated stable isotope in cell culture (SILAC) screen to identify the spatially restricted mtp53-targeted proteome of chromatin. We used a bioinformatics approach to compare the cytoplasmic and chromatin data sets (see Fig. 1 for the work flow). Recent work

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suggests that a key regulatory role for mtp53 on chromatin is to
regulate transcription by chromatin remodeling,12 but we
hypothesized that DNA repair and DNA replication could also be
critical targets.

To our knowledge, there has been no direct evidence of GOF
mtp53 regulating chromatin-mediated DNA replication and repair.
Herein, we identified a mtp53-PARP-MCM chromatin axis by an
unbiased bioinformatics screen of spatially segregated cytoplas-
ic vs. chromatin SILAC data from R273H mtp53 knockdown in
TNBC cells. The enzyme PARP1 catalyzes the transfer of ADP-
ribose to target proteins and plays a role in many cellular
processes including transcription, DNA replication, and DNA
repair.14,15 Herein, we validate the mtp53-PARP-MCM axis and
found that blocking PARP1 may be an excellent therapeutic target
for certain mtp53-expressing TNBCs.

RESULTS

Gain-of-function mtp53 influences 3403 chromatin proteins
Stable isotope labeling in cell culture (SILAC) of the MDA-MB-468.
shp53 cell line was carried out and mtp53 R273H was depleted by
inducible shRNA expression in two independent reciprocal
experiments. A work flow diagram (Fig. 1) shows the experimental
approach that included cell fractionation and LC-MS/MS of heavy
and light extract mixed at a 1:1 protein concentration ratio. For
one experiment the mtp53 was depleted in the heavy label
conditions (13C6 L-Lysine-2HCl and 13C6

15N4 L-Arginine-HCl) and
for the other mtp53 was depleted in the light label conditions.
Chromatin fractionation was adapted from the Mendez and
Stillman protocol.6,16 Following gel electrophoresis we used liquid
chromatography and tandem mass spectrometry (LC-MS/MS) to
identify the mtp53 protein targets associated with the chromatin
fraction. We sequenced over 70,000 chromatin-associated
peptides and compared the heavy/light ratio resulting from the
depletion of mtp53 to determine how R273H knockdown reciprocally influenced the 3403 representative proteins. The chromatin mtp53 SILAC data were examined for gene set
enrichment and then compared to the cytosol mtp53 targets
determined in our previously published results.6 We carried out a
bioinformatics comparison of the influence of mtp53 depletion on
proteins in the cytosol to those affected on the chromatin.

Gene set enrichment analysis indicates that the hexomeric pre-
replicative MCM2–7 complex is the most highly enriched mtp53-
associated chromatin complex

The gene set enrichment analysis was performed using the GSEA
software (GSEA; version 2.0.14)17 to determine how chromatin
associated proteins were influenced by mtp53. A defined set of
genes associated with the proteins that showed concordant
differences between the biological states of the mtp53 present, vs.
the mtp53 absent, was determined with pathways defined by the
Reactome Pathway Database (version 4.0).18 The GSEA analysis of
the chromatin fraction revealed a total of 27 Reactome pathways
that were positively associated with mtp53 at a
P value < 1%. Interestingly, a key pathway was the pre-replicative
complex, chromatin enriched proteome pathway, which is a novel
finding for mtp53 GOFassociations. The proteins in this pathway
are (in rank order): MCM2, MCM3, MCM6, ORC1, MCM4, MCM5,
MCM7, RPA2 and POLA2 (Fig. 2). The GSEA proteomic chromatin
enriched pathway sets are shown in their entirety at the link:
http://diverge.hunter.cuny.edu/~weigang/silac-chromatin-gsea/.

The first three positive GSEA pathways in the list corresponded
with electron transport, which did not directly correlate with a
chromatin-associated pathway; we hypothesize this resulted from
insoluble cellular components that were associated with the
chromatin pellet. The fourth GSEA pathway associated with generic transcription pathways, which are currently the focus of many mtp53 GOF studies. However the fifth pathway identified a very specific chromatin associated pre-replicative pathway that has not yet been studied for mtp53 involvement. The GSEA analysis also demonstrated 17 Reactome pathways that were negatively associated with mtp53 abundance at a P value < 1%. By clicking on enrichment results in html format you will be directed to the positive association protein sets and negative association protein sets (Fig. 2). We are providing open access to this powerful data set.

Distributions of mtp53 associated changes in the cytosol and on the chromatin indicate that the hexomeric MCM2–7 complex proteins reside in the double positive quadrant

The chromatin SILAC data were then compared to our previously published cytosol set http://diveger.hunter.cuny.edu/~weigang/silac-chromatin-gsea/ (chromatin) and http://diveger.hunter.cuny.edu/Polotskaia_etal_2014/supp-table-s1/ (cytosol) providing a new powerful data set we have identified a potentially important mtp53 protein pathway that is involved in regulation of DNA replication.

Comparison of the nuclear and cytosol proteomes displays a double positive mtp53 influence on the MCM 2-7 hexomeric complex

The mPAI for the entire mtp53-influenced proteome in the cytosol vs. the mtp53-influenced proteome on the chromatin were graphed as coordinates of the chromatin proteome on the Y-axis and the cytosol proteome on the X-axis. This resulted in a scatter plot with four quadrants demonstrating differentially influenced mtp53 associated proteins. Figure 4 shows a representative image with all the dots as grey shades, p53 as a prominent red dot, the MCM2–7 helicase subunits as green dots (zoomed in in upper right), and PARP as a blue dot. An interactive searchable scatter plot is part of the Supplementary Data http://diveger.hunter.cuny.edu/~weigang/mpai-browser/. Each dot represents a protein and its mPAI in the cytosol and chromatin. The four p53-influenced quadrants are (a) double positive in the top right, (b) chromatin positive and cytosol negative in the top left, (c) double negative in the bottom left, and (d) cytosol positive with chromatin negative in the bottom right. The mtp53 protein is by definition a double positive signal and it is highlighted as a red dot (TP53, Fig. 4). The MCM2–7 pre-replication complex proteins are shown in green and were all situated as double positives (Fig. 4). The PARP1 protein appeared in the upper left quadrant and is highlighted as a blue dot. PARP1 showed a negative association with mtp53 in the cytosol and a positive association on the chromatin, consistent with our previous experimental results. The center of the scatter plot corresponds to proteins that are unchanged by p53 knockdown. While the majority of proteins are unchanged by the knockdown of mtp53 key proteins and mtp53 levels were high and redistributed to the cytosol when the mtp53 was low as expected and the mPAI for PARP reflected this as a negative value for the cytosol and a positive number for the chromatin (Fig. 3). In support of our previous data, we determined that PARP had a positive mPAI on the chromatin of 1.2 and a negative mPAI in the cytosol of −2.3. Therefore in addition to providing a new powerful data set we have identified a potentially important mtp53 protein pathway that is involved in regulation of DNA replication.
most proteins are not significantly impacted by mtp53 depletion, showing $-1 < \text{mPAI} < 1$ (shaded in light gray). As a positive control, the mTP53 protein level (in red) shows significantly high positive mPAI values. As another positive control, the PARP1 levels (in blue) show contrasting mPAI values between the two fractionations, consistent with previous experimental results.6 The six components of the MCM2–7 complex (in green) show significant positive association with mtp53 in both cytosol and nucleus pathways including those involved in DNA replication and repair are strongly implicated.

Mutant p53 interacts with members of the MCM hexomeric complex on chromatin

To verify that the R273H mtp53 levels influenced multiple MCM hexomeric proteins on chromatin in different cells, we reduced GOF mtp53 levels in MDA-MB-468 cells and HT-29 cells and used Western blot analysis to examine MCM2, MCM4, and MCM7 (Fig. 5). When mtp53 was decreased the chromatin-associated levels of MCM2, MCM4, and MCM7 were also decreased (Fig. 5a). To further examine this interaction in situ and to determine if the mtp53 was co-localized with the MCM2–7 we used proximity ligation assay (PLA) with confocal microscopy detection.10 To our knowledge we are the first group to use antibodies in PLA to detect the interaction of mtp53 and MCM2. Strong nuclear co-localization of mtp53 and MCM2 was apparent and this signal was drastically reduced by the knockdown of mtp53 (Fig. 5b). The results from the PLA documented an interaction between mtp53 and MCM2 that was restricted to the subcellular nuclear zone. Our data showed that mtp53 R273H interacted with MCM2 in the nucleus and made us interested in seeing if the interaction of missense mtp53 with MCM2 was a more general phenomenon. In order to address the interaction of other mtp53 isoforms and wild-type p53 with MCM2 we compared the MDA-MB-468 PLA signal to those seen in a number of other cell lines (Supplemental Figure 1). The confocal microscope settings were kept constant in order to have a direct comparison. We observed a strong PLA signal between R280K mtp53 and MCM2 in MDA-MB-231 cells and this was reduced by mtp53 knockdown. We also observed a strong PLA signal between R248Q mtp53 and MCM2 in HCC70 cells, which again was reduced by mtp53 knockdown. Interestingly, we detected some MCM2 interacting with the low level wtp53 in MCF-7 cells and this reaction was stable. Therefore the high concentration of different missense mtp53 on the chromatin in cancer cells corresponds to a strong PLA signal with MCM2, and even low-level wtp53 can be found in close proximity to MCM2. A previously published immunoprecipitation screen of mtp53 R175H found an interaction with MCM proteins that was reported only in the Supplementary Data section.20 We found that exogenously expressed mtp53 R175H, and to a much lesser extent wild-type p53, interacted with both MCM2 and MCM4 (Fig. 5c). Mice with the analogous human R175H knockin mutation (Trp53R172H/Trp53R172H) develop lymphomas.21,22 We also found that the mtp53 in these mouse tumors interacted with MCM4 (Fig. 5d). In mice with mtp53 R172H, the protein is low in normal tissue and is only found stable and highly expressed in tumor tissue.23 Therefore it is not surprising that there was very little mtp53 evident in the input, or immunoprecipitation samples from normal tissue (Fig. 5, lanes 1, 2, 7, and 8). The fact that we observed a stronger interaction between mtp53 R175H and MCM2, and a weaker interaction between wtp53 and MCM2, corresponds with our observations for comparative PLA analysis for multiple breast cancer cell lines (Supplementary Figure 1). The MCM4 interaction in the co-immunoprecipitation was more difficult to assess due to poor antibody specificity, but nevertheless looked strongest for mtp53.
Damaging agent temozolomide in BRCA1 mutant cells. It has been shown that wtp53 expression decreases sensitivity of breast cancer cells to PARP inhibition and ciprofloxacin blocks the MCM2–7 complex. We asked if increased cytotoxicity of PARP inhibition could be detected in the presence of mtp53 if DNA was damaged by alkylation. We predicted that there would be synergistic activation of apoptosis of the breast cancer cell lines with mtp53 in the presence of talazoparib plus temozolomide because this alkylating agent has been shown to provoke PARP trapping. From these data we conclude that all forms of p53 can be found in close proximity to MCM proteins but that a higher level of oncogenic mtp53 in cancer cells corresponds to a much more robust signal for the proximity interaction with MCM proteins. The fact that we do not see strong enrichment for co-immunoprecipitation of MCM proteins, but a strong proximity interaction suggests that the mtp53–MCM interaction is not due to a strong direct protein–protein interaction.

Activation of apoptosis and PARP trapping is mitigated by knockdown of mtp53 or inhibition of MCM2–7. We previously saw that the inhibition of PARP was more cytotoxic in the presence of mtp53 than in its absence. We hypothesized that this might be due to a mtp53–PARP–MCM interaction at damaged DNA. Synergistic activity is seen when the PARP inhibitor talazoparib is used in combination with the DNA damaging agent temozolomide in BRCA1 mutant cells. It has been shown that wtp53 expression decreases sensitivity of breast cancer cells to PARP inhibition and ciprofloxacin blocks the MCM2–7 complex. We asked if increased cytotoxicity of PARP inhibition could be detected in the presence of mtp53 if DNA was damaged by alkylation. We predicted that there would be synergistic activation of apoptosis of the breast cancer cell lines with mtp53 in the presence of talazoparib plus temozolomide because this alkylating agent has been shown to provoke PARP trapping. We previously saw that the inhibition of PARP was more cytotoxic in the presence of mtp53 than in its absence. We hypothesized that this might be due to a mtp53–PARP–MCM interaction at damaged DNA. Synergistic activity is seen when the PARP inhibitor talazoparib is used in combination with the DNA damaging agent temozolomide in BRCA1 mutant cells.

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DISCUSSION

High levels of mtp53 are found in over 50% of all human tumors from patient samples. Somatic mutations in only three genes occur at greater than 10% incidence across all different subtypes of breast cancers, and one of these is mutation in the TP53 gene. More than 80% of TNBCs contain mtp53 protein. As far back as 1984 it was reported that the "oncogene" p53 cooperated with ras to transform cells; however, we still do not use mtp53 as a diagnostic or treatment-mediated paradigm. TNBCs may serve as
The GOF mtp53 proteins have a target genes that promote tumorigenesis, to the inhibition of p53 oncogenic activities that range from transcriptional activation of activity, many missense cancer metastasis because of loss of p53 tumor suppressor caspase 3/7 green biochemical functions exist for mtp53. Improved detection evidence including data from our lab indicates that other MS.33,34 We are the only group to study mtp53-proteome cellular fractionation experiments of SILAC followed by LC-MS/MS.35,36 We carefully analyze the proteomic data we designed an algorithm to critical determinant. Synergistic activity is seen with the PARP inhibitor talazoparib plus temozolomide hypothesis that mtp53 status (with specific BRCA1/2 status dictate the sensitivity to PARP inhibitors36 and we hypothesize that mtp53 status (with specific hot spot mutations) is a critical determinant. Synergistic activity is seen with the PARP inhibitor talazoparib in combination with the DNA modifier temozolomide.34 PARP is recruited to DNA damage sites in replication fork to directly show that the synergistic activity of mtp53 depletion also depleted chromatin-associated PARP and all members of the MCM2-7 hexomeric complex. We are the first to show that mtp53 influenced MCM chromatin levels in multiple cancer cell lines and directly associated with MCM2 in nuclei as seen by confocal microscopy PLA (Fig. 5). TNBCs are resistant to a number of different treatments; temozolomide is one of the chemotherapeutic drugs TNBCs are resistant to.35 It is clear that properties in addition to BRCA1/2 status dictate the sensitivity to PARP inhibitors36 and we hypothesize that mtp53 status (with specific hot spot mutations) is a critical determinant. Synergistic activity is seen with the PARP inhibitor talazoparib in combination with the DNA modifier temozolomide.34 PARP is recruited to DNA damage sites in replication fork to directly show that the synergistic activity of mtp53 depletion also depleted chromatin-associated PARP and all members of the MCM2-7 hexomeric complex. We are the first to show that mtp53 influenced MCM chromatin levels in multiple cancer cell lines and directly associated with MCM2 in nuclei as seen by confocal microscopy PLA (Fig. 5). TNBCs are resistant to a number of different treatments; temozolomide is one of the chemotherapeutic drugs TNBCs are resistant to.35 It is clear that properties in addition to BRCA1/2 status dictate the sensitivity to PARP inhibitors36 and we hypothesize that mtp53 status (with specific hot spot mutations) is a critical determinant. Synergistic activity is seen with the PARP inhibitor talazoparib in combination with the DNA modifier temozolomide.34 PARP is recruited to DNA damage sites in replication fork to directly show that the synergistic activity of mtp53 depletion also depleted chromatin-associated PARP and all members of the MCM2-7 hexomeric complex. We are the first to show that mtp53 influenced MCM chromatin levels in multiple cancer cell lines and directly associated with MCM2 in nuclei as seen by confocal microscopy PLA (Fig. 5).
finding MCM2-7 is involved in DNA repair also found BAG2 interacting with the complex. This suggests that stable mtp53 may help recruit MCM2-7 and PARP proteins to chromatin in order to help cancer cells survive during replication stress. While PARP inhibitors have been used to target breast cancers with BRCA1 mutations, they have not been approved for use in cancers that have mutation in the TP53 gene. Breast cancers with BRCA1 mutations include many TNBCs, however PARP inhibitors have not shown a direct correlation of effectiveness directly related to BRCA1 or BRCA2 mutations in TNBCs. MDA-MB-468 cells and HCC70 cells do not have BRCA mutations and they are more sensitive to PARP inhibitors than some breast cancer cell lines that have BRCA mutations. Importantly both of these TNBC cell lines express high levels of mtp53, however a correlation between mtp53 status and PARP activity before now had not been determined. Recent work has shown that the cytotoxicity of PARP inhibitors requires that the inhibitors trap the PARP enzyme onto the chromatin. Importantly, we found that in the presence of mtp53, but not in its absence, combination treatment with the PARP inhibitor talazoparib plus the DNA damaging agent temozolomide resulted in efficient PARP trapping and apoptosis induction (Fig. 6).

It is of interest and important to determine if the temozolomide plus talazoparib combination strategy works in vivo with a specificity for tumor cells possessing specific p53 missense mutations. We found that while mtp53 is highly associated with MCM2 on the chromatin (Fig. 5b and Supplementary Figure 1A), the mutation R273H associated with the highest level of synergistic killing by the combination treatment, and the sensitivity for cells with other p53 missense mutations varied (Supplementary Figure 1B). There have been demonstrations of remarkably synergistic activity of the PARP inhibition by talozolomide plus temozolomide in a subset of pediatric Ewing Sarcoma xenografts. The genomic landscape of Ewing Sarcoma shows an aggressive subtype with TP53 mutations. The p53 status has been reported for many of the cell lines used for the Ewing Sarcoma xenograft models, and there is not a direct correlation between the p53 mutation status and those cell lines that are sensitive to combination treatment vs. those that are not. Cancer cell sensitivity to combination treatment may be specific for certain p53 missense mutations in collaboration with other driver or passenger mutations. We previously documented that the depletion of mtp53 in MDA-MB-231 cells does not reduce MCM protein on the chromatin. Herein, we saw that there was an interaction between mtp53 and MCM2 in MDA-MB-231 cells; however combination temozolomide plus talazoparib treatment of MDA-MB-231 cells did not cause synergistic killing (Supplementary Figure 1B). It is possible that missense mtp53, in different contexts, influences PARP and MCM in different ways. Experiments are needed to elucidate the relationship between different p53 missense mutants, and accessory proteins, for influencing PARP and MCM2-7 structure and function.

Homologous-recombination-deficient tumors are dependent on DNA-replication repair mechanisms that are more sensitive to PARP inhibitors. It is possible the certain missense mutants of p53 block homologous-recombination in humans, as the p53 in C. elegans inhibits nonhomologous end joining while promoting high fidelity homologous recombination. Our results describe the close proximity between mtp53 and the replication initiator mini chromosome maintenance complex MCM2-7 on replicating DNA. However, they suggest that each missense mutation has to be evaluated for its specific activity. Researchers have found a way to reactivate mtp53 to become wild-type like, but this reactivation is allele specific for R175H. The ability to target a characteristic of multiple mtp53 proteins will enable using the newfound mtp53 activities to be used against tumorigenesis. Our results implicate an interaction of stable mtp53 at replication forks, and with PARP on the chromatin that can be used to sensitize cancer cells to die.

We saw that the processivity of the MCM2-7 complex was required for synergistic mtp53-dependent induction of apoptosis by the combination of talazoparib plus temozolomide (see model in Fig. 6G). The MCM2-7 multi-subunit helicase participates in driving DNA replication and improves replication under stressful conditions. This may be the connection between mtp53 and MCM2-7 facilitating the synthetic lethal function of PARP inhibition in treating TNBC. The disruption of p53 by mutation often allows the subverted protein to interact with normal partners of wild-type p53 but differentially influences the outcome. It remains to be determined if PARP and MCM2-7 will be added to the list of proteins that are influenced by wild-type and mtp53 in opposing ways or if this is a new paradigm. Our findings demonstrate a connection between mtp53 expression in TNBC and the ability to target cells with the combination therapeutic drug protocol previously intended for BRCA1 mutated cancers. Taken together, our findings suggest that certain mtp53 missense mutations drive PARP trapping and then MCM2-7 helps to facilitate the increased cytotoxicity of PARP inhibitors plus temozolomide. This data also suggests that the treatment of TNBC, with specific mtp53 proteins, by PARP inhibitors plus temozolomide may have promising therapeutic effects and therefore the use of mtp53 status in TNBC may be a predictive marker for combination PARP-trapping therapy response.

METHODS

Statistical analysis

We quantified the degree of under- and over-expression of proteins from the reciprocal knockdown experiments in the different sub-cellular fractions of the breast cancer cells by defining a mPAI. The mPAI was defined as

\[
mPAI = \log_2\left(\frac{\text{H}}{\text{T,exp}_1}\right) - \log_2\left(\frac{\text{H}}{\text{T,exp}_2}\right),
\]

where \((\text{H})_{\text{exp}_1}\) is the ratio of peptide abundance in an experiment in which the control cells were labeled with the light isotope and the mtp53 knockdown cells were labeled with the heavy isotope, and \((\text{H})_{\text{exp}_2}\) is the corresponding ratio in the reciprocally labeled experiment. The use of logarithm with base two converts these ratios to the unit of fold changes between the control and the knockdown cells. When abundance of a protein is not affected by mtp53 knockdown, both H/L ratios are expected to be close to one, resulting in an mPAI ~ 0. For a protein with abundance increased by the presence of mtp53, \((\text{H})_{\text{exp}_1}\) is expected to be >1 and \((\text{H})_{\text{exp}_2}\) < 1, resulting in an mPAI > 0. Conversely, mPAI was expected to be <0 for a protein with abundance decreased by the presence of mtp53.

Reagents. Doxycyclin, aprotinin, leupeptin, DTT, temozolomide and ciprofloxacin were obtained from Sigma, Talazoparib BMN 673 from Selleckchem. CellEvent Caspase-3/7 Green and ReadyProbes Cell Viability Imaging Kit Blue/Red for Live Cell Imaging were obtained from Life Technologies. Duolink in situ red kit goat/rabbit (Sigma) was used for PLA assay.

Cell lines. MDA-MB-468, H1299 and HT-29 cell lines were obtained from ATCC and cultured in DMEM or McCoy’s (for HT-29) medium (Invitrogen), supplemented with 10% FBS (Gemini, West Sacramento, CA, USA) and 50 U/ml penicillin and 50 µg/ml streptomycin (Mediatech). Cell lines with the inducible p53 knockdown were generated and described previously. To induce shRNA expression cells were treated with 8 µg/ml of doxycyclin (Dox) for the time periods indicated in the figure legends, fresh medium with Dox was supplemented every 48 h.

Antibodies. Anti-human p53 mouse 1:1:1 mix of hybridoma supernatants pAb421, pAb240, and pAb1801 (N-terminus, Central and C-terminus regions respectively), rabbit anti-Actin (Sigma); mouse anti-Fibrillarin (AbCam), mouse anti-PARP-1 (Santa Cruz), rabbit anti-PARP (Millipore/
Calbiochem), anti-MCM2, MCM4, MCM7 (Cell Signaling), secondary anti-body: anti-mouse and anti-rabbit HRP-conjugated (Sigma).

**Sub-cellular fractionation.** Cells were harvested and fractionation was performed using the Stillman protocol. Briefly, cells were scraped from the plates, rinsed with cold PBS twice and pelleted by centrifugation in 50 ml tubes at 1000 rpm 5 min. Cell pellets were resuspended in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 M sucrose, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin, 8.5 μg/ml aprotinin) with 0.1% Triton X-100. After 5 min incubation on ice, the cells were transfected to Eppendorf tubes and spun down at 3600 rpm for 5 min at 4 °C. The supernatant was spun down for an additional 5 min at 13,000 rpm at 4 °C to clarify (Cytoplasmic Fraction). Pellets were washed two times with Buffer A by centrifugation at 3600 rpm for 5 min at 4 °C. The supernatant was collected and sonicated three times for 30 s, followed by 30 s rest on ice (Chromatin Fraction). Samples were stored at −80 °C.

**Gel electrophoresis and immunoblotting.** Proteins were separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane. The Gel electrophoresis and immunoblotting and sonicated three times for 30 s followed by 30 s rest on ice (Chromatin Fraction). Samples were stored at −80 °C.

Quantitative proteomics by stable isotope labeling in cell culture (SILAC) mass spectrometry For SILAC mass spectrometry, we used Protein Quantitation Kit—DMEM (Pierce) with 13C6, 15N5 L-Lysine-2HCl and added to the media a second amino acid 13C, 15N L-Arginine-HCl (Pierce) for double labeling. Cells were passaged for at least five cell doublings by splitting cells when required and isotope incorporation efficiency was determined by MS analysis. MDA-468, shp53 R273H depleted and non-depleted cells were cultured in media containing either non-labeled or labeled amino acids, harvested, fractionated, cytoplasmic, or chromatin fractions were mixed at 1:1 ratio, separated by 10% SDS-PAGE electrophoresis, stained with GelCode Blue Stain Reagent (Thermo Scientific), and 15 gel sections excised with in situ tryptic digestion of polypeptides in each gel slice performed as described. The tryptic peptides were desalted using a 2 μl bed volume of Poros 50 R2 (Applied Biosystems, CA) reversed-phase beads packed in Eppendorf gel-loading tips. The purified peptides were diluted to 0.1% formic acid and each gel section was analyzed separately by microcapillary liquid chromatography with tandem mass spectrometry using the NanoAcquity (Waters) with a 100-μm-inner-diameter × 10-cm-long c18 column (1.7 um BEH130, Waters) configured with a 180-μm × 2-cm trap column coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Key parameters for the mass spectrometer were: AGC 3 E6, resolution 70,000. Tandem mass spectrometry fragmentation spectra were searched for protein identification using the Andromeda search engine (http://maxquant.org/) against the reversed and concatenated IPI_HUMAN protein database (v3.87). One unique peptide was required for high-confidence protein identifications and a minimum ratio count of two peptides (one unique and one razor) were required for SILAC ratio determination. Normalized SILAC ratios (H/L) were used for subsequent analysis. All MS/MS samples were analyzed using MaxQuant (Max Planck Institute of Biochemistry, Martinsried, Germany; version 1.3.0.3) at default settings with a few modifications. The default was used for first search tolerance and main search tolerance: 20 and 6 ppm, respectively. Labels were set to Arg10 and Lys6. MaxQuant was set up to search the reference human proteome database downloaded from Uniprot on April 2, 2013. Maxquant performed the search assuming tryptic digestion with up to two missed cleavages. Peptide, Site, and Protein FDR were all set to 1% with a minimum of 1 peptide needed for identification but two peptides needed to calculate a protein level ratio. The following modifications were used as variable modifications for identifications and included for protein quantification: Oxidation of methionine, acetylation of the protein N-terminus, phosphorylation of serine, threonine and tyrosine residues, and propionamide for acrylamide adducts on cysteine. Raw data files are publicly available via the Chorus data repository (https://chorusproject.org) with project I.D. number 1266. Original MaxQuant result files can be provided upon request.

**RNA interference and transfection.** For siRNA experiments, HT-29 cells were seeded at 60% confluence in media without penicillin−streptomycin and allowed to attach overnight. Cells were transfected with 100 nM of p53 or non-targeted siRNA smart pool from Dharmacon for 6 h using Lipofectamine 2000 (Invitrogen) as per manufacturers protocol. At the end of the incubation period equal volume of McCoy’s media with 40% FBS was added, next morning fresh media with 10% FBS was added and cells were allowed to grow for 72 h. Cells were harvested by scraping into the media, washed with PBS and lysed for chromatin fractionation.

**Live cell imaging** Cells were seeded at 2×10⁵ per well in a 12-well glass bottom plate (MatTek, Ashland, MA, USA). Detection of apoptotic cells was performed using the CellEvent™ Caspase 3/7 Green Detection Reagent (Life Technologies). After treatment, cells were stained with 50 μl CellEvent Caspase-3/7 green ready probes reagent and 50 μl ReadyProbes Cell Viability Imaging Kit Blue/Red (Life Technologies) for 15 min at room temp. z-stack images of stained cells were taken by confocal microscopy using a Nikon A1 confocal microscope with 20x objective. Active caspase-3/7: green fluorescence, Propidium iodide: red fluorescence, Nuclear DNA: blue fluorescence.

**In situ PLA** Cells were seeded at 2×10⁵ per well in a 12-well glass bottom plate (MatTek). After removing the media, cells were rinsed with cold PBS three times, fixed in 4% formaldehyde for 15 min and permeabilized in 0.5% Triton x-100 in PBS for 10 min at room temperature. After washing three times in PBS and one time in distilled water for 2 min, cells were then carried out PLA assay using Duolink in situ red kit goat/rabbit (Sigma Aldrich) according to the manufacturer’s instructions. Briefly, cells were incubated in the blocking buffer for 30 min at 37 °C in a humidified chamber and then incubated with primary antibodies diluted in the antibody diluents overnight at room temperature in a humidified chamber. On the following day, cells were washed in Buffer A for 5 min three times and incubated with the PLA probes (anti-rabbit minus and anti-Goat plus) for 60 min at 37 °C in a humid chamber. This was followed by 5 min wash in Buffer A for two times. The ligation reaction was carried out at 37 °C for 60 min in a humid chamber followed by two times 2 min wash in Buffer A. Cells were then incubated with the amplification mix for 100 min at 37 °C in a darkened humidified chamber. After washing with 1× Buffer B for 10 min for two times and a 1 min wash with 0.01x buffer B, cells were mounted with mounting media containing 4',6-diamidino-2-phenylindole (DAPI). z-stack images were taken using Nikon A1 confocal microscope with 60x objective oil immersion. The acquisition software is Nikon elements. The primary antibodies were rabbit anti-p53 (Cat# A300-247A) and goat anti-MCM2 (Cat# A300-122A) from Bethyl Laboratories.

**Immunoprecipitation (IP) assays** IP assays were performed as previously described to determine mtp53 binding proteins in human cancer cells and mouse tumor tissues. In brief, 1×10⁶ p53 null H1299 cells were transfected with expression vectors of H1299 (MatTek). After removing the media, cells were rinsed with cold PBS three times, fixed in 4% formaldehyde for 15 min and permeabilized in 0.5% Triton x-100 in PBS for 10 min at room temperature. After washing three times in PBS and one time in distilled water for 2 min, cells were then carried out PLA assay using Duolink in situ red kit goat/rabbit (Sigma Aldrich) according to the manufacturer’s instructions. Briefly, cells were incubated in the blocking buffer for 30 min at 37 °C in a humidified chamber and then incubated with primary antibodies diluted in the antibody diluents overnight at room temperature in a humidified chamber. On the following day, cells were washed in Buffer A for 5 min three times and incubated with the PLA probes (anti-rabbit minus and anti-Goat plus) for 60 min at 37 °C in a humid chamber. This was followed by 5 min wash in Buffer A for two times. The ligation reaction was carried out at 37 °C for 60 min in a humid chamber followed by two times 2 min wash in Buffer A. Cells were then incubated with the amplification mix for 100 min at 37 °C in a darkened humidified chamber. After washing with 1× Buffer B for 10 min for two times and a 1 min wash with 0.01x buffer B, cells were mounted with mounting media containing 4',6-diamidino-2-phenylindole (DAPI). z-stack images were taken using Nikon A1 confocal microscope with 60x objective oil immersion. The acquisition software is Nikon elements. The primary antibodies were rabbit anti-p53 (Cat# A300-247A) and goat anti-MCM2 (Cat# A300-122A) from Bethyl Laboratories.

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