Rescue of embryonic stem cells from cellular transformation by proteomic stabilization of mutant p53 and conversion into WT conformation

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p53 is a well-known tumor suppressor that is mutated in over 50% of human cancers. These mutations were shown to exhibit gain of oncogenic function compared with the deletion of the gene. Additionally, p53 has fundamental roles in differentiation and development; nevertheless, mutant p53 mice are viable and develop malignant tumors only on adulthood. We set out to reveal the mechanisms by which embryos are protected from mutant p53-induced transformation using ES cells (ESCs) that express a conformational mutant of p53. We found that, despite harboring mutant p53, the ESCs remain pluripotent and benign and have relatively normal karyotype compared with ESCs knocked out for p53. Additionally, using high-content RNA sequencing, we show that p53 is transcriptionally active in response to DNA damage in mutant ESCs and elevates p53 target genes, such as p21 and btx2. We also show that the conformation of mutant p53 protein in ESCs is stabilized to a WT conformation. Through MS-based interactome analyses, we identified a network of proteins, including the CCT complex, USP7, Aurora kinase, Nedd4, and Trim24, that bind mutant p53 and may shift its conformation to a WT form. We propose this conformational shift as a novel mechanism of maintenance of genomic integrity, despite p53 mutation. Harnessing the ability of these protein interactors to transform the oncogenic mutant p53 to the tumor suppressor WT form can be the basis for future development of p53-targeted cancer therapy.

The tumor protein 53 (p53) transcription factor (encoded by the human gene *TP53/TRP53*) is a key tumor suppressor and a master regulator of genomic stability, cell cycle, DNA repair, senescence, and apoptosis (1). p53 function is frequently compromised during tumorigenesis, usually as a result of somatic mutations, which occur in more than 50% of human cancers (2). These mutants were shown to exhibit gain of oncogenic functions in addition to the loss of WT activity, leading to an aggressive malignant phenotype (2). Most *TP53* mutations can be classified into two main categories: DNA contact and conformational mutations. The first group is composed of mutations in residues that directly bind the DNA, the second group of mutations causes distortion of the core domain folding and inhibits p53 from binding the DNA and transactivating its target genes. These mutations affect p53 conformation in a dynamic fashion, which at least partially depends on its binding partners in a cell context-dependent manner (3).

Over the years, researchers have developed several mouse models as tools for investigating p53, including p53 KO mice (4) and mice knocked in for mutant p53 (Mut) (5, 6). These models showed the role of p53 as a regulator of developmental and differentiation processes. For instance, p53 KO mice were found to display developmental abnormalities, such as upper incisor fusion, ocular abnormalities, polydactyly of the hind limbs, and exencephaly (7). On the cellular level, ES cells (ESCs) were found to express high levels of p53 mRNA and protein, which are reduced during embryonic development (8, 9). ESCs are extremely sensitive to DNA damage and readily undergo either

apoptosis or differentiation in an attempt to eliminate sub-optimal cells from the stem cells pool (10). When p53 is activated in ESCs, it transactivates its target genes, *p21* and *Mdm2*, and represses the expression of *Nanog*, which is required for self-renewal, leading to the differentiation of the cells (8). Despite the important role of WT p53 in maintaining ESCs genomic integrity, mice homozygous for Mut p53 are viable. These mice develop aggressive tumors in adulthood, but the malignant properties of Mut p53 are restrained in the ESCs stage, leading to mature embryos.

In this study, we set out to examine the effect of a conformational mutation of p53 on ESCs. We show that, in ESCs, Mut p53 protein is stabilized to a WT conformation and preserves its transcriptional activity and the genomic stability of the cells. Using mass spectrometry (MS)-based interactome analysis, we identified a conformation-specific proteomic network, including the eight members of the CCT complex, USP7, Aurora kinase (Aurka), NEDD4, and TRIM24. We suggest that these proteins act as stabilizers of Mut p53 into a WT conformation and can be the basis for future Mut p53-targeted cancer therapeutics.

Results

WT and Mut p53 ESCs Exhibit Similar Characteristics. To study the function of Mut p53 in ESCs, we established novel mouse ESC lines with different p53 status. We focused on the R172H conformation mutation, which is homologous to the R175H hotspot mutation in humans (Fig. S1A). The established cell lines include WT p53 (WT) and p53^{R172H/R172H} (Mut) as

Significance

Mutations in p53 lead to cell transformation through the elimination of the WT tumor suppressor activities and the gain of oncogenic ones. In contrast, mouse embryos develop normally, despite the expression of mutant p53. Here, we report that, in ES cells, mutant p53 conformation is shifted to a WT form, leading to transcriptionally active p53 and increased genomic integrity of these cells. Using MS-based proteomics, we were able to isolate a network of interacting proteins that bind mutant p53 and stabilize it to the WT form. These results can serve as a potential platform for p53-based cancer therapy development in the future.

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well as p53 KO (KO) and p53^{WT/R172H} (Het) ESCs as controls. We examined the stemness characteristics of three clones of each genotype. We used WT and Mut mouse embryonic fibroblasts (MEFs) as controls for gene expression in somatic cells. Surprisingly, we found no significant differences in basal *Nanog* or *Oct4* expression between the WT and Mut ESCs (Fig. 1A and Fig. S1B).

Mut p53 is known to accelerate proliferation of somatic cells (5); we, therefore, examined whether these effects are also apparent in ESCs. Unlike Mut MEFs, which displayed accelerated proliferation compared with WT MEFs, in ESC, we found no difference in doubling time (Fig. 1B). To test the effect of p53 mutation on the pluripotent capacity of the ESCs, we induced their in vitro differentiation into cells of the three germ layers. Both WT and Mut ESC lines gave rise to cells of the three germ layers, indicating their in vitro pluripotent potential (Fig. S1C). We proceeded to test the in vivo pluripotent potential of the new ESC lines and injected the cells s.c. into nude mice. This method is used to evaluate the pluripotent potential of cells and their ability to give rise to teratomas and assess the malignant potential of the cells by examining the tumors obtained, their differentiation capacity, and invasiveness. Pathological examination of the tumors revealed that they were all well-differentiated benign teratomas, suggestive of their nonmalignant nature (Fig. 1C). Similarly, examination of the KO and Het ESCs showed no difference in their differentiation or their malignant potential (Fig. S2A and B). Thus, despite the mutation in p53, the ESCs remain pluripotent and intact and gave rise to benign teratomas similar to those teratomas derived from cells containing WT p53.

The high genomic integrity of ESCs and the known role of p53 in DNA maintenance led us to investigate the effect of p53 status on their genomic stability. Karyotype analysis of the WT ESCs was close to normal as expected; Mut and Het ESCs displayed minor karyotype abnormalities, with 59 and 49 chromosomes per cell on average, respectively. In contrast, KO ESCs showed the highest incidence of karyotype abnormalities, with cells having more than 150 chromosomes and an incidence of translocation (Fig. 2, Fig. S2C, and Table S1). Genomic stability was further assessed with respect to the incidence of loss of heterozygosity

(LOH) in Het ESCs. These cells represent the genotype of Li-Fraumeni Syndrome patients, who carry a germ-line mutation in p53. Previous studies that investigated tumors from Li-Fraumeni Syndrome patients showed that ~60% of the tumors exhibited LOH in the p53 locus because of increased genomic instability (11). Testing of LOH in the Het ESCs revealed that these cells do not undergo LOH and maintain both alleles even at late passage, such as passage 42 (Fig. S2D). Overall, these results suggest an active role for p53 in preserving genome integrity in ESCs. Importantly, these data imply that Mut p53 possesses tumor-suppressive functions in ESCs similar to the WT protein.

Mutant p53 Protein Acquires WT Conformation in ESCs. The high similarity of the WT and Mut p53 ESCs suggested that the conformational mutation is not manifested in these cells. We therefore assessed the conformation of the Mut p53 protein by immunoprecipitation with conformation-specific antibodies. pAb240 binds a region in the core domain of p53 that is exposed in a denatured or Mut conformation but not accessible when the protein is in the WT form (12). pAb246 (and pAb1620 in human p53) only recognizes the WT conformation of the p53 protein (13, 14). Analysis of the protein conformation of p53 in the Mut ESCs revealed almost a 1:1 balance between the WT and Mut conformations, despite the homozygote p53 mutation (Fig. 3A). Similarly, in the Het ESCs, the majority of the p53 protein was stabilized to the WT conformation (Fig. S3D). In contrast, examination of Mut p53 conformation in somatic cells, including MEFs, mesenchymal stem cells (MSCs), and cells from the spleen, showed significantly higher Mut conformation than WT conformation (Fig. 3B and C and Fig. S3E). Thus, in contrast to the ESCs, somatic cells do not exhibit a similar shift of Mut p53 to a WT conformation.

Next, we examined whether this p53 conformational shift in ESCs depends on the pluripotent state of the cells. We treated the cells with retinoic acid (RA) for 6 d to induce differentiation. After treatment, the cells had a significant decrease in the expression levels of their pluripotency markers *Nanog* and *Oct4* (Fig. S3F), which coincided with a shift from the WT conformation to Mut conformation of Mut p53 (Fig. 3D).

Fig. 1. Mut p53 and WT p53 ESCs exhibit similar differentiation capacity in vivo. (A) QRT-PCR measuring *Nanog* in WT ESCs, Mut ESCs (three clones each), and WT and Mut MEFs. Results indicate the mean $\pm$ SD of duplicate runs. Relative expression refers to *Nanog* normalized to the *HPRT* house-keeping gene. (B) Population doubling of WT and Mut ESCs and WT and Mut MEFs. (C) Representative H&E section of a typical teratoma generated from WT and Mut ESCs. (Upper) WT ESCs 325-4. (Left) Mesodermal differentiation: cartilage. (Center) Ectodermal differentiation: well-differentiated nervous tissue. (Right) Endodermal differentiation: a cyst lined by ciliated epithelium (arrows) with interspersed goblet cells (arrowheads). (Lower) Mut ESCs 503-12. (Left) Mesodermal differentiation: striated muscle fibers. (Center) Ectodermal differentiation: well-differentiated nervous tissue. (Right) Endodermal differentiation: a cyst lined by ciliated epithelium (arrows).

Fig. 2. ESCs harboring Mut p53 exhibit genomic stability. (A–C) Representative pictures of spectral karyotyping of (A) WT ESCs 325–1, (B) Mut ESCs 503–1, and (C) KO ESCs 125–1. (Right) Chromosomes 3 and 16 display insertions from chromosome 10. (D) A bar plot of the average chromosome number of each genotype (two clones of each genotype were analyzed; 10 metaphases of each one).

Mutant p53 Exhibits Transcriptional Activity After DNA Damage.

Because p53 activity is induced on stress signals, we examined whether the Mut protein presents the transactivation functions of the WT protein under these conditions. First, we found that Mut p53 maintains its WT conformation after DNA damage with Doxorubicin or UV irradiation (Fig. 4A and B). Second, we performed an RNA sequencing experiment comparing WT, KO, and Mut ESCs that were not treated, differentiated, or treated by Doxorubicin or UV irradiation. These results revealed a cluster of genes activated after DNA damage in both WT and Mut ESCs but not KO ESCs. This cluster was also elevated in WT ESCs after differentiation but not in Mut ESCs, when p53 is in the mutant conformation (Fig. 4C). The gene cluster contains known p53 transcriptional targets, such as *Cdkn1a* (p21), *Btg2*, *Perp*, and *Rap2b*. These results were also validated by quantitative real-time RT-PCR (QRT-PCR) performed on WT, KO, and Mut ESCs treated by UV irradiation (Fig. S4C–F). Using ChIP, we further show direct binding of Mut p53 to the promoters of *Cdkn1a* and *btg2* after UV treatment (Fig. 4D and Fig. S4G and H).

Interaction Proteomics Reveal a Stabilizing Network of Mut p53. We hypothesized that cellular factors in the pluripotent cells contribute to the stabilization of the WT conformation of p53. Therefore, we incubated cell extracts from KO ESCs with extracts of Mut MEFs. Incubation of these lysates induced an increase in

the WT conformation compared with the control (Fig. 5A). Furthermore, we found that this conformational change also occurs in SKBR-3 cells, a human adenocarcinoma cell line that expresses Mut p53, after incubation with ESCs extract (Fig. 5B). These results show that ESCs harbor factors that can stabilize both mouse and human Mut p53 into a WT form. The presence of such factors is independent of p53 status but dependent on the pluripotent state of the cells.

We set out to identify the Mut p53 stabilizing factors in ESCs using unbiased MS-based proteomic approach. To that end, we analyzed the interaction network of the different conformations of p53 in WT and Mut ESCs compared with somatic cells from the spleen. We immunoprecipitated WT and Mut conformation of p53 and used p53 KO cells as controls for background binding. Overall, we identified 144 specific interactors in ESC or spleen with no overlap between the tissues. As expected, known binders of p53, such as MDM2, MDM4, TRP53BP1, and TRIM24, were found to bind to the WT conformation of WT ESCs (Fig. S5A). The comparison of the interactors in the different samples revealed proteins that bind specifically to the WT conformation of Mut ESCs, and are proposed to have a role in the conformational change of p53 (Fig. 6A, Fig. S5B and C, and Dataset S1). Mut p53 interactome in ESCs included 59 proteins, among them a group of chaperones, such as the CCT complex (all eight members), DNAJA1, DNAJC7, PFDN2, and HSPA8, that might be involved in the stabilization of p53. Additionally, we identified proteins associated with ubiquitin and ubiquitin-like modifiers, including TRIM24, NEDD4, USP7, CUL1, FBXO15, FBXO3, UBXN7, USP9X, GPS1, and COPS7A. Finally, Mut p53 binders included regulators of posttranslational modifications: AURKA, MAPK1, CSNK2A1, MELK, PPM1G, SIRT1, and SIRT4. MS analysis further showed that the WT conformation of p53 is highly phosphorylated on Serine 312 in ESCs (Fig. 6B). This site

Fig. 3. Mut ESCs but not differentiated cells exhibit a shift in p53 conformation to a WT form. (Left) Immunoprecipitation of p53 with conformation-specific antibodies αWT (PAb246) and αMut (PAb240) and (Right) band quantification of (A) WT and Mut p53 ESCs (p53 input is displayed in Fig. S3A), (B) WT and Mut MEFs (p53 input is displayed in Fig. S3B), (C) WT and Mut spleen cells (p53 was not detected in the input). (D) Mut ESCs induced to differentiate by 400 nM RA for 6 d (p53 input is displayed in Fig. S3C).

Fig. 5. ESCs extracts shift Mut p53 conformation to the WT form in differentiated cells. (A, Left) Mut MEFs protein lysate or Mut MEFs lysate mixed with KO ESCs lysate was immunoprecipitated as described in Fig. 3. (A, Right) Band quantification (p53 levels were not detected in the input). (B, Left) Immunoprecipitation of SKBR-3 cells mixed with KO ESCs with conformation-specific antibodies αWT (PAb1620; human-specific) and αMut (PAb240). (B, Right) Band quantification (p53 levels were not detected in the input).

phenotype of Mut p53 expressing ESCs differs from the malignant phenotype seen in Mut induced pluripotent stem cells (21). Presumably, the conformational switch of Mut p53 occurs only in the pluripotent state after full reprogramming; thus, genomically unstable Mut MEFs are reprogrammed and not eliminated by WT p53 activity.

Our results provide a novel mechanism in ESCs aimed at maintaining their integrity and proper functions at both the genomic and proteomic levels, thus assuring the safety of the entire embryo. This mechanism may apply to additional p53 mutations, including DNA contact mutations. In support of this hypothesis, drugs such as PRIMA1, which are speculated to act by restoring WT conformation, affect the R175H conformational mutant and the R273H DNA contact mutant in humans (22). We, therefore, propose that these p53 binders may serve as a more global mechanism of proteomic integrity, which may stabilize additional valuable proteins in ESCs.

Mut p53 is an attractive target for novel cancer therapy given the high frequency of p53 mutations in tumors and especially in light of studies showing that the reconstitution of WT p53 in vivo triggers the rapid elimination of tumors (23). We speculate that these interactors can be exploited in the future for the targeted conversion of Mut p53 into WT p53 protein in tumors, thus reconstituting the tumor-suppressive response of p53.

In sum, our findings show stabilization of the Mut p53 protein in pluripotent cells, leading to the maintenance of genomic integrity in these cells. We suggest a proteomic interactome network to be responsible for this stabilization and propose it as the basis for future anticancer therapeutics.

Materials and Methods

Ethics Statement. Animal protocols were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Mice Strains. The following mouse strains were used in this study: C57BL/6 containing WT p53, KO p53, Het p53, or Mut p53 alleles (provided by Guillermina Lozano, MD Anderson Cancer Center, Houston) and *Hfh11nu* Nude mice (Harlan).

Cell Cultures. Mouse ESCs were generated as described in ref. 24. ESCs were cultured in DMEM supplemented with 15% (vol/vol) FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 1,000 units/mL leukemia inhibitory factor (ESG1107;

Millipore), and penicillin and streptomycin. Primary MEFs were prepared from 13.5-d-postcoitum embryos. MSCs were prepared from bone marrow and grown in MSC medium (murine MesenCult Basal Media, 20% (vol/vol) murine mesenchymal supplement; StemCell Technologies). Splenocytes were harvested from the spleen and treated with red blood cells lysis buffer (Sigma).

Teratoma Formation and Analysis. Teratoma formation assay was performed by s.c. injection of ESCs into Nude mice (10^6 cells/100 μL with Matrigel matrix

Fig. 6. Interaction proteomics reveal a stabilizing network of Mut p53. (A) MS analyses showing interactors of the WT conformation of p53 in ESCs. (B) Serine 312 phosphorylation detected by MS.

[BD] at a ratio of 1:1). The tumors were removed 3–16 wk after injection, fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin blocks. Sections were stained with H&E. The designation of a tumor as a benign teratoma was based on histological criteria.

Population Doubling Time and Growth Area Measurement. Proliferation rates of the various MEFs and ESCs were evaluated by calculating population doubling time. Cells (4×10^5) were plated in 6-well plates in triplicates. The cells were counted every 3 or 4 d and replated at the same density. This procedure was repeated six to seven times.

QRT-PCR. QRT-PCR was performed as previously described in ref. 25. The values for the specific genes were normalized to the *HPRT* housekeeping gene. Primers are listed in [Table S2](#).

Spectral Karyotype Analysis. ESCs were plated in a 6-well plate for 48 h. Chromosome resolution agent was added to the culture for 1 h followed by 30 min of Colcemid (0.1 $\mu\text{g/mL}$). Cells were trypsinized and lysed with hypotonic buffer, and they were fixated in glacial acetic acid:methanol (1:4). Chromosomes were simultaneously hybridized with 21 (19 + 2) combinatorially labeled chromosome dyeing probes and analyzed using GenASi Spectral HiSKY (Applied Spectral Imaging Ltd.).

RNA Sequencing. Three cell lines of each genotype (WT, Mut, and KO) were used: untreated, differentiated for 8 d by RA treatment (1 μ M), and treated with Doxorubicin (0.27 μ g/mL) for 12 h or collected 7 h after UV irradiation (20 J/m²). RNA was prepared using the RNeasy Microkit (Qiagen); 1 μ g RNA from each sample was used for preparation of an mRNA TrueSeq library in the Israel National Center for Personalized Medicine (Weizmann Institute of Science), which was processed by the HiSeq 2000 Sequencing System (Illumina) in single-end 50-nt reads. RNA sequencing reads were aligned to the University of California Santa Cruz mm10 genome using Tophat (26). The expression of each gene was calculated as the number of reads uniquely mapped to its location divided by a normalization factor calculated as the median number of total reads in each sample. Genes with expression values below 30 and genes that did not change across the samples were removed from the analysis. Log₂ transformation was applied on the remaining set of genes. We imputed the data to fill missing points by creating a Gaussian distribution of random numbers, with a width of 0.3 of the measured values and 1.8 SD downshift to match the overall Gaussian distribution at low signal values. To extract genes that are highly expressed after DNA damage in a p53-dependent manner, we took a three-step approach: (i) Welch test between WT not treated and WT UV [false discover rate (FDR) = 0.01, S0 = 1]; (ii) Welch test between KO UV and WT UV (FDR = 0.05, S0 = 0.5); and (iii) Welch test between Mut UV and KO UV (FDR = 0.1, S0 = 0.5). We performed hierarchical clustering after z-score normalization of the significantly changing genes.

ChIP. ChIP was performed as previously described in ref. 25. p53 was immunoprecipitated using the PAb246, and beads only were used as controls for nonspecific binding. Primers are given in [Table S2](#).

Immunoprecipitation. Cells were lysed with 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40 (NP40), and 1x Protease inhibitors. PAb240, a monoclonal anti-Mutp53 antibody, and PAb246 (or PAb1620 for human p53), a monoclonal anti-Wtp53 antibody (provided by David Lane, University of Dundee, Dundee, UK), were conjugated to protein G beads (Sigma) followed by the addition of cell lysates (1 mg protein). For immunoblotting, immunoprecipitated material was washed and resuspended in SDS sample buffer, and then, it was subjected to Western blot analysis. For MS analysis, beads were washed three times with a buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM glycerol, and 1% NP40 and three additional times without NP40; washes were followed with on-bead trypsin digestion as described previously (27).

Sample Preparation and MS Analysis. For global proteomic profiling, cells were lysed in 4% SDS, 100 mM Tris-HCl (pH 7.5), and 100 mM DTT. Proteins were trypsin-digested according to the filter-aided sample preparation protocol (28). Liquid chromatography-MS/MS analyses for global proteome profiling and coimmunoprecipitation experiments were performed on the EASY-nLC1000 UHPLC coupled to the Q-Exactive mass spectrometer (Thermo Scientific). Peptides were separated on a 50-cm PepMap column with a 240-min water-acetonitrile gradient for the proteome profiles and a 140-min gradient for the immunoprecipitation samples. MS data were analyzed with MaxQuant (version 1.3.10.17) with an FDR threshold of 0.01. For the analysis of modifications, the database search included serine/threonine/tyrosine phosphorylation and lysine acetylation as variable modifications of p53.

To extract specific binders, we imputed the data to fill missing points by creating a Gaussian distribution of random numbers with a width of 0.3 of the measured values and 1.8 SD downshift to match the overall Gaussian distribution at low signal values. We then used the Welch test with a permutation-based FDR threshold of 0.05. MS results can be found in [Fig. S5C](#) and [Dataset S1](#).

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