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איבוד ההטרוזיגוטיות של p53 בתהליך התמרה סרטנית של תאים ממוינים ותאי גזע

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Losing the brakes: p53 loss of heterozygosity in malignant transformation of differentiated cells and stem cells

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Declaration

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Abstract

The function of the tumor suppressor protein p53 (TP53) is compromised in approximately half of human cancers cases, usually as a result of somatic mutation in its genomic sequence. Mutant p53 does not only lose its tumor suppressive function, but rather it gains new oncogenic activities that confer the cells with many cancer's hallmarks. Usually, p53 is initially mutated in a single allele, then the resulting heterozygous cells may lose the remaining wild type (WT) allele, through a process termed loss of heterozygosity (LOH). Importantly, LOH is considered to be one of the tumorigenesis cornerstones. In recent years the role of cancer stem cells in malignancies is being uncovered. Yet, not much is known about the formation of cancer stem cells**.** As part of my PhD, I aimed to decipher the molecular mechanism underlying p53 deregulation as a result of p53 LOH, which leads to malignant transformation of differentiated cells and stem cells.

To this end, we examined p53 LOH in several complementary cellular models; mouse embryonic fibroblasts (MEFs), embryonic stem cells (ES), induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs). We discovered that differentiated cells robustly undergo p53 LOH, which is attenuated upon reprogramming into iPSCs. The few iPSCs clones which underwent p53 LOH appeared to be malignant upon injection into immune-deficient mice. In addition, all heterozygous bone marrow (BM) derived MSC isolates, which were cultivated *in vitro*, underwent p53 LOH in an age-dependent manner, i.e. MSCs derived from adult mice completed the p53 LOH process in a shorter latency compared to MSCs derived from adolescent mice. Moreover, cultivation of BM derived MSC isolates after p53 LOH acquired them with the ability to give rise to malignant sarcomas, when injected into immune-compromised mice. Interestingly, we could also detect p53 LOH in BM mesenchymal progenitors *ex-vivo.* Surprisingly, in these BM progenitors we observed a significant preference to the loss of the mutant allele compared to the loss WTp53 allele.

To examine whether the mechanism underlying this phenotype involves clearance of cells that lost WTp53 by the immune system, we established a heterozygous p53 immune deficient mice colony. Our data suggested that hampered immune system did not affect p53 LOH bi-directionality. However, immune-compromised p53 heterozygous mice showed shorter tumor

free survival and p53 LOH in all spontaneous tumors, suggesting that the immune system might attenuate p53 LOH and tumor development.

We next aimed to reveal the molecular mechanism underlying p53 LOH. Utilizing expression profiling and genomic sequencing, we discovered that the underlying mechanism of p53 LOH is homologous recombination and we detected elevation in DNA repair genes during p53 LOH culmination. Moreover, genome-wide analysis of differentiated cells and stem cells (MEFs, iPSCs and ESCs) revealed many regions across the genome that underwent LOH, among them a large region that includes p53 sequence.

Our study suggests that p53 serves as a barrier to CSCs formation both from dedifferentiation of somatic cells or transformation of adult stem cells.

Finally, our data extend our knowledge as for the role of p53 LOH in malignant transformation of stem cells and might be utilized for rationale based drug design.

תקציר

חלבון מדכא הסרטן, p53, פגום ביותר מחמישים אחוז ממקרי הסרטן באדם, בדרך כלל כתוצאה ממוטציה נקודתית ברצף הגנומי שלו. p53 המוטנטי לא רק מאבד את יכולתו לדכא סרטן אלא רוכש תכונות אונקוגניות חדשות שמעניקות לתא סממנים שמאפיינים התמרה סרטנית. בדרך כלל, אלל אחד של הגן עובר מוטציה ולאחר מכן התא ההטרוזיגוטי לגן זה מאבד את האלל התקין השני בתהליך שנקרא איבוד ההטרוזיגוטיות. איבוד ההטרוזיגוטיות מהווה את אחד מאבני הדרך בהתמרה סרטנית. בשנים האחרונות, חשיבותם של תאי הגזע הסרטניים במחלות ממאירות נחשף. למרות זאת, רב הנסתר על הידוע בנוגע להתפתחותם של תאי גזע סרטניים. בעבודת הדוקטורט שלי ניסיתי לפענח את המנגנון המולקולרי של דה-הרגולציה של p53 בהתמרה סרטנית כתוצאה מאבוד ההטרוזיגוטיות של p53 בתאים ממוינים ובתאי גזע. לצורך העניין, חקרנו את אבוד ההטרוזיגוטיות של p53 במספר מערכות תאיות משלימות; פיברובלסטים עכבריים עובריים, תאי גזע עובריים, תאי גזע פלוריפוטנטים מושרים ותאי גזע מזאנכימאליים. אנחנו גילינו שתאים ממוינים מאבדים את ההטרוזיגוטיות של p53 בתרבית, איבוד זה מעוכב ברגע שמתכנתים-מחדש אותם לכיוון של תאי גזע פלוריפוטנטים מושרים. מיעוט מהקלונים של תאי הגזע הפלוריפוטנטים המושרים אשר אבדו את ההטרוזיגוטיות של p53 יצרו גידולים ממאירים, כאשר הוזרקו לעכברים מדוכאי מערכת חיסון. בנוסף, כל הבידודים של תאי גזע מזאנכימלים שגודלו בתרבית איבדו את ההטרוזיגוטיות של p53 באופן תלוי-גיל, כלומר: תאים שבודדו מחיות בגיל ההתבגרות משך הזמן עד שאיבדו את ההטרוזיגוטיות של p53 היה ארוך יותר מתאים שבודדו מחיות בוגרות. אכן, כעבור משך זמן מסוים בתרבית לאחר איבוד ההטרוזיגוטיות של p53 כל הבידודים של תאי גזע מזאנכימאליים יצרו גידולים ממאירים כשהוזרקו לעכברים מדוכאי מערכת חיסון. למרבה העניין, הבחנו באיבוד ההטרוזיגוטיות של p53 בתאי אב מזאנכימאליים ממוח העצם. להפתעתינו, בתאי אב אלו הייתה העדפה לאיבוד האלל המוטנטי לעומת האלל התקין של p53. על מנת לבחון אם המנגנון שמתווך את התופעה הזאת קשור לסילוק התאים שאיבדו את האלל התקין של p53 על ידי מערכת החיסון, ביססנו מודל עכברי הטרוזיגוט לp53 מדוכא מערכת חיסון. המידע שלנו מצביע שמערכת חיסון פגומה אינה משפיעה על כיווניות איבוד ההטרוזיגוטיות של p53. למרות זאת, עכברים מדוכאי מערכת חיסון והטרוזיגוטים לp53 הראו זמן הישרדות ללא גידולים קצר יותר לעומת עכברים בעלי מערכת חיסון ובנוסף כל הגידולים הספונטניים של העכברים מדוכאי מערכת החיסון איבדו את ההטרוזיגוטיות של p53 לעומת שני שליש מהגידולים של העכברים בעלי מערכת חיסון. ממצאים אלו מרמזים שמערכת החיסון יכולה לעכב את אבוד ההטרוזיגוטיות של p53 והתפתחות הסרטן.

בהמשך ניסינו לפענח את המנגנון המולקולרי של איבוד ההטרוזיגוטיות של p53. לצורך כך עשינו שימוש באיפיון של ביטוי הגנים וריצוף הגנים וגילינו שהמנגנון שבאמצעותו מתרחש איבוד ההטרוזיגוטיות הוא רקומבינציה הומולוגית. אנו הבחנו בעלייה בביטוי גנים שקשורים לתיקון נזקי דנא בשיאו של תהליך איבוד ההטרוזיגוטיות של p53. יתר על כן, ריצוף של כל הגנום של תאים ממוינים ותאי גזע חשף שאיבוד הטרוזיגוטיות של p53 הוא חלק קטן מאד ממקטע גדול שעובר את התהליך ובנוסף ישנם אירועים דומים ברחבי הגנום. לפי עבודת הדוקטורט הזאת

ניתן להסיק כי ללא קשר למקורם של תאי הגזע הסרטניים; התמרה סרטנית של תאי גזע בוגרים או התמיינות לאחור של תאים ממוינים, p53 משמש כמחסום להתפתחותם של תאי גזע סרטניים. לבסוף, מסקנות עבודה זו מרחיבות את הידע המדעי על איבוד ההטרוזיגוטיות של p53 בהתמרה סרטנית של תאי גזע ותאים ממוינים ויכולה לשמש לפיתוח תרופות מכוונות מטרה כנגד מחלת הסרטן.

List of Abbreviations

AP	Alkaline Phosphatase
Aprt	Adenine Phospho-Ribosyl Transferase
BM	Bone Marrow
BMNC	BM Nucleated Cells
Braca1/2	Breast Cancer 1/2
CFU-f	Colony Forming Unit - fibroblast
cMYC	v-myc avian Myelocytomatosis viral oncogene homolog
CSCs	Cancer Stem Cells
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant Negative
EF1a	Elongation Factor 1-Alpha
ESCs	Embryonic Stem Cells
FCS	Fetal Calf Serum
GOF	Gain of Function
H&E	Hematoxylin and Eosin
Hprt	Hypoxanthine-guanine Phospho-Ribosyl Transferase
HR	Homologous Recombination
HZ	Heterozygous
IC	Immune-Competent
ID	Immune-Deficient
IGV	Integrative Genomic Viewer
iPSCs	induced Pluripotent Stem cells
Klf4	Krüppel Like Factor 4
KO	Knock Out
LFS	Li Fraumeni Syndrome
LIF	Leukemia Inhibitory Factor
LOH	Loss of Heterozygosity
MEFs	Mouse Embryonic Fibroblasts
MSC	Mesenchymal Stem Cells
Mut	Mutant
NHEJ	Non-Homologous End Joining
Oct4	Octamer 4
QRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
Rb1	Retino Blastoma 1
ROS	Reactive Oxygen Species
SCs	Stem Cells
SKY	Spectral Karyotype Analysis
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
Sox2	SRY-type high mobility group box 2
SRY	Sex Determining Region Y
STEMCCA	Stem Cell Cassette
Tfrc	Transferrin Receptor

TICs	Tumor Initiating Cells
TSG	Tumor Suppressor Gene
WT	Wild Type

Introduction

p53 from "guardian of the genome" to master regulator of homeostasis

p53 (human *TP53*, mouse *Trp53*) is one of the most important tumor suppressor genes. It functions in response to a variety of cellular stresses, including oncogene activation and DNA damage. Activated p53 suppresses cellular transformation mainly by inducing growth arrest, apoptosis and DNA repair in damaged cells. Hence, p53 is considered as the "guardian of the genome" (Lane, 1992). In addition, p53 was shown to be involved in various differentiation processes acting either by suppressing or facilitating cell differentiation, depending on the cell type (Molchadsky et al., 2008; Shaulsky et al., 1991). In recent years, novel p53 activities in cellular pathways have been unveiled, including autophagy (Tasdemir et al., 2008), metabolism (Goldstein and Rotter, 2012; Vousden and Ryan, 2009) and innate immunity (Miciak and Bunz, 2016), both at the cellular and non-cell autonomous level (Charni et al., 2016; Lujambio et al., 2013) placing p53 as a master regulator of homeostasis.

Mutant p53 in human cancer

In approximately half of all human cancers p53 function is compromised, usually as a result of a point mutation. Mutant p53 forms not only lose their tumor suppressive function but also were shown to hold a dominant negative (DN) effect over the WTp53 protein, and to gain new oncogenic properties that are independent of WTp53. These new features were termed "gain-of-function" (GOF) (Brosh and Rotter, 2009; Shetzer et al., 2016). Mutant p53 GOF notion was first demonstrated in 1984, whereby introduction of mutant p53 was shown to transform cells lacking p53 (Wolf et al., 1984). However, the most compelling evidence for mutant p53 GOF was shown in a mutant p53 knock-in mouse model, which exhibited high incidence of metastatic tumors compared to knock out (KO) p53 mice (Lang et al., 2004; Olive et al., 2004)

In clinic, p53 status is considered as a landmark of tumor progression (Hollstein et al., 1991; Vogelstein et al., 2000). Accordingly, p53 mutations are associated with drug resistance and clinically poor prognosis (Shetzer et al., 2014b; Wallace-Brodeur and Lowe, 1999).

Typically, in tumor cells following a mutation in one of p53's alleles, the remaining allele is lost in a LOH process, which is one of the tumorigenesis cornerstones. LOH of the WTp53 allele occurs in the majority of tumor cells harboring a p53 mutation (Levine et al., 1991). However, p53 may exhibit haplo-insufficiency, namely a single-copy loss-of-function mutation can be sufficient to cause a malignant phenotype, as well (Berger and Pandolfi, 2011).

Nevertheless, a recent study that performed p53-based genomic and transcriptomic meta-analyses using data from the Cancer Genome Atlas estimated that over 93% of heterozygous (HZ) p53 sporadic tumors undergo p53 LOH or copy neutral LOH (Parikh et al., 2014). This data suggest that haplo-insufficiency of p53 is feasible, yet very rare.

In addition to somatic mutations of p53, there is a rare type of cancer predisposition syndrome associated with germline p53 mutations termed, the Li-Fraumeni syndrome (LFS) (Malkin et al., 1990). LFS is characterized by the appearance of a wide spectrum of tumors, including bone and soft-tissue sarcomas, acute leukemia, early onset of breast cancer, brain cancers such as glioblastoma, and adrenocortical tumors occurring over a wide age range (Varley et al., 1997a). This highlights the diversity in the manifestation of p53 mutations, implying that the specific cancer type and the mutation occurrence may be interdependent (Rivlin et al., 2011). Consistent with p53 being a tumor suppressor gene that conformed to the two-hit hypothesis, tumors from the LFS patients were analyzed for LOH of the p53 gene, with the expectation that tumors might select for loss of p53. Indeed, Varley *et al*., (Varley et al., 1997a) demonstrated that approximately 40-60% of the initially analyzed tumors exhibited LOH in the p53 locus. The remaining tumors bypass the suppressive effect of the WT allele by diverse mechanisms such as p53 promoter hypermethylation (Kang et al., 2001), increased activity of the p53 negative regulator-mdm2 (Leite et al., 2001), by impairing other components of the p53 pathway (Zheng et al., 2006) or by the enhanced oncogenic potential of the p53 missense mutations (Rivlin et al., 2011). This implies that, gain of function mutants or those showing dominant negative features may be sufficient to induce tumor formation in the presence of the WTp53 allele, especially in context of other genetic or environmental insults (Berger et al., 2011; Buganim et al., 2010; Varley et al., 1997a).

<u>Mesenchymal stem cells as the proposed cells of origin of LFS tumors</u>

LFS patients and LFS mouse models predominantly develop tumors of mesenchymal origin (Lang et al., 2004; Malkin et al., 1990). During recent years the notion that sarcomas may arise from faulty MSCs has been proposed, placing MSCs as candidate cells of origin for several sarcoma types (Mohseny and Hogendoorn, 2011). MSCs represent a population of heterogeneous multipotent cells, which can be isolated from many adult tissues throughout the body and are able to self-renew and differentiate into different cell types of mesodermal origin (Pittenger et al., 1999; Zipori, 2009). Increasing evidences suggest that MSCs that acquire

mutations in oncogenes or tumor suppressor genes (TSG) may function as tumor initiating cells (TICs) leading to *de novo* tumor formation. In this regard MSCs might be the TICs capable of initiating sarcomagensis (Rodriguez et al., 2012), as was shown for hematopoietic stem cells, which may serve as TICs for hematopoietic malignancies (Reya et al., 2001).

Induced pluripotent stem cells

Another system that allows to study the biology of stem cells is the iPSCs. In 2006, the specific factors required for reprogramming differentiated cells into pluripotency were identified (Takahashi and Yamanaka, 2006). The original quartet of factors includes Oct4, Sox2, Klf4 and c-Myc. The simultaneous introduction of these factors to somatic cells leads to their reprogramming into pluripotent cells. Subsequent studies demonstrated that by using Oct4 or Nanog as a selection marker it is possible to obtain iPSCs with ESCs-like features, i.e. cells that retain the potential to differentiate into all three germ layers *in vitro*, form teratomas, a differentiated and non-malignant tumors, when injected into immune-deficient mice and produce chimeric live pups, when injected into blastocysts or germ cells. Several studies, including our own, showed that p53 is a negative regulator of the reprogramming process (Krizhanovsky and Lowe, 2009; Sarig et al., 2010). In addition, our lab showed a novel GOF property for mutant p53, which markedly enhanced the efficiency of the reprogramming process compared to p53 deficient cells (Sarig et al., 2010). However, as opposed to WTp53 or p53 deficient cells that gave rise to typical iPSCs and teratomas, mutant p53 reprogrammed cells exhibited genomic instability and malignant tumor-forming potential. This suggests that mutant p53 possess tumorigenic function and its presence is not safe for the purposes of regenerative medicine.

One of ESCs hallmarks is their ability to maintain high genomic fidelity. Indeed, the mutation frequency in ESCs was found to be significantly lower than that of embryonic fibroblasts (Cervantes et al., 2002). In this respect, our lab revealed that in ESCs, mutant p53 proteins are stabilized towards WTp53 conformation by different chaperons, hence conferring the cells with non-malignant phenotype, suggesting a novel mechanism of maintaining ESCs genome stability (Rivlin et al., 2014). An early study that aimed to characterize iPSCs claimed that they are indistinguishable from ESCs (Wernig et al., 2007). However, in recent years, several studies questioned the assumption that iPSCs are as genomically stable as ESCs. Indeed, iPSCs at early passage had more copy number variations (CNVs), aneuploidy and deletions of TSGs in

comparison to both MEFs and ESCs. However, prolong culturing selects against mutated cells driving the clones into a high genomic fidelity state, similarly to ESCs (Hussein et al., 2011; Laurent et al., 2011; Mayshar et al., 2010). Another study showed that iPSCs harbor point mutations in protein coding sequences. Half of these mutations were originated from their parental somatic cells and half were created during the reprogramming process and culturing time (Gore et al., 2011). These studies suggest that iPSCs are not as genetically stable as ESCs, and highlight the threat of cancerous transformation when utilizing reprogramming processes for regenerative medicine.

The questions such as what are the effects of p53 LOH in differentiated cells and stem cells as well as whether the cell-state affects p53 LOH remained largely unanswered. In this PhD study I suggest that differentiated cells robustly undergo p53 LOH, while shifting them towards pluripotent state attenuates this process, at large. p53 LOH is crucial for transforming iPSCs. In addition, despite being stem cells, MSCs exhibit less genomic fidelity than ESCs and undergo p53 LOH in an age-dependent manner. Namely, MSCs derived from adult mice completed the p53 LOH process in a shorter latency compared to MSCs derived from adolescent mice. The p53 LOH process allowed additional transforming events, which eventually led to induction of malignant tumors. Furthermore, we observed that MSCs undergo bi-directional p53 LOH in the BM, i.e. they may lose either the WT or the mutant p53 allele with significant preference of losing the mutant allele, suggesting LOH as a DNA repair mechanism. Analysis of transgenic mice model demonstrated that this preference remains in immune-deficient animals, suggesting that the adaptive immune system probably is not involved in regulation of this process. Yet, the immune system was found to be involved in p53 LOH prevalence in spontaneous tumors and tumor free survival, suggesting that the immune system might attenuate p53 LOH and tumor development. Utilizing genomic sequencing analysis, we revealed many regions across the genome that underwent LOH, among them a large region that includes p53 sequence and that homologous recombination underlies these LOH events. Our work suggest that regardless of its cell of origin, i.e. either they evolve from dedifferentiation of somatic cells or transformation of adult stem cells, p53 serve as a barrier to CSCs formation. These data might contribute to future development of tailor-made therapy, which targets the p53 LOH networks in cancer stem cells.

Materials and Methods

Mice strains

The following mice strains were used in this study: C57BL/6 containing either $p53^{WT/WT}$ (WTp53), $p53^{WT/R172H}$ (HZp53) or $p53^{R172H/R172H}$ (Mutp53) alleles (kindly provided by Prof. G. Lozano) *Hfh11nu* Nude mice and NOD.CB17-prkdc-SCID/NCrHsd (Harlan, Israel). C57BL/6 HZp53 for $p53^{R172H}$ were crossed with NOD.Cg-$Rag1^{tm1Mom}Il2^{rgtm1Wjl}$/SzJ ID mice (The Jackson Laboratory, Sacramento, CA) to create mice which are ID and IC HZp53. Animal protocols were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Cell cultures

MEFs prepared as previously described (Shetzer et al., 2014a) and were maintained in DMEM (Biological Industries) supplemented with 10% FCS and antibiotics.

MSCs and CFU-fs were grown in MSC medium, containing murine MesenCult™ Basal Media (StemCell Technologies) supplemented with 20% murine mesenchymal supplement (StemCell Technologies), 60μg/mL penicillin, 100 μg/ml streptomycin and 50 μg/ml kanamycin. Cells were incubated at 37ºC in a humidified atmosphere of 10% CO_2. Fresh medium was added twice a week. iPSCs were maintained on irradiated MEFs in ESCs medium: DMEM (Biological Industries) containing 15% FCS, 5 mg recombinant human LIF (Millipore; LIF1005), 1 mM glutamine (Biological Industries), 1% nonessential amino acids (Biological Industries), 0.1 mM β-mercaptoethanol (Invitrogen), 60μg/mL penicillin and 100μg/ml streptomycin (Biological Industries).

Generation and characterization of iPSCs

The EF1a–STEMCCA lentiviral vector, a kind gift of Dr. Mostoslavsky, G. (Sommer et al., 2009), allows for constitutive expression of the four proteins *Oct4, Klf4, Sox2,* and *mCherry* from a single polycistronic transcript. Lentiviruses were produced in 293T packaging cells as previously described (Mostoslavsky et al., 2006). Forty eight hours post infection, $3.5*10^5$ cells were plated in 10 cm plates, on top of a feeder layer of irradiated MEFs (irradiated with 60 gray gamma irradiation), and medium was replaced to ESCs medium. At this stage the medium was supplemented with 2I: small-molecule inhibitors CHIR99021 (GSK-3binhibitor, 3mM; Axon

Medchem) and PD0325901 (ERK1/2 inhibitor, 1mM; TOCRIS). Emerging colonies were selected by morphology. Each colony was isolated either mechanically or by incubating for 15 minutes in a trypsin filled glass cylinder on the emerging clone. The colonies were then transferred to 12-well plates containing ESCs+2I medium and separately passaged. A few passages later, 2I was removed gradually from the medium. Alkaline phosphatase activity was performed as previously described (Kochupurakkal et al., 2008). Colony number was determine using Image-Pro® Plus analysis software. Further characterization and verification of the nature of the reprogrammed clones were performed by Quantitative Reverse Transcription-PCR (QRT-PCR) as described below in detail.

Preparation of BM cells suspension

BM nucleated cells (BMNCs) cells were obtained from femurs and tibias of 6-8 weeks old, 4-13 months old WTp53, HZp53 and Mutp53 mice. The bone was flushed with PBS containing 2% FCS (Biological Industries LTD). The cells were dissociated to single-cell suspension and were centrifuged at 300 g for 5 minutes at room temperature (R.T). Red blood cells (RBC) were removed by RBC lysis buffer (R7757, Sigma).

Production and characterization of MSC isolates

For the standard MSC production the pellet BM cells were re-suspended to single-cell suspension and seeded in 6-well plates containing MSC medium (ratio of one mice to one well). The medium was replaced every 3 days to remove the non-adherent cells. Once the adherent cells had reached confluence, the cells were trypsinized using Trypsin B solution (0.05% EDTA, 0.25% trypsin), centrifuged for 5 minutes at 300 g, 4ºC, re-suspended in their medium and split 1:2. Expression of MSC isolates surface markers were analyzed using the following antibodies: anti-CD11b–PE, anti-CD45.2–PE, anti-CD31-PE, anti-CD34-PE, anti-Ter119-PE, anti-Sca1-PE, Rat IgG2b isotype control–PE, Rat IgG2a isotype control–PE and Mouse IgG2a isotype control (eBioscience). 10^6 MSC cells were harvested and incubated for 10 minutes on ice with the Fc blocker antibody of anti-CD16/CD32, following one hour incubation on ice with the specific antibodies listed above. Cells were subjected to flow cytometry analysis using a LSRII flow cytometer (Becton Dickinson Immunocytometry Systems).

Evaluation of MSC differentiation potential:

Adipogenesis

Cells were seeded at a concentration of $2x10^4$ cells/well in a 24-well plate. The next day, adipogenic medium containing 10 μg/ml insulin (Sigma), 0.5mM IBMX (Sigma) and 1×10^{-5} M dexamethasone (Sigma) was added. The cells were grown for 1-3 weeks, with medium replacement twice a week. Adipogenesis was detected by Oil red O staining. For Oil red O quantification, 4% IGEPAL CA 630 (Sigma) in isopropanol was added to each well. Light absorbance was measured in 492 nm.

Osteogenesis

Cells were seeded at a concentration of $2x10^4$ cells/well in a 24-well plate. The next day, osteogenic medium containing 50 μg/ml L-ascorbic acid-2 phosphate, 10 mM glycerol 2-phosphate disodium salt, and 1×10^{-7}M dexamethasone (all from Sigma) were added. The cells were grown for 1-3 weeks with medium being replaced twice a week. Osteogenic differentiation was detected by Alizarin red staining. For Alizarin red quantification, 0.5 N hydrochloric acid (HCl) and 5% SDS were added to each well. Light absorbance was measured in 405 nm.

Population doubling time and growth area measurement

Proliferation rates of the various MEFs and iPSCs were evaluated by calculating population doubling time. Cells ($5*10^5$) were plated in in 6 cm plates in duplicates. The cells were counted every 3 or 4 days and re-plated at the same density. This procedure was repeated 5 times. MSCs proliferation rates were evaluated by defining the number of times that the cells were transferred to a larger growth area at defined time points. For instance, if the growth area increased times two the effective growth area increased by one unit of 6 cm plate (28.3 cm^2).

Single-cell cloning

iPSCs and MSCs isolates cells were serially diluted to reach 1-5 cells per well. The cells were plated on gelatin coated 96 well plates. The colonies were examined by microscope to ensure that they originated form a single-cell. Two to three weeks later colonies were subjected to genomic DNA genotyping.

Colony forming units-fibroblasts (CFU-Fs) assay

BMNCs of HZp53 adolescent (age of 5-12 weeks old) and adult (age of 13-60 weeks old) were plated at cell densities of $20x10^6$- $30x10^6$ in 10 cm BD falcon plates. The cells were grown in MSC medium as described above and re-fed once a week without further treatment. At day 14, un-fixed colonies were subjected to genomic DNA genotyping.

***In vivo* tumorigenesis assays**

iPSC clones were trypsinized and re-plated with ESCs medium for 15 minutes. The non-adherent cells were collected, resuspended in PBS and injected sub-coutaneously into 6-8 weeks old *Hfh11nu* Nude mice (10^6 cells /100µl, with Matrigel matrix at a ratio of 1:1 (Becton Dickenson FAL354232)). The tumors were removed 2-16 weeks post injection, fixed in 4% paraformaldehyde, decalcified and embedded in paraffin. Selected sections, derived from 3 distinct tumor levels were stained with hematoxylin and eosin (H&E).

7-8 weeks old age NOD.CB17-prkdc-SCID/NCrHsd mice were injected subcutaneously with $3x10^6$ MSC cells expressing WTp53, HZp53 or Mutp53. Mice were sacrificed when their tumors reached a diameter of 1-1.5 cm or after 120 days after inoculation. Upon tumor removal, half the tumor was mechanically disaggregated on mesh to establish MSC–transformed cell lines. The remaining portion of the tumor was used for histological analysis by H&E staining.

Western blot and immunoprecipitation analysis

Cells were lysed in 1X passive lysis buffer (Promega), 1 mM Tris-HCl (pH 7.5), 5 Mm MgCl2, 0.13 mM CaCl2, 25µg / µl DNase (Sigma), incubated for 1 h at 37°C and 0.5X TLB buffer was added (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails I and II (Sigma) for 15 minutes on ice, followed by centrifugation. BCA reagent (Pierce) was used to determine Protein concentration. Fifty µg protein of each sample were separated by SDS-gel electrophoresis, and transferred to nitrocellulose membranes. The following primary antibodies were used: anti-mouse *p53* monoclonal 1c12 (Cell Signaling Technology), anti-p21 polyclonal (Santa Cruz) and anti-GAPDH mab374, (Chemicon). The protein-antibody complexes were detected using horseradish peroxidase–conjugated secondary antibodies and the Amersham ECL western blotting detection reagents (GE Healthcare). For

immunoprecipitation either PAb240, a monoclonal anti-Mutp53 antibody (a kind gift from Dr. D. Lane) , PAb246, a monoclonal anti-WTp53 antibody (a kind gift from Dr. D. Lane) or control IgG antibody (Sigma) were incubated overnight at 4ºC with the lysate followed by the addition of 30 µl protein A beads for 2 h at 4°C. The immunoprecipitated material was washed and pellets were resuspended in SDS sample buffer and subjected to western blot analysis.

Genomic DNA extraction

DNA was extracted using QUICK gDNA Miniprep (Zymo Research, Irvin, CA, USA), according to manufacturer's protocol. The genomic DNA was eluted in double distilled water.

Genomic sequencing

Prior sequencing, a polymerase chain reaction (PCR) analysis was performed using 5ng genomic DNA and primers surrounding the p53 R172H mutation site. Forward primer: TCCCAGTCCTCTCTTTGCTG. Reverse primer: CTCGGGTGGCTCATAAGGTA. PCR reactions consisted of 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s, purified with HiYield gel/PCR DNA fragment extraction kit (RBCBioscience, Xindian City, Taiwan). Twenty ng of DNA was sequenced at the sequencing unit of Weizmann Institute of Science with either forward or reverse primer above.

Quantifying Copy Numbers in Genomic DNA Using the TaqMan Copy Number Assay

Copy number genotyping was performed using RT-PCR–based copy number analysis (TaqMan Copy Number Assays, Applied Biosystems) for *p53* (exon 1- intron 1 Chr.11:69394017, Applied Biosystems) and custom *loxP* site mutant *p53* (intron 4, Applied Biosystems). Analyses were initially performed on a subset of mouse tip fibroblasts of known genotypic origin. For each single-well reaction using 20 ng genomic DNA and 1x TaqMan Universal PCR Master Mix, a 1x TaqMan Copy Number Assay, which contained forward primer, reverse primer, and FAM dye-labeled MGB probe specific for the gene of interest, was run simultaneously with a 1xTaqMan Copy Number Reference Assay, which contained forward primer, reverse primer, and a VIC dye-labeled TAMRA probe specific for transferrin receptor (Tfrc) according to the manufacturer's instructions. PCR was performed in 96-well plates using a PCR system (7300 Real-Time PCR System, Applied Biosystems). Samples were assayed using triplicate wells for

each gene of interest. Copy numbers were estimated (CopyCaller Software version 2.0, Applied Biosystems) using the ΔCt relative quantification method. A maximum likelihood algorithm was used to estimate the mean ΔCt expected for copy number 1 (CN =1) based on the probability density distribution across all samples, and this parameter was used in subsequent copy number calculations for each given gene. This analytical method was used to calculate the relative copy number of a target gene normalized to Tfrc, a reference of known copy number (CN = 2).

Genomic DNA genotyping

Genotyping was performed by PCR analysis using 50 ng of genomic DNA and primers surrounding the *lox*P site (See Supplementary S1). Forward primer: ACCTGTAGCTCCAGCACTGG. Reverse primer: ACAAGCCGAGTAACGATCAGG. PCR reactions consisted of 35 cycles of 95°C for 60 s, 60°C for 60 s, and 72°C for 180 s and run on 2% agarose electrophoresis gel.

QRT-PCR

Total RNA was isolated using the Nucleospin II kit (Macherey Nagel) according to the manufacturer's protocol. An aliquot of 2 μg of total RNA was reverse-transcribed using Bio-RT (BioLab) and random hexamer plus oligo-dTprimers (NEB). QRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 instrument (Applied Biosystems). The values for the specific genes were normalized to HPRT housekeeping gene control. Specific primers were designed for the following genes: Pmaip1: forward: GCAGAGCTACCACCTGAGTTC, reverse: CTTTTGCGACTTCCCAGGCA , Cong1: forward: ACAACTGACTCTCAGAAACTGC , reverse: CATTATCATGGGCCGACTCAAT, Ercc5: forward: TGCTGGCCGTGGATATTAGC, reverse: GCCGGTGGAATAATGTGAGAAGA, Mgmt: forward: TGCTCTCCATCACCCTGTGTT, reverse: AACACCTGTCTGGTGAATGAATCTT, Rad51: forward: AAGTTTTGGTCCACAGCCTATTT, reverse: CGGTGCATAAGCAACAGCC, Brca1: forward: CGAATCTGAGTCCCCTAAAGAGC, reverse: AAGCAACTTGACCTTGGGGTA, Brip1: forward: TACTCTGGCTGCAAAGTTATCTG, reverse: TCGTGCATCTACATGGTGGAC, Mre11a: forward: CCTCTTATCCGACTACGGGTG, reverse: ACTGCTTTACGAGGTCTTCTACT Crabp1: forward:

CAGCAGCGAGAATTTCGACGA, reverse: CGCACAGTAGTGGATGTCTTGA, Hoxb7: forward: AAGTTCGGTTTTCGCTCCAGG, reverse: ACACCCCGGAGAGGTTCTG.

Flow cytometric determination of apoptosis by annexin V/propidium iodide double staining.

Cells were analyzed for phosphatidylserine exposure by an annexin-V FITC/propidium iodide Annexin using V FLUOS staining kit (Roche) according to manufacturer's protocol.

DNA Library Preparation and Sequencing

Exome capturing was carried out with Agilent SureSelect V4 All Exon Mus musculus kit according to the manufactures protocol. In brief, 2-5 μg of gDNA were fragmented to ~170 bp (PE) insert-size with a Covaris S2 device. 500 ng of Illumina adapter-containing libraries were hybridized with the exome baits at 65°C for 24h. Each enriched final paired-end library was sequenced using multiplexing of six samples on two Illumina Hiseq2000 lanes.

Mapping and analysis

Illumina sequence data were aligned to the mm10 mouse reference genome assembly using BWA (0.5.9, (Li and Durbin, 2009)) duplicate and nonuniquely mapping reads were excluded. We subsequently detected SNVs and InDels as described in (Jones et al., 2013; Rausch et al., 2012) adjusting the pipeline by using mouse genome annotations for Mapability, simple tandemrepeats, repeatmasker, segmental duplications, dbSNP137 as well as mm10 SNVs by ENSEMBL.

cDNA Microarray

Total RNA was extracted using Tri-Reagent (MRC Inc.) according to manufacturer's protocol, and submitted for analysis to the Micro-Array unit of Weizmann institute of science, Rehovot, Israel. Agilent chips were used as a platform for RNA loading. The limma package (Smyth and Speed, 2003) was used for microarray processing. Background was subtracted using the function backgroundCorrect and normalization within and between arrays was performed using the functions normalizeWithinArrays and normalizeBetweenArrays, respectively. Spots with

the same probes were averaged. Analysis of variance (ANOVA) including contrasts was applied to the data set using Partek Genomic Suite 6.5 (Inc. St. Charles, MO).

Spectral karyotype analysis (SKY)

Half a million cells were plated in a 10 cm plate and were cultivated for 48 h following replacement of medium and additional incubation period of 24 h. Colcemid (0.1 µg/ml) was added to the culture for 4 h. Cells were trypsinized and lysed with hypotonic buffer following fixation in glacial acetic acid:methanol (1:4). The chromosomes were simultaneously hybridized with 24 combinatorially labeled chromosome painting probes and analyzed using the SD200 spectral bioimaging system (Applied Spectral Imaging Ltd.).

p53 SNP genomic quantitative melt curve genotyping

The p53 R172H SNP was detected using the SimpleProbe TaqMan assay (Roche, Switzerland) according to the manufacturer's protocol. Known percentage of WTp53 and Mutp53 DNA samples were mixed to create a six points' standard curve for the unknown samples. The fluorescence was measured using the LightCycler® 480 instrument (Roche). An algorithm was applied to analyze the heterozygous percentage of cells in the population, based on the slope between the WTp53 and the Mutp53 picks. The formula for calculating the percentage of HZp53, Mutp53 and WTp53 cells in the population is:

for %Mutp53 allele in the population>50:
(%Mutp53 allele in the population-50)*2= %Mutp53 cells in the population100-%Mutp53 cells in the population= %HZp53 cells in the population
for %Mutp53 allele in the population<50:
(50-%Mutp53 allele in the population)*2= %WTp53 cells in the population
100-%WTp53 cells in the population= %HZp53 cells in the population

Melt-curve genotyping analysis

Analysis was performed using an algorithm that was developed with the help of Alex Kagan from the physics department. This algorithm detects the melting temperatures local maxima for the WTp53 and Mutp53 alleles and calculates their ratio. This ratio is then compared to the standard curve, resulting in high resolution genotyping of the unknown samples.

Tumor samples

A cohort of mice was monitored for signs of illness or obvious tumor burden. Moribund mice were sacrificed, and tumors were fixed with 4% paraformaldehyde in phosphate-buffered saline. Tissues were paraffin embedded and sectioned at 10 μm. All sections were stained with H&E prior to pathological analysis.

Statistical analysis

Unless specified otherwise, all statistical analyses were performed using Graphpad Prism.

Results

Chapter 1: Reprogramming into pluripotency attenuates p53 LOH associated malignant phenotype

MEFs robustly undergo p53 LOH that enables malignant transformation

The main objective of my research is the identification and characterization of p53 LOH-associated cellular transformation in various contexts. To accomplish this, I utilized the LFS model of mutant p53 R172H knock-in mice (Figure 1). The p53 heterozygous mice (HZp53) represent the human LFS patients harboring in their germ-line heterozygous p53 mutation (Lang et al., 2004). In order to follow p53 status, we established three complementary methods: (1) PCR amplification around *loxp* insertion in the mutant p53 allele (Figure 1B). (2) Sanger sequencing around p53 missense mutation (Figure 1C). (3) Quantitative genotyping utilizing Taqman probe that anneals both WT and mutant p53 alleles in different temperatures with a designated algorithm that calculates the percentage of cells within the population that underwent p53 LOH (Figure 1D). As a first step we examined p53 LOH in differentiated cells. MEFs derived from 13.5 dpc embryos of WTp53, HZp53 and Mutp53 genotypes were cultivated *in vitro,* proliferation rate and p53 genotype was tracked. We first examined whether HZp53 MEFs undergo p53 LOH *in vitro* and found that p53 LOH occurred in all examined MEFs at day 12 (passage 7, Figure 2B and C). This correlated with a distinct increase in their proliferation capacity (Figures 2A) and with a decrease of classical WTp53 target gene, p21 mRNA and protein levels (Figures 2D and E). These results indicated loss of WTp53 function. Our results suggest that in MEFs with one copy of WTp53 exhibited controlled cell growth, whereas Mutp53 facilitated cell proliferation only upon the completion of WTp53 LOH.

Next, to test whether p53 LOH facilitates induction of tumors of MEFs *in vivo*, both early and late passages of HZp53 MEFs were injected into immune-compromised mice. As controls, early and late passages of Mutp53 MEFs as well as early passage of WTp53 MEFs (before senescence occurring around passage 7) were injected. Only late passages of both HZp53 and Mutp53 gave rise to malignant fibrosarcomas (Figure 2F), indicating that the p53 LOH, similarly to homozygous p53, enables other genomic alterations to occur that leads to malignant transformation.

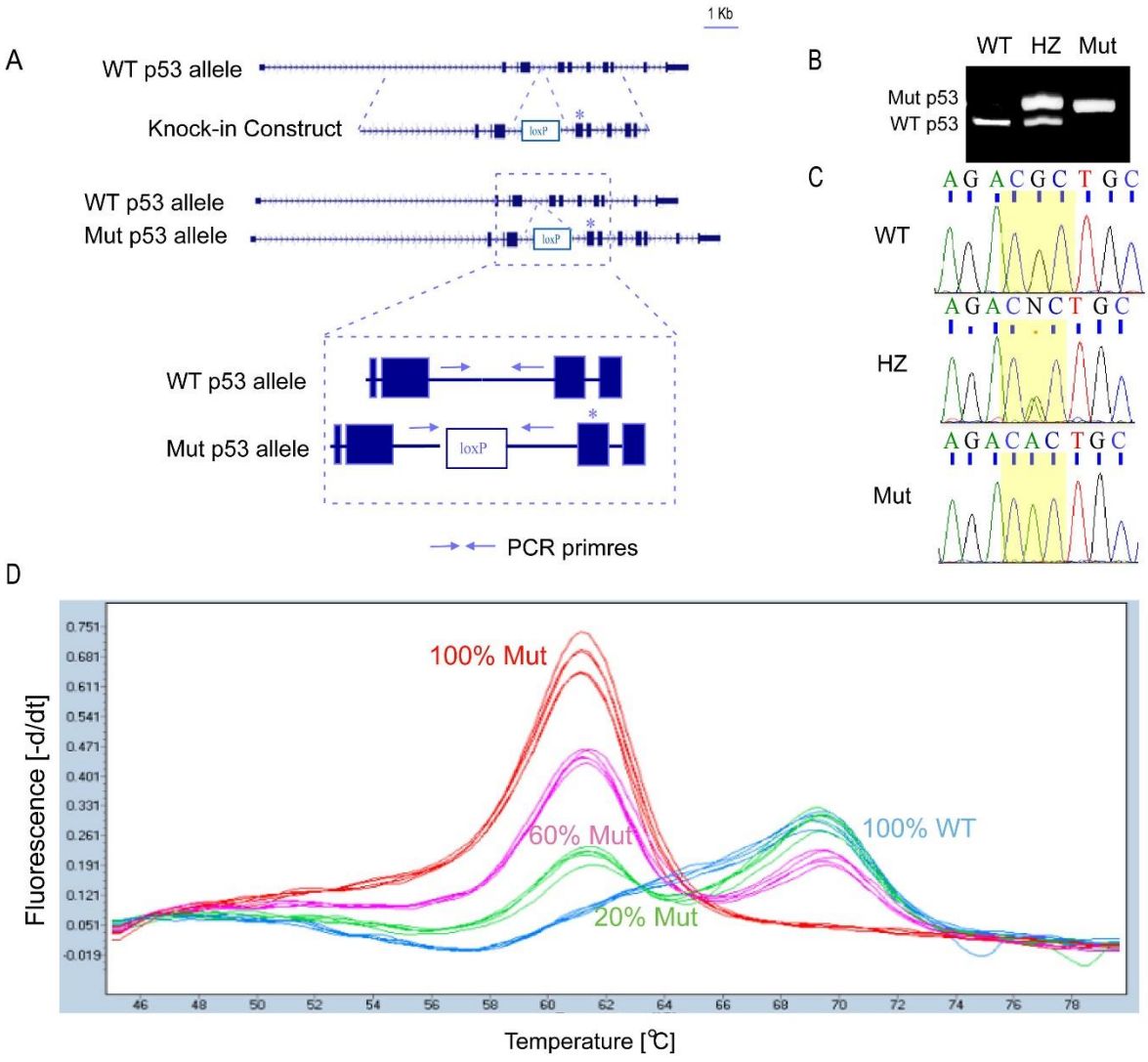


Figure 1. Analyses of p53 heterozygosity.
(A) Schematic representation of a p53 genomic sequence of Mutp53 knock-in mice. *loxP site* was inserted in intron 4. A missense mutation in exon 5 is indicated by an asterisk. The arrows in the blow-up depict the primers annealing sites. (B) PCR analysis of MEFs derived from mice with different p53 status: WTp53, HZp53 and Mutp53 with the primers set shown in A. (C) Sequencing analysis of MEFs showing the guanine-to-adenine substitution between WTp53 and Mutp53 and the presence of both bases in the heterozygous cells. (D) Schematic representation of a p53 quantitative genotype. The first negative derivative of the fluorescence against the temperature is presented. The peak represents the melting temperature of each sample. The different curves are for different ratios of WTp53 and Mutp53 alleles in the population: homozygous for Mutp53 (red), 60% of the population is Mutp53 (pink), 20% of the population is Mutp53 (green), homozygous for WTp53 (blue).

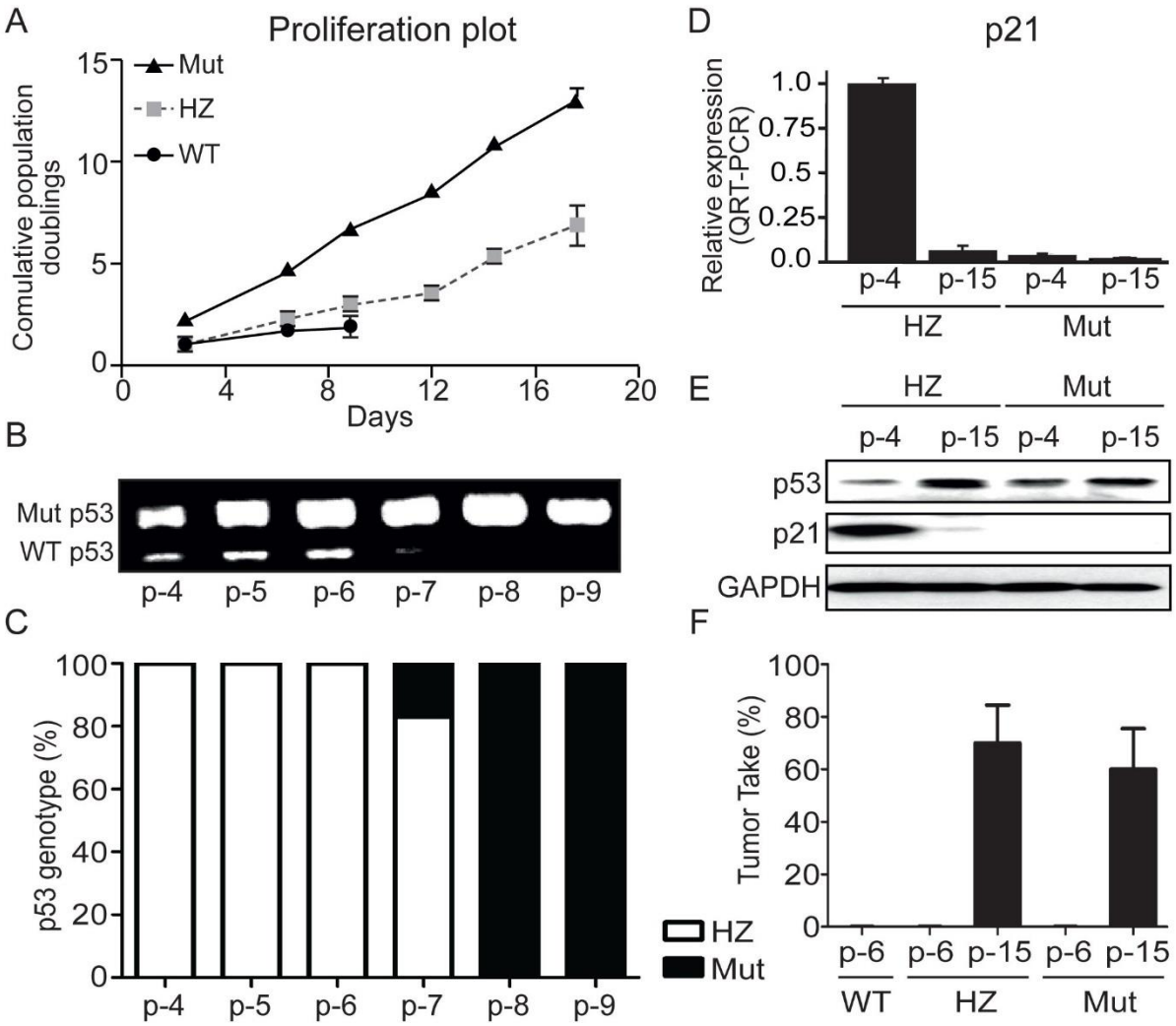


Figure 2. MEFs undergo p53 LOH.
MEFs were generated from WTp53, HZp53 and Mutp53 mice embryos and propagated *in vitro*. (A) Cumulative population doublings were calculated and plotted. (B) PCR analysis of HZp53 MEFs in correlation with the proliferation plot. This experiment was performed 3 times in duplicates from three independent MEF preparations. (C) A representative Taqman PCR genotype analysis of HZp53 MEFs in correlation with the proliferation plot. Computer algorithm assessing the percentage of HZ and Mut (homozygous) p53 cells in the population according to the ratio between WT and Mutp53 alleles. (D) Relative mRNA expression of p21 in HZp53 and Mutp53 MEFs at early and late passages as measured by QRT-PCR. (E) Western blot analysis of p21 and p53 protein levels in HZp53 and Mutp53 MEFs at early and late passages (F) WTp53, HZp53 and Mutp53 MEFs at different passage were injected sub-cutaneous into immune-deficient mice. Tumor take is presented (n=10 in each group).

Reprogramming attenuates p53 LOH process

Next to study p53 LOH in stem cells, we harnessed Yamnaka's reprogramming technology to reprogram MEFs into pluripotent state. We used three reprogramming factors (Klf4, Sox2 and Oct4) to reprogram MEFs into iPSCs (Nakagawa et al., 2008), since c-Myc exhibits oncogenic properties, it was not included in our system. Interestingly, the early reprogramming kinetics of the HZp53 cells were comparable to those of WTp53 cells, as shown by the early reprogramming marker alkaline phosphatase (Figure 3A) and the rate of appearance of Nanog-expressing colonies (Figure 3B). The p53 status did not influence proliferation or apoptosis (Figures 3C, D and E), suggesting that in the steps of reprogramming of HZp53 iPSCs, the WTp53 dominates over the Mutp53. Next, we followed and analyzed the effect of reprogramming on the heterozygous state of the clones. Unlike MEFs, 76% of the analyzed iPSCs (24 different isolated clones from three different experiments) retained their WTp53 allele (Figure 4A). This remarkable finding, which suggests that the reprogramming process attenuates p53 LOH, encouraged us to examine whether bona fide ESCs show a similar phenotype. For this aim, we adopted mouse ESCs heterozygous for p53, established from the same mice colony. These ESCs showed markers of pluripotency and were also able to differentiate *in vitro* into the three germ layers as well as to form benign teratomas, when injected subcutaneously into nude mice (Rivlin et al., 2014). Indeed, we could not detect any LOH in our HZp53 ESCs (Figure 5), thus showing that pluripotent state of the cells can attenuate p53 LOH.

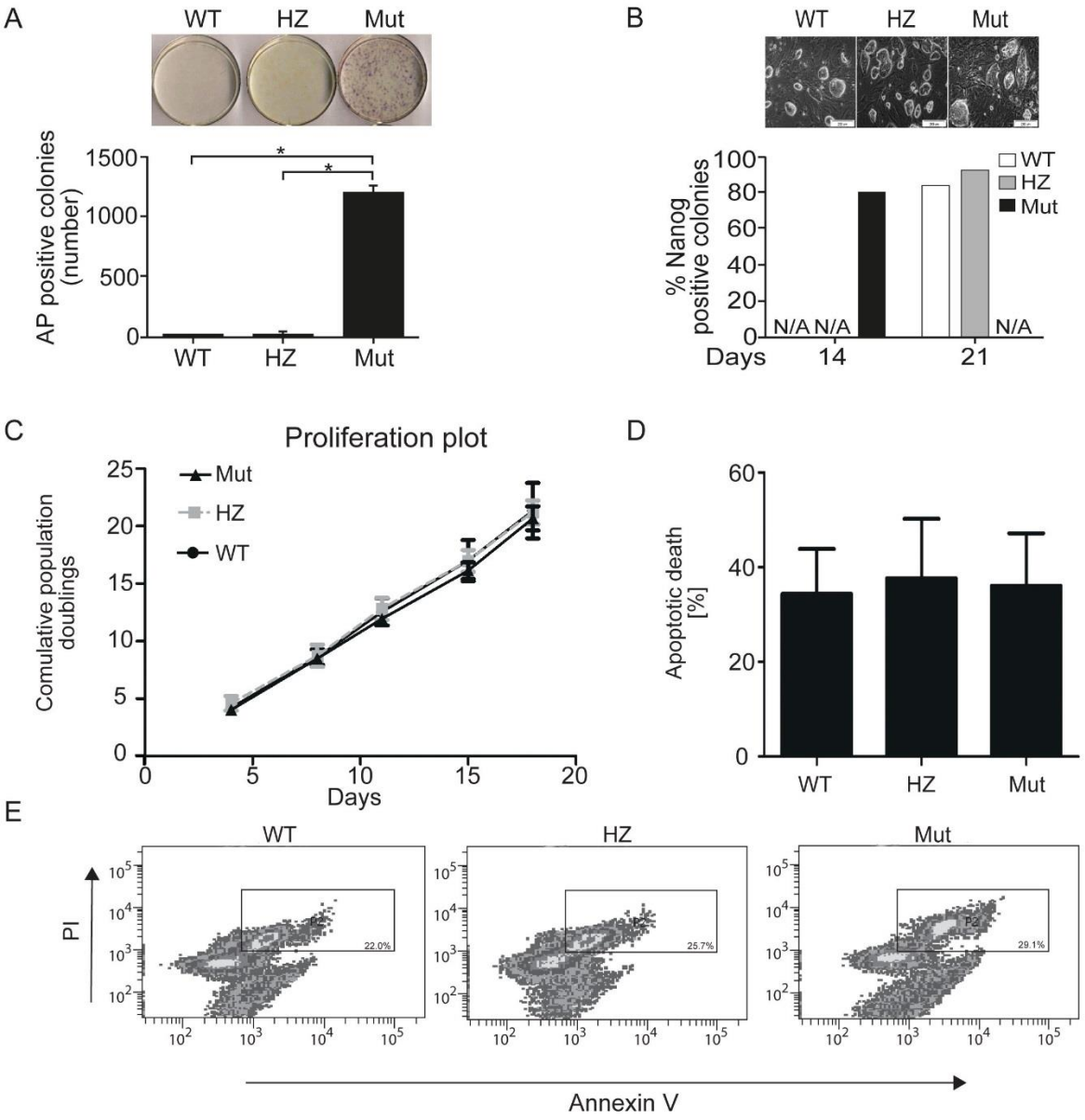


Figure 3. The reprogramming kinetics of HZp53 MEFs are similar to WTp53 MEFs, rather than Mutp53 cells.

(A) WT, HZp53 and Mutp53 MEFs were infected with lentiviruses encoding Oct4, Sox2 and Klf4. Cells were plated and assayed for alkaline phosphatase (AP) activity, 4 weeks post infection. (B) Upper panel: Light microscopy images, depicting typical ESCs morphology obtained from WTp53, HZp53 and Mutp53 expressing iPSC clones. Lower panel: percentage of iPSC clones in which the relative expression levels of Nanog reached at least 50% of Nanog expression levels in ESCs. These clones were harvested at 14 and 21 days post infection and subjected for QRT-PCR analysis. (C) Cumulative population doublings of WTp53, HZp53 and Mutp53 iPSCs were calculated and plotted. (D) Percentage of apoptotic death was determined using Annexin-V PI staining flow cytometry analysis. (E) A representative Annexin-V PI staining flow cytometry analysis of WTp53, HZp53 and Mutp53 is presented.

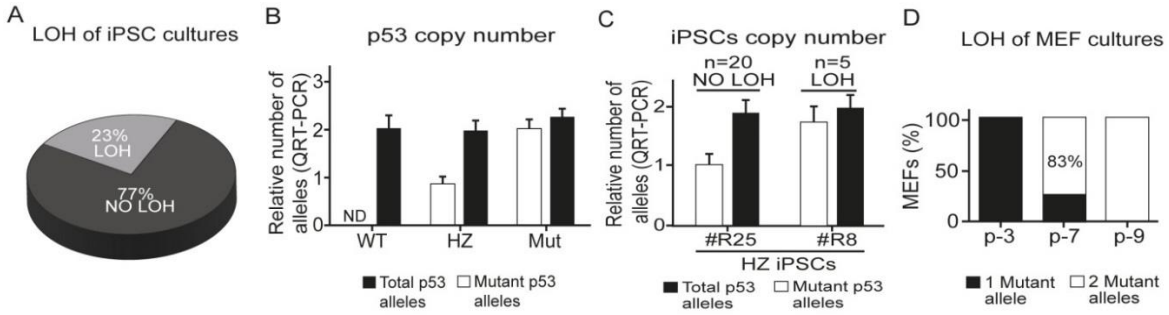


Figure 4. Reprogramming attenuates p53 LOH, which occours via duplication of the mutant allele.

(A) A diagram summarizing the distiribution of iPSC clones that underwent LOH. n=26. The presented data indicates summary of three independent experiments (B) A representative Taqman Q-PCR of the genomic DNA copy number of WTp53, HZp53 and Mutp53 MEFs. Each well is normalized to TFRC. Probes against total p53 or mutant p53 were designed to identify exon1 and *loxP* site, respectively. (C) A representative Taqman Q-PCR of p53 copy number in HZp53 iPSCs clones at p-12, ~70 days post infection (#R25 and #R8). Probes and normalization were as in B. n=25. (D) A plot summarizing copy number of the mutant alleles of HZp53 MEFs in culture in passages 3, 7 and 9.

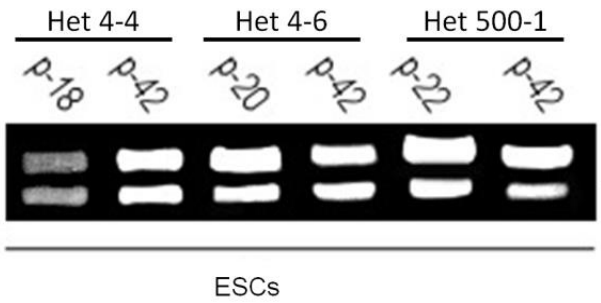


Figure 5. HZp53 ESCs do not undergo p53 LOH.

PCR analysis of three p53 HZ ESC lines (4-4, 4-6 and 500-1) in early passage (p-18-20) and following prolonged culturing (p-42).

<u>Duplication of the Mutp53 allele underlies p53 LOH process, which results in genomic instability</u>

We next decided to investigate the mechanism underlying LOH occurred in MEFs and iPSCs. To distinguish between the homozygous (two mutated alleles) and hemizygous (one null and one mutated allele) states, we designed specific probes that anneal either specifically to the mutated p53 allele (targeting the *loxP* site sequence in intron 4), or to all p53 alleles (targeting the first exon of p53). Using these probes we performed a Taqman Q-PCR copy number assay

to assess the number of mutant p53 alleles compared to the total number of p53 alleles (Figure 4B). As shown in Figure 4C and D, both MEFs and iPSC clones that underwent LOH duplicated the mutant allele and became homozygous cells rather than hemizygous cells.

We next evaluated the effect of p53 LOH on the genomic stability of the generated iPSC clones. To that end, we performed spectral karyotyping (SKY) of iPSCs that did not undergo p53 LOH, which largely exhibited a normal karyotype (Figure 6A), whereas those that underwent p53 LOH showed translocations and irregular chromosome numbers (Figure 6B). This together with our observation that HZp53 ESCs did not undergo p53 LOH under prolonged culturing (Figure 5) suggest that both ESCs and iPSCs which are known to have high genomic fidelity possess the ability to avoid p53 LOH which occurs via duplication of the mutant allele.

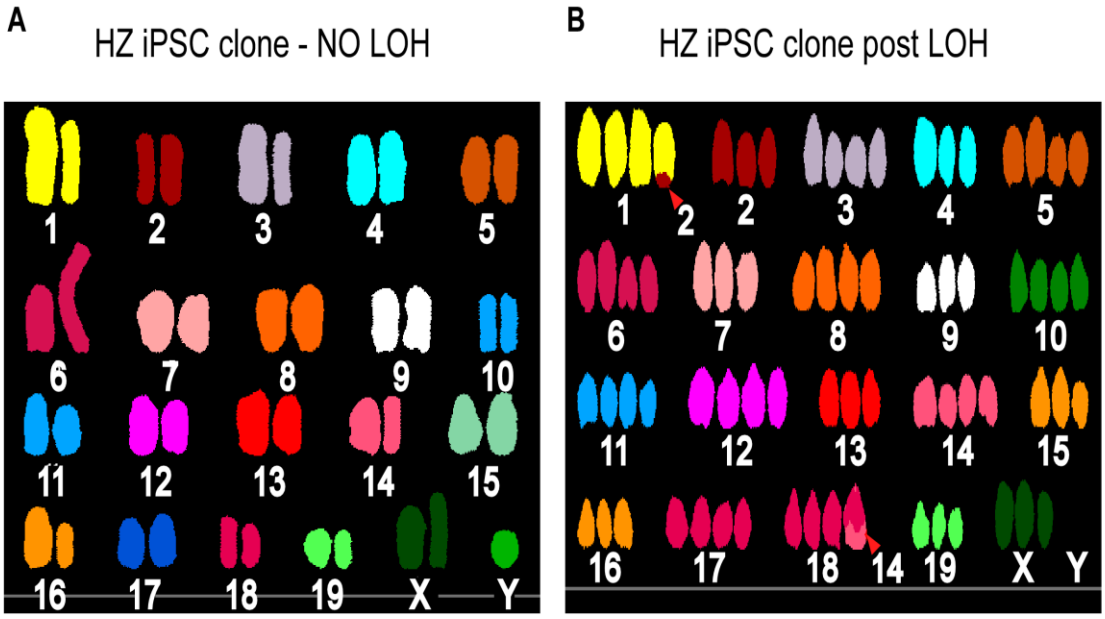


Figure 6. Following p53 LOH, iPSCs exhibit abnormal karyotype.
(A) A representative SKY analysis of HZp53 IPSC clone. Two iPSC clones were examined (6 metaphases each). (B) A representative SKY analysis of HZp53 clone, which underwent p53 LOH indicates translocations of chromosome 2 to 1 and 14 to 18. One iPSC clone was examined (6 metaphases).

p53 LOH induces tumor aggressiveness

A study conducted in our lab revealed that mutant p53 possesses a GOF activities in reprogrammed cells (Sarig et al., 2010) both *in vitro* and *in vivo*. While injection of WTp53 iPSCs into immunosuppressed mice gave rise to benign teratomas, mutant p53 iPSCs gave rise to malignant tumors. To follow the biological significance of the various p53 genotype iPSCs *in vivo,* we injected the established clones subcutaneously into nude mice. As expected, injection of Mutp53 iPSCs gave rise to poorly differentiated tumors of either mesoderm or low level of ectoderm appearance (Figure 7D). Notably, the iPSC clones that underwent LOH *in vitro* (prior to their injection) showed tumors with limited capacity to differentiate (Figure 7C), which was similar to Mutp53 iPSCs. On the contrary, injection of HZp53 iPSCs that preserved the WTp53 allele gave rise to benign teratomas, suggesting they retain a high capacity to differentiate into the three germ layers (Figure 7A), similarly to the WTp53 iPSCs clones. Moreover, some of the clones which were injected in a heterozygous state underwent p53 LOH *in vivo,* as estimated by the finding that only mutant allele was detected *ex vivo* (Figure 7B.1). These clones exhibited heterogenic phenotypes within the tumor, i.e. some regions displayed high differentiation level, while other regions of the tumor seemed to be undifferentiated (Figure 7B.2-4). Remarkably, the same clones that lost heterozygosity *in vitro* also lost heterozygosity *in vivo* (data not shown), which can imply that either they were predisposed to p53 LOH, or that the p53 LOH occurred in single cells prior to their injection and these cells underwent clonal expansion both *in vitro* and *in vivo*. This result suggests that p53 LOH is a crucial event that leads to malignant phenotype in iPSCs.

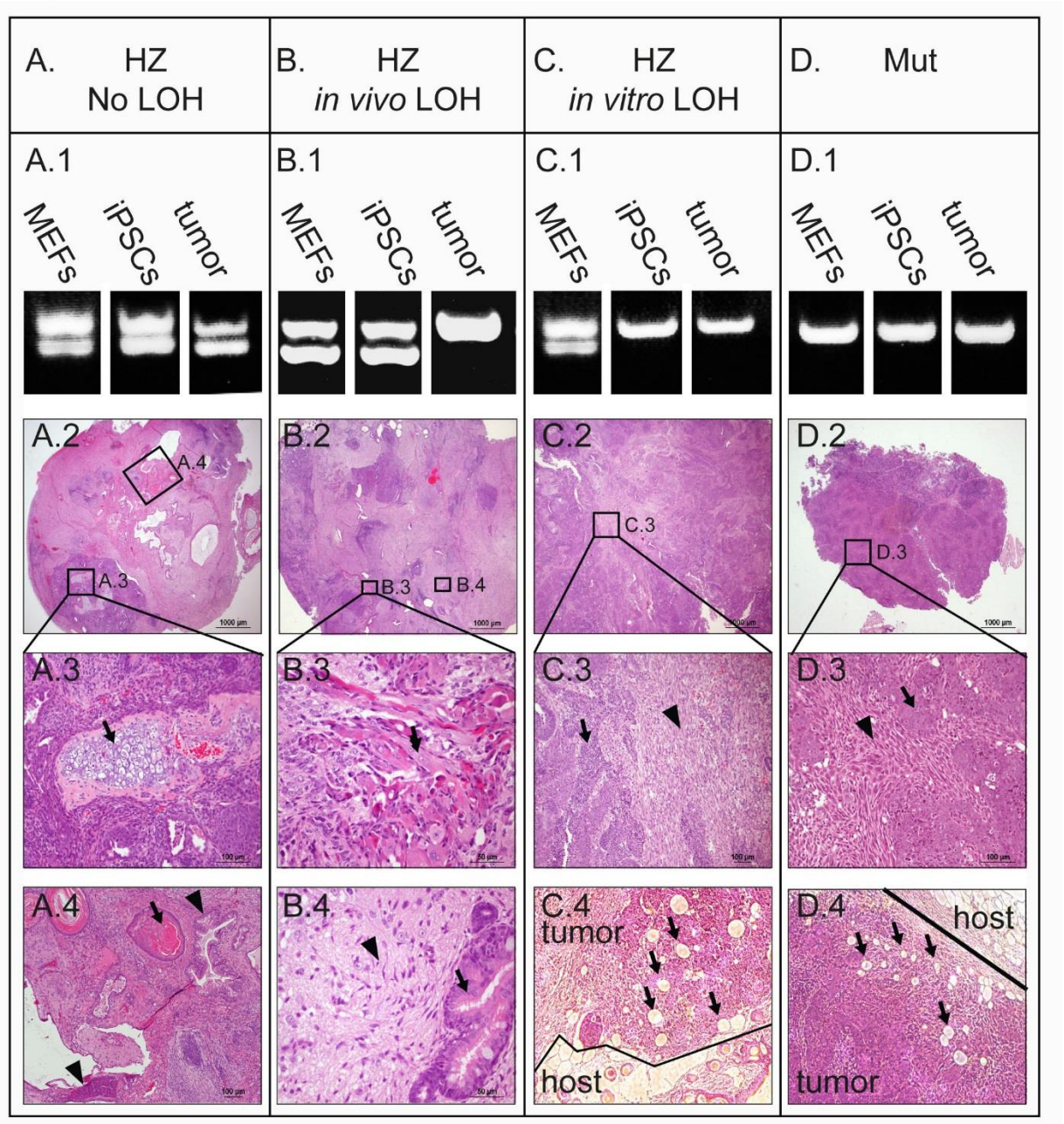


Figure 7. p53 LOH is crucial for malignant transformation of iPSCs.
The generated iPSCs were injected subcutaneously into nude mice. When tumors reached 1cm^3 mice were sacrificed and tumors were analyzed by H&E staining. (A) Representative sections of HZp53 tumors, n=10. (A.2) A highly-differentiated teratoma, x2. Squares indicate areas that were analyzed in higher magnification. (A.3) X20 magnification of square A.3 in A.2. An arrow points to an island of cartilage surrounded by osteoid (mesoderm). (A.4) X10 magnification of square A.4 in A.2. An arrow points to keratin (ectoderm) and an arrowhead to respiratory epithelium with goblet cells and cilia (endoderm). The arrowhead at the bottom identifies exocrine pancreatic glands (endoderm).

(B) Representative sections of tumors derived from HZp53 clones that underwent LOH *in vivo*, n=4. (B.2) Predominantly poorly differentiated teratoma, x2. The squares indicate areas that were analyzed in higher magnification. (B.3) X40 magnification of square B.3 in B.2. An arrow points to skeletal muscle fibers (mesoderm). (B.4) X40 magnification of square B.4 in B.2. An arrow points to respiratory epithelium with goblet cells and cilia (endoderm) and an arrowhead to very well-differentiated nervous tissue (ectoderm). (C) Representative sections of HZp53 clones that underwent LOH *in vitro*, n=5. (C.2) Mostly poorly differentiated tumor, x2. The square indicates an area that was analyzed in higher magnification in C.2. (C.3) X20 magnification of square C.3 in C.2. An arrow points to poorly differentiated epithelium interpreted as nervous tissue (ectoderm) and an arrowhead points to poorly differentiated stroma (mesenchyme), with features of sarcoma. (C.4) The neoplastic cells engulfed pre-existing tissue elements. In this case - lipocytes (arrowheads), x20. (D) Representative sections of Mutp53 clones, n=8. (D.2) A poorly differentiated tumor, x2. The square indicates an area that was analyzed in higher magnification in J. (D.3) X20 magnification of square D.3 in D.2. An arrow points to poorly differentiated epithelium interpreted as nervous tissue (ectoderm) and an arrowhead points to poorly differentiated stroma (mesenchyme), with features of sarcoma. (D.4) The neoplastic cells engulfed pre-existing tissue elements. In this case - lipocytes (arrowheads), x20.

Chapter 2: BM-derived MSCs undergo p53 LOH, which enables malignant transformation

BM MSCs undergo p53 LOH in an age dependent manner

In order to study the LOH process in a more physiological stem cells system, in collaboration with Prof. Dov Ziporri, Dr. Ronit-Aloni-Grinstein, Sivan Kagan and Gabriela Koifman, we investigated the MSCs, as a model of adult stem cells. An increasing body of evidence suggests that SCs or progenitor cells represent a target population that initiates tumorigenesis in various solid tumor types. The notion that sarcoma may arise from defective MSCs together with the observation that LFS is characterized by occurrence of familial sarcoma prompted us to investigate whether p53 status in MSCs regulates their tumorigenic potential. Thus, we investigated whether BM derived MSCs may represent the cells of origin of sarcoma upon p53 LOH, and the molecular events that underlie the p53 LOH process in MSCs. Of note, in LFS patients tumors are usually not diagnosed before 15 years of age (Malkin et al., 1990). Thus, it was important to compare young and adult age groups.

To that end, MSCs were isolated from adolescent and adult mice of various p53 genotypes. BM cells were cultivated and the plastic adherent cells were propagated to confluence. The stromal cell cultures established were free of hematopoietic and endothelial cell contamination, as estimated by FACS analysis (Sup. Figures 1 and 2). The MSC isolates were further characterized by their ability to differentiate into adipocytes and osteocytes (Sup. Figures 3 and 4). Time dependent changes in the growth area along culturing were measured. Comparison of cell proliferation rates indicated that the WTp53 MSC isolates exhibited the slowest growth rate, while Mutp53 MSCs exhibited the most rapid cell proliferation rate. HZp53 MSCs exhibited a bi-phasic growth curve. At first, their growth rate was similar to WTp53 MSCs, however within a few passages *in vitro,* they presented a switch in their growth rate and acquired an enhanced growth rate, similarly to that observed with Mutp53 isolates (Figure 8A). To examine whether a p53 LOH process occurs in the HZp53 MSC isolates as a function of *in vitro* culturing time, we performed p53 genotyping of all the individual HZp53 MSC isolates. While MSC isolates derived from adolescent mice completely lost the WTp53 allele at p-12, MSCs isolated from adult mice exhibited WTp53 LOH at p-5 (Figures 8B and C). This indicates that MSCs, despite being stem cells, have lower genome stability and fidelity compared to embryonic pluripotent stem cells.

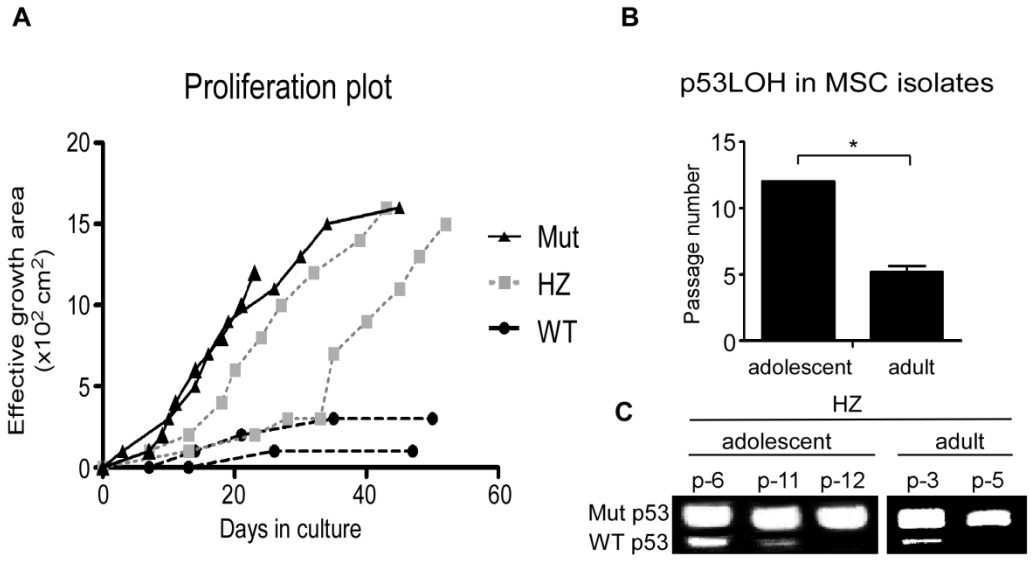


Figure 8. p53 LOH in MSC isolates is age-dependent.

MSC isolates derived from WTp53, HZp53 and Mutp53 mice were established. (A) Proliferation plot presenting effective growth area along time in culture. Each curve represents MSC isolate derived from 2 mice. Two isolates were prepared for each mouse genotype. (B) p53 status of MSC isolates derived from 2 adolescent and 4 adult mice was determined by PCR genotyping in each passage. The plot represents the mean passage number at which p53 LOH completion was detected. (C) A representative figure of p53 PCR genotype analysis of MSC isolates derived from adolescent and adult mice.

MSCs that underwent p53 LOH give rise to tumor initiating MSCs

To evaluate the biological outcome of the p53 LOH, we examined the tumorigenic potential of the various MSC isolates as a function of their p53 status. To that end, we injected subcutaneously into immune-compromised NOD/SCID mice HZp53 MSC isolates as well as WTp53 and Mutp53 MSC isolates (as a control) derived from 8-10 weeks old mice. It should be noted that isolates were injected at about passages 13-15 of *in vitro* culturing, shortly after the HZp53 isolates completed the p53 LOH process (Figure 9A). All ten mice injected with homozygous Mutp53 MSCs developed tumors within 65 to 99 days post injection (Figure 9A and B). Interestingly, no tumors were detected in mice inoculated with HZp53 MSC isolates derived from adolescent mice, despite losing WTp53 allele. However, when we examined the tumor formation potential of HZp53 isolates derived from adult mice (age 59 weeks), which underwent WTp53 LOH around p-5 (Figure 8C), all mice developed tumors within 53-104 days (Figures 9A and C). These tumors exhibited histological characteristics similar to tumors formed by Mutp53 MSCs from adolescent mice (Figures 9B and C). Histological analysis of the tumors indicated typical features of sarcoma with an invasive edge (Figures 9B and C). These results might imply that the process of p53 LOH enables additional transforming events to occur. To evaluate this, MSCs derived from adolescent mice were injected ten passages post p53 LOH (passages 20-21) and MSCs derived from adult mice were injected shortly after p53 LOH (passages 7-8). Indeed, MSCs in both age groups did not induce tumors shortly after p53 LOH, whereas ten passages post p53 LOH both age groups were tumorigenic. However, the adolescent MSCs showed lower tumor take (70%) and twice as much tumor formation latency, mean=72.5 days vs. mean=151 days. Mice injected with WTp53 MSCs at passage 20-21 exhibited no signs of morbidity or mortality during 180 days after the inoculation. Together,

these results indicate that p53 LOH contributes to the malignant transformation of MSCs, yet is not sufficient on its own.

A

MSC strain	Mice age (weeks)	Passage	Tumor take	Days to tumor detection (mean)
WT	8-10	20-21	0/10	ND
HZ		13-15	0/10	ND
HZ		20-21	7/10	106-187 (151)
Mut		13-15	10/10	65-99 (83)
HZ	59	7-8	0/10	ND
HZ		13-15	10/10	53-104 (72.5)

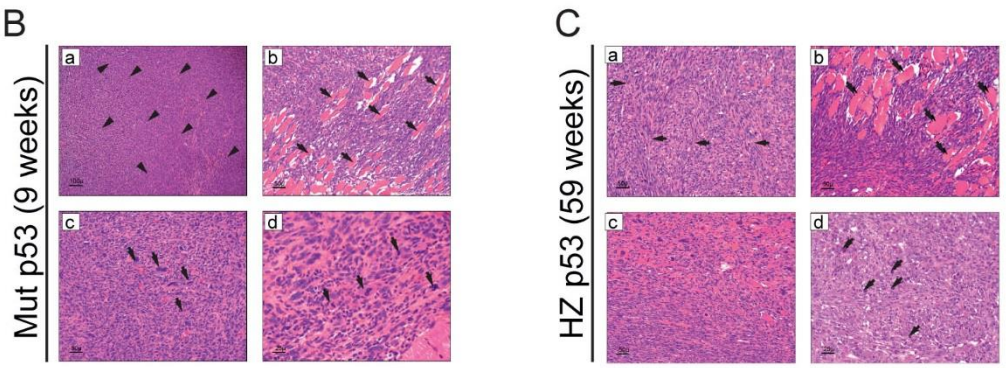


Figure 9. HZp53 MSC isolates ten passages following p53 LOH formed malignant sarcoma upon injection into immune-deficient mice

$3*10^6$ cells of two different MSC isolates from WTp53, HZp53 and Mutp53 adolescent mice and HZp53 from adult mice were injected subcutaneously into 5 NOD-SCID mice per group (total of 10 mice per each p53 genotype). Tumors were removed and stained by H&E. Tumors exhibited histologic features typical of sarcoma. (A) Table summarizing tumor take of the different MSC isolates injected. (B+C) Representative sections from Mutp53 (B) and HZp53 (C) MSC isolates. (a) Arrowheads indicate neoplastic cells arranged in interlacing fascicles, in an arrangement similar to fibrosarcoma, x20. (B) x10 (C). (b) A typical invasive edge is presented. The neoplastic cells engulfed pre-existing tissue elements. In this case - skeletal myofibers (arrowheads), x20. (c). Marked difference in the overall size (anisocytosis) and in nuclear size (anisokaryosis) of neoplastic cells. Several larger cells were identified (A, arrowheads), x20. (d). The mitotic rate was high (arrows indicate mitotic figures), x40.

Homologous recombination underlies p53 LOH process in MSCs, iPSCs and MEFs

To unravel the gene expression patterns associated with p53 LOH process in MSCs, we performed mRNA profiling by cDNA microarray. To this end, we analyzed mRNA of MSC isolates derived from adolescent mice at passage 9, in which the p53 LOH process has already begun, but was not yet completed (Figure 8) and HZp53 isolate at passage 2, with no detectable p53 LOH. Our analysis yielded 11 clusters. Cluster 1, the 'downregulated cluster' (Figure 10A-C) is composed of genes that are down-regulated upon p53 LOH and is enriched for known 'p53 signaling' genes, such as Fas, Ccng1, Cdkn1a, Pmaip1, Mdm2 and Zmat3 (Figure 10B; $P=2.76x10^{-7}$), as well as for the p53-dependent DNA repair genes Ercc5 and Mgmt. Reduced expression of p53 target genes and the above DNA repair genes in HZp53 p-9 isolates confirms a progressive loss of functional WTp53 in these cells. Cluster 2 represents a group of 633 genes that are upregulated upon p53 LOH (Figure 10D). We evaluated whether this 'upregulated cluster' contains genes previously described as proliferation related genes (Brosh and Rotter, 2010; Whitfield et al., 2006) and found an overlap of 25 genes (Figure 10E). Functional annotation revealed that the 'upregulated' cluster is enriched for genes of the HR DNA repair pathway (HRDRP; $P=9.14*10^{-4}$). Notably, the HRDRP genes do not overlap with the 'proliferation cluster' genes, with one exception (Trip13). We validated the elevated expression of Rad51, Brca1, Brip1 and Mre11a in p-9 HZp53 MSCs (Figure 10G). Fanconi Anemia proteins (Fanci, Fanca, Fancb and Fancd2) that execute cross-linked DNA repair, known to engage HR, (Yang et al., 2005) also appeared in the upregulated cluster (Figure 10D), as well as Xrcc5 (Ku80) and Mre11a, known to have a role in non-homologous end joining (NHEJ; Figure 10F). Figure 10F illustrates a 'heat map' of expression levels of all the upregulated genes involved in HRDRP and additional DNA repair pathways, suggesting that HRDRP is one of the major mechanisms leading to LOH (Tischfield, 1997). This indicates that a burst of DNA repair genes is associated with p53 LOH process in MSCs.

In order to examine whether homologous recombination underlies the molecular mechanism involved in this process in iPSCs and MEFs as well, we performed a whole-exome sequencing of four HZp53 iPSC clones and two HZp53 MEFs batches. The obtained results confirmed that three of the iPSCs clones retained their p53 heterozygosity, whereas one underwent p53 LOH and all MEFs displayed a p53 LOH pattern. Interestingly, a single-nucleotide variant (SNV) in Efnb3, an adjacent gene upstream of Trp53 remained heterozygous in all examined samples

regardless whether p53 LOH occurred or not (Figure 11). We concluded that homologous recombination (HR) underlies the mechanism of p53 LOH process. Moreover, it is intriguing to speculate that a fragile site lies in between Efnb3 and Trp53.

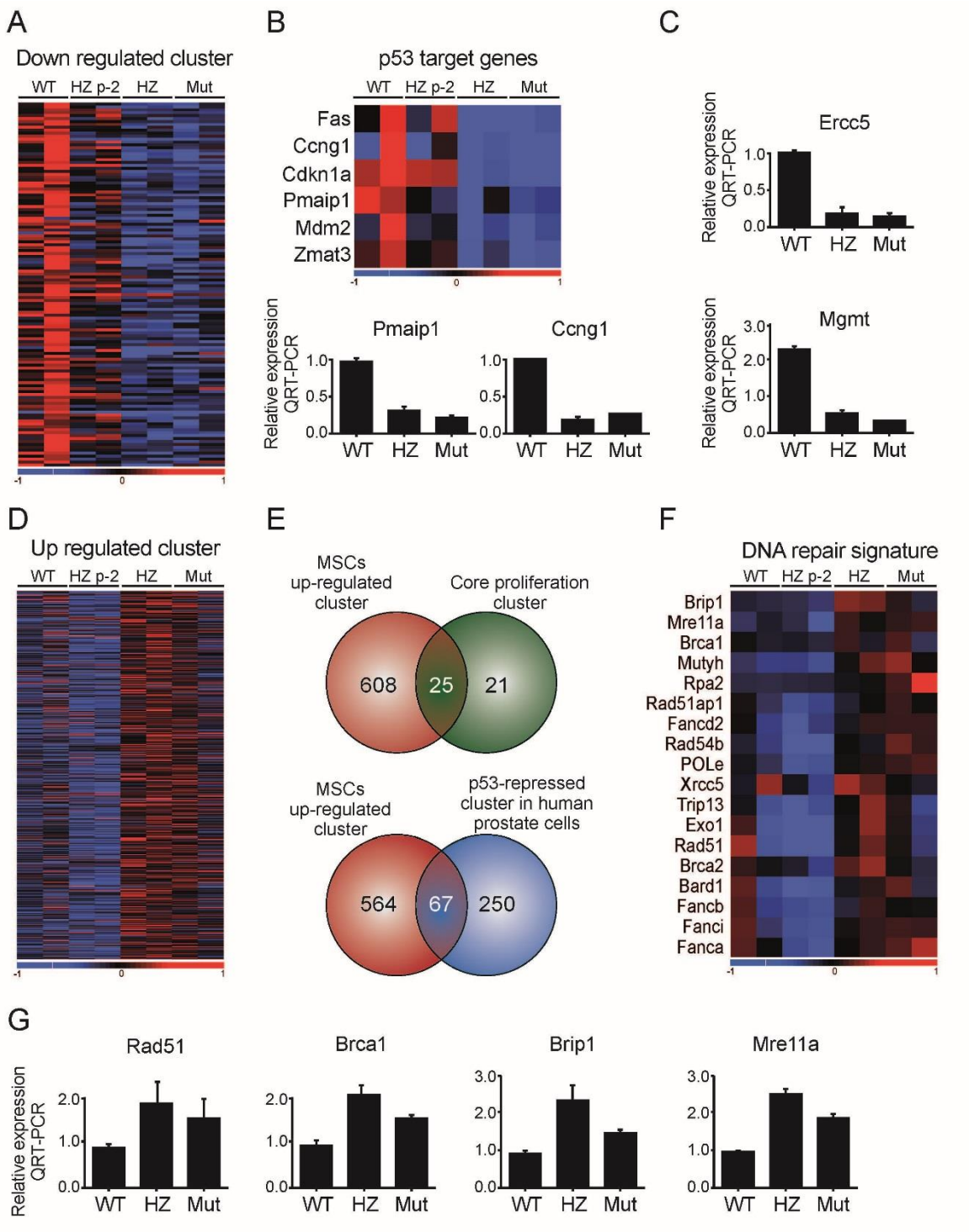


Figure 10. Downregulation of p53 target genes and upregulation of HR DNA repair genes coincide with p53 LOH in MSC isolates.
Genome-wide expression screen to identify changes associated with p53 LOH. Samples originated from MSC isolates duplicates of WTp53 p-9, HZp53 p-2, HZp53 p-9 and Mutp53 p-9. The various samples were hybridized to the Agilent mouse Genome Array and the relative mRNA abundance of 55681 mRNA species was monitored. After standard preprocessing steps, 4400000 expression values were collected from eight microarrays. Of these, 4524 genes exhibited a greater than twofold change between any two conditions, and were clustered according to their Pearson correlations. (A) Heat-map representation of Agilent microarray data depicting the downregulation of 133 genes between WTp53 (WT) and HZp53 p-2 (HZ p-2) to HZp53 p-9 (HZ) and Mutp53 (Mut) (cluster 1). (B) Heat-map representation of known p53 targets from cluster 1 and validation of gene expression of Pmaip1 and Ccng1 by QRT-PCR. (C) Validation of the expression changes in DNA repair genes from cluster 1 (Ercc5 and Mgmt) by QRT-PCR. (D) Upregulation of 633 genes between WTp53 (WT) and HZp53 p-2 (HZ p-2) to HZp53 p-9 (HZ) and Mutp53 (Mut) (cluster 2). (E) 'Upregulated cluster' was compared with the 'core proliferation cluster' (right hand). *P<=0.001. A Fisher exact test was used to compare this overlap. (F) Heat-map representation of genes that were implicated as homologous recombination (HR) DNA repair pathway genes ($P=9.14x10^{-4}$) using IPA functional annotation, genes associated with non-homologous end joining (NHEJ), and genes associated with Fanconi Anemia cross-linked DNA repair known to engage HR. (G) QRT-PCR validation of expression changes in Rad51, Brca1, Brip1 and Mre11a. QRT-PCR results of each gene were normalized to Hprt. All samples were collected at p-9 unless indicated otherwise. Bars represent mean±S.D.

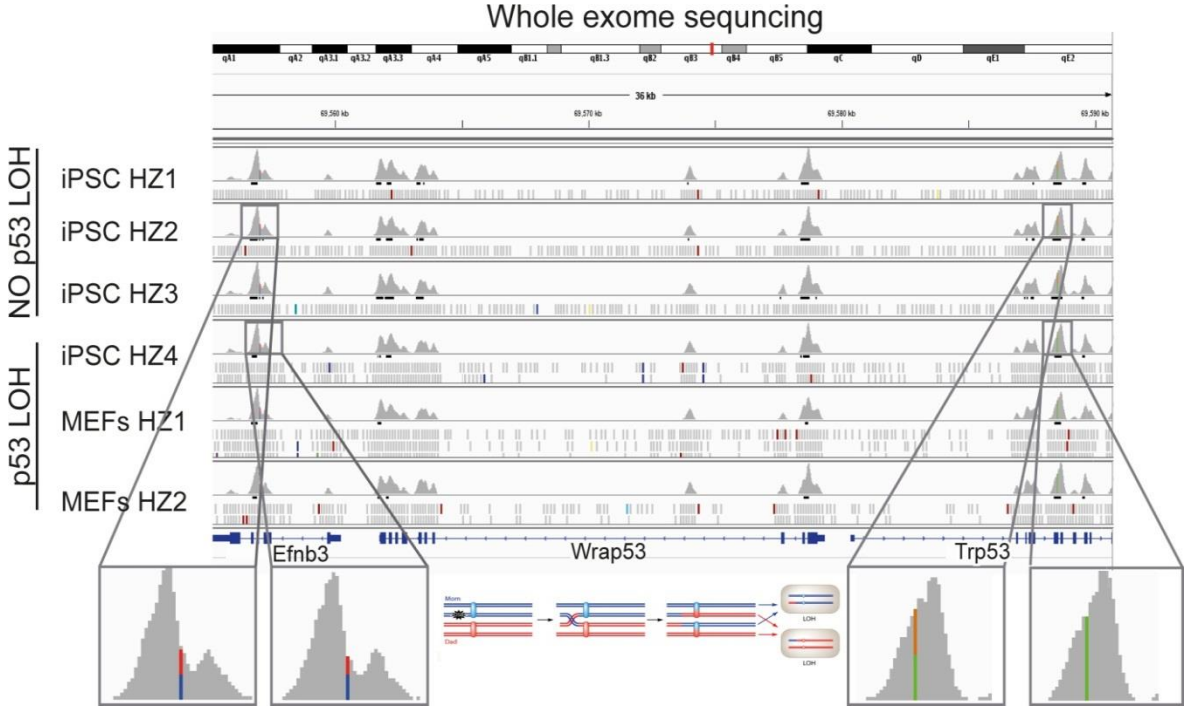
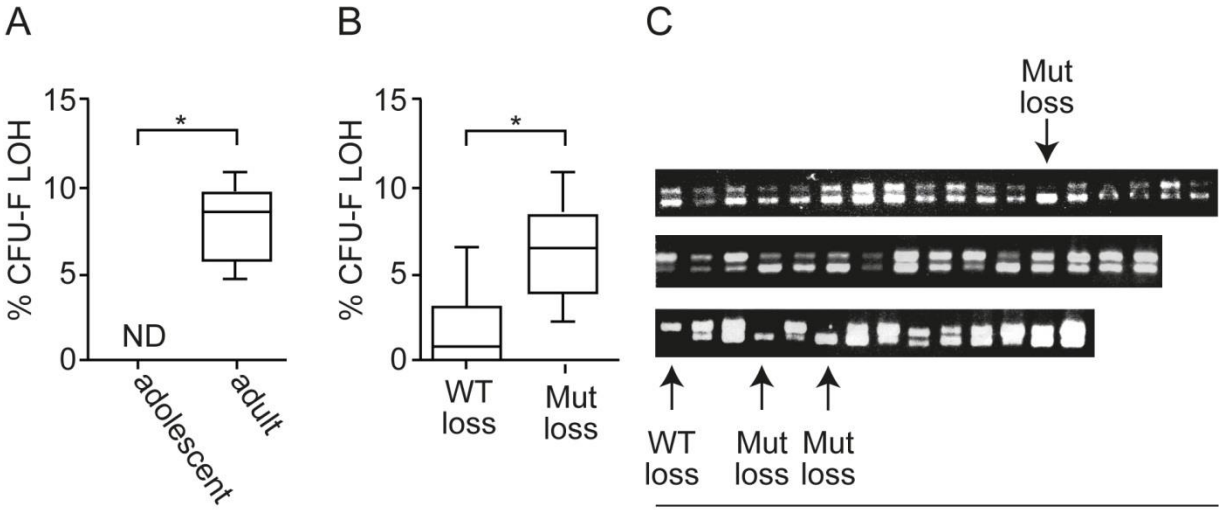


Figure 11. Homologous recombination underlies p53 LOH process.
Four HZp53 iPSC clones at p-11 and two HZp53 MEF preparations at p-10 were subjected to whole exome sequencing. Integrative Genomic Viewer (IGV) image of a 36Kb section of chromosome 11 of the six samples is presented. Insets depict heterozygous SNVs in Efnb3 found in iPSC HZ2 and HZ4, while these same cells are either heterozygous at a SNV in Trp53 (iPSC HZ2, two colors) or homozygous (iPSC HZ4, single color). At the bottom is shown a schematic representation of p53 LOH through homologous recombination.

Bi-directional p53 LOH in BM progenitors

Next, we aimed to elucidate at which stage of tumorigenesis the p53 LOH process occurs. Thus, we examined whether p53 LOH can take place in the BM of healthy HZp53 mouse. To this end, precursor cells from the BM were isolated and tested *ex-vivo* for p53 status by genotyping. BM progenitors were isolated by the colony forming units fibroblast assay (CFU-F). This is the basic method to evaluate mesenchymal precursors, where each colony is derived from a single initiating cell. To obtain CFU-Fs, BM from WTp53, HZp53 and Mutp53 adolescent mice at the age of two months was plated under specific conditions to allow formation of MSCs colonies. The highest number of CFU-F colonies, was obtained from the Mutp53 mice and the lowest were from the WTp53 mice cells (Data not shown). A slightly higher score than the one achieved for WTp53 mice was recorded with the HZp53 mice. To analyze whether progenitors derived from the BM of HZp53 mice have undergone p53 LOH, we screened unfixed live CFU-Fs for p53 genotype status. As mentioned above, in LFS patients tumors are usually not diagnosed before 15 years of age (Malkin et al., 1990). Thus, it was important to compare young and adult age groups. Hence, we assessed p53 LOH in BM progenitors originating from adolescent (4-12 weeks) and adult (13-59 weeks) mice. Notably, we could not detect p53 LOH in adolescent HZp53 mice. However, CFU-Fs derived from BM of adult HZp53 mice exhibited p53 LOH at varying frequencies (4-11% per mouse), as shown in Figure 12A and D. Surprisingly, we found that in addition to the loss of the WTp53 allele (WTp53 LOH), the majority of the LOH events were targeting the loss of the Mutp53 allele (Mutp53 LOH) (Figure 12B). These results suggest p53 LOH as a DNA repair mechanism in young age that later its dysfunction might lead toWTp53 loss and tumorigenesis.



D

Mouse number	Mice age	Number of screened CFU-F	Total LOH (%)	Loss of WT allele (%)	Loss of mutant allele (%)
1	Adolescent (5-12 weeks)	74	0	0	0
2		70	0	0	0
3		39	0	0	0
4		82	0	0	0
5	Adult (13-60 weeks)	94	6.4	0	6.4
6		120	9.1	1.7	7.5
7		46	10.8	0	10.8
8		88	4.5	0	4.5
9		47	8.4	2.1	6.3
10		45	8.8	6.6	2.2

Figure 12. WTp53 and Mutp53 LOH in BM mesenchymal cells
BM was isolated from HZp53 adolescent and adult mice and CFU-Fs formation was assessed. Two weeks later, colonies were genotyped by PCR. (A) Percentage of BM-derived CFU-F colonies that underwent p53 LOH, derived from adolescent and adult mice. (B) Percentage of CFU-F colonies derived from BM of adult mice that lost either their WTp53 or Mutp53 allele. Box plots represent median, 25th percentile, 75th percentile and extreme values. (C) Raw unprocessed data of PCR-genotyped CFU-F colonies derived from mouse no. 9. WT or Mut LOH is marked accordingly. (d) Table summarizing the results obtained from genotyped CFU-Fs presented in panels A and B.

Bi-directional p53 LOH occurs in single-cell sub-cloning of iPSCs

In order to resolve whether the bi-directional p53 LOH is a unique phenomenon in BM progenitors, single-cell sub-cloning of iPSC clone that retained its heterozygosity was performed. Genomic analysis of 156 iPSC single-cell sub-clones indicated that 153 (98%) of the sub-clones kept their HZp53 genotype and only 3 of them (2%) underwent p53 LOH (Figure 13A). This further supporting the conclusion that reprogramming indeed attenuates p53 LOH. Surprisingly, the 3 sub-clones that underwent p53 LOH have lost the Mutp53 allele and exhibited only the WTp53 allele. We could not detect any single cell sub-clones that have lost the WTp53 allele (Figure 13A). The detection of WTp53 LOH in some of the original iPSC clones coupled with the detection of Mutp53 LOH only in single cell sub-clones derived from iPSCs suggests that iPSCs, at large, can undergo a bi-directional p53 LOH, however this is a very rare event.

Bi-directional p53 LOH occurs in single-cell sub-clones derived of MSCs isolates

Our results indicated that p53 LOH in total population of HZp53 MSCs always leads to the loss of the WTp53 allele. In contrast, as shown above, only by single cell sub-cloning of iPSCs we were able to detect Mutp53 LOH, which led us to conclude that iPSCs may undergo bi-directional p53 LOH. We therefore examined the frequency of bi-directional p53 LOH in MSCs using single-cell culturing of MSCs. Indeed, genotyping of 220 single cell sub-clones of early passages of MSC isolates identified some sub-clones that exhibited the loss of the Mutp53 allele (1.4%), while most of them showed the loss of the WTp53 allele (69.4%) (Figure 13B). Thus, suggesting that bi-directional p53 LOH is not restricted to a specific type of stem cells.

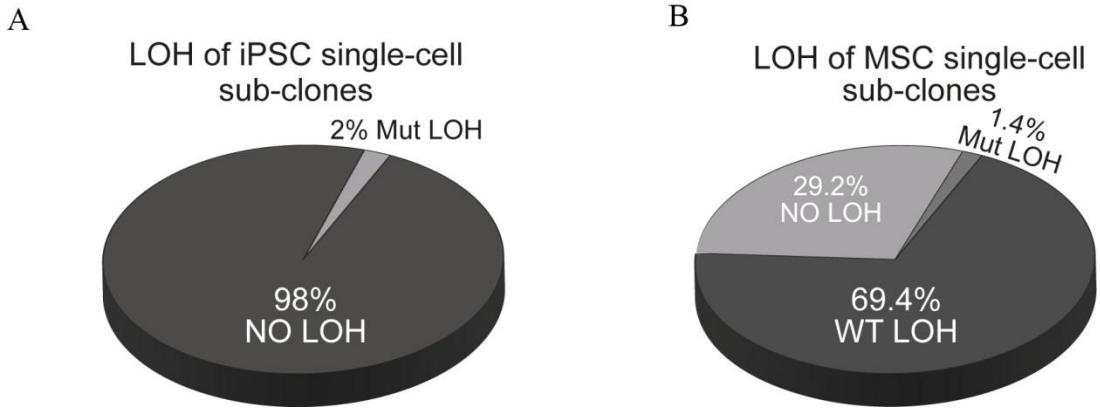


Figure 13. Bi-directional p53 LOH is not restricted to a specific stem cell type.

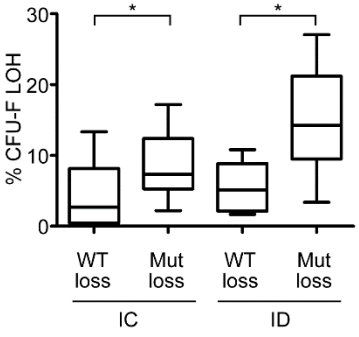
Two HZp53 iPSC clones were single-cell sub-cloned in 96 well-plates. After 2-3 weeks, plates were genotyped by PCR. (A) Summary of 156 single-cell sub-clones is presented in a pie chart (F) Two HZp53 MSC isolates derived from adolescent mice were sub-cloned at a density of either 1 or 5 cells per well in 96 well-plates, and PCR-genotyped. (B) Summary of the data from three independent experiments of 220 single-cell sub-clones is presented in a pie chart. The diagram summarizes the percentage of sub-clones that did not undergo LOH (NO LOH), sub-clones that lost the WTp53 (WT LOH) and sub-clones that lost the Mutp53 (Mut LOH).

The adaptive immune system does not affect p53 LOH bi-directionality in CFU-Fs

Two possible explanations for the observed bi-directionality of p53 LOH are considerable. The first, a specific cellular repair mechanism dictates a preference to lose the mutant allele and retain the WTp53 allele. The second possible explanation is that LOH is a stochastic event, however when WTp53 is lost the cell undergoes cellular alterations that are recognized by the immune system that in turn eliminate these cells, which results in more cells that lost the mutant p53 allele. To this end we examined whether the immune system recognizes WTp53 LOH cellular alterations and as a consequence executes clearance of these cells. For this purpose, immunodeficient (ID) mice, that lack *Rag1*, which is involved in V(D)J recombination, but not in homologues recombination, were utilized. These mice also lack *Il-2rγ*, which is required for T, B and NK cells maturation. Therefore, these mice exhibit an impaired adaptive and innate immune system. Indeed, these ID mice have diminished number of Th, Tc, B, macrophages and NK cells in their spleen (Pearson et al., 2008). The mice were crossed with our Mutp53 R172H Knock-In mice in order to generate HZp53 ID mice. First, we validated the absence of T, B and NK cells in the BM. Our FACS analysis indicated that the established mice exhibited a diminished number of Th and Tc cells, as well as a reduced number of B and NK cells, similarly to the original ID mice colony (Sup Figure 5). Next, p53 LOH incidence and the effect of the immune system on its directionality were assessed in CFU-Fs derived from both immune-competent (IC) and ID mice, using quantitative genotyping. CFU-Fs derived from HZp53 ID mice underwent p53 LOH in a similar frequency as IC mice (Figure 14). We observed that p53 LOH directionality was not affected by the immune system (Figure 14). This indicates that reduction in T, B and NK cells do not affect p53 LOH directionality.

Despite the undeniable significance of the immune system in tumor surveillance (Koebel et al., 2007), many reports showed that mostly in carcinogenic-induced mice models, immune-deficient animals have shorter tumor-free survival compared to matched immune-competent animal, but not in spontaneous forming tumor mice models (Rogers et al., 2013), implying that this phenomenon is dependent on the model used. Thus, it was of essence to examine the immune system effect on spontaneous tumor latency and p53 LOH status in spontaneous tumors of our mice cohort. For this aim, mice were monitored until tumor formation, once tumor reached 1-1.5 cm, the animal was sacrificed and p53 LOH status of the tumor detected. ID mice showed significantly shorter tumor-free survival compared to IC mice, mean=50 weeks vs. mean=65 weeks, respectively (Figure 15A). This is in agreement with *Rag1* deficient p53 deficient mice (Nacht and Jacks, 1998), indicating that B and T cell are crucial for immune-surveillance in the absence of p53. Surprisingly, all ID derived tumors underwent p53 LOH (n=8) compared to 66.7% in IC derived tumors in our cohort (Figure 15B), similarly to published data (Lang et al., 2004; Olive et al., 2004). This may indicate that the immune system can recognize and clear cells that underwent p53 LOH, whereas in its absence, those cells will form tumors in shorter latency. In an individual with a competent immune system other pathways are utilized in the transformation process which take longer period of time to form tumors.

A



B

Mouse number	Group	Number of screened CFU-F	Total LOH (%)	Loss of WT allele (%)	Loss of mutant allele (%)
1	immune-deficient	78	17.9	6.4	11.5
2		118	5.1	1.7	3.4
3		23	23.0	3.8	19.2
4		43	18.6	2.3	16.3
5		49	18.6	8.2	12.2
6		37	18.6	10.8	27.0
7	immune-competent	39	14.1	9.0	5.1
8		40	18.2	3.0	15.1
9		44	9.6	2.4	7.2
10		57	23.3	13.3	10
11		63	18.1	5.1	12.9
12		63	26.5	9.4	17.2

Figure 14. Hampered immune system does not affect bi-directional p53 LOH.
BM was isolated from HZp53 immuno-competent (IC) and immune-deficient, *Rag1* null and *Il2rγ* null mice (ID) and CFU-F formation was assessed. Two weeks later, colonies

were quantitatively genotyped by Q-PCR. (A) Percentage of BM-derived CFU-F colonies that underwent p53 LOH, derived from IC and ID mice that lost either WTp53 or Mutp53 allele. Box plots represent median, 25th percentile, 75th percentile and extreme values (B) Table summarizing the results obtained from genotyped CFU-Fs presented in panel A.

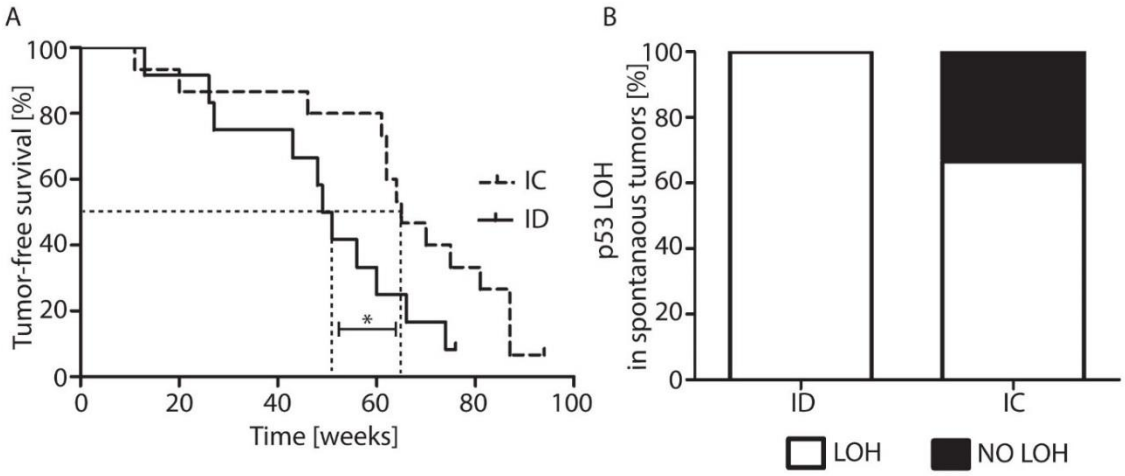


Figure 15. HZp53 ID mice have shorter tumor free survival and 100% p53 LOH in spontaneous tumors.
Tumor appearance was monitored in HZp53 immuno-competent (IC) and immune-deficient, *rag1* null and *il2rγ* null mice (ID) and quantitatively genotyped by Q-PCR. (A) Tumor-free survival curve of IC (n=16) and ID (n=12). (B) Percentage of tumors derived from IC mice (n=9) and ID (n=8) that show p53 LOH or p53 LOH – free in spontaneous tumors. LOH determined when >30% of tumor cells underwent p53 LOH as measured by quantitative genotyping.

Chapter 3: Multiple large LOH events across the genome

Trp53 locus is part of at least 41 mb genomic region that underwent LOH in MEFs and iPSCs

Ample data obtained following analyzing tumors derived from LFS patients suggest that a catastrophic genome rearrangements event, termed chromotripsis, is linked to mutant p53 (Rausch et al., 2012). To obtain a more comprehensive understanding of the changes at the genome level and whether the ability to undergo p53 LOH represents a more global phenomenon in the genome, we performed a whole genome sequencing of p53 heterozygous iPSC clones and their parental MEFs, in collaboration with the group of Prof. Lichter and Dr. Zapatka at the DKFZ. Heterozygous ESCs that do not undergo LOH were used as control (4#4, 4#6). For this reason, next generation whole genome sequencing of 2 ESCs isolates, 2 iPSC clones that did not undergo p53 LOH (Het6, Het7), 3 iPSC clones that underwent p53 LOH (Het 4, L8Het7, R24) and 5 passages of MEFs isolates (3, 6, 8, 11, 14) representing different phases of the p53 LOH processes. DNA sequences, mapped to mouse reference genome GRCm38, for all samples were analyzed. Freebayes was used to discover SNPs and INDELs. The resulting variants were filtered using SnpSift.jar. Known variants were annotated according to snpEff.jar. Only SNPs (single alternate allele), QUAL > 100 were selected for use in the analysis. Among all variants, 877688, 768040 (87.51%) annotated (rs identifier), while the rest were novel. Surprisingly, large difference in the number of SNPs per chromosome was detected. Furthermore, comparing our results to the mouse genome project revealed regions with high number of densely populated SNPs. These hyper-variable blocks, which contain most of the SNPs showed 93.61% similarity to 129S1_SvimJ mouse strain, where the remaining blocks showed similarity to C57BL_6NJ. This similarity stems from the method by which this mice colony were generated (Lang et al., 2004). The original ESCs that were used to knock-in mutant p53 R172H, were of 129S1_SvimJ origin. Those cells were introduced into C57BL_6 derived blastocyst and the offspring were then crossed with C57BL_6 mice. Resulting in genomic regions that retained SNPs of both colonies dozens of generations after the transgenic colony was generated.

In order to detect LOH event, we examined minor allele frequency of the various samples in the different chromosomes. In chromosome 11 we could recapitulate p53 status as was examined

by quantitative genotyping (Figure 16). Each dot in the image represents a SNP. The middle line represents 0.5 minor allele frequency, which is heterozygosity. Any elevation or decrease would reflect LOH. The background comparison is C57BL, thus the value of one represents the common variant of this mouse strain, whereas zero, the rare variant represents the second mouse strain, 129S1_S. The LOH event can be best visualized in the MEFs. Along the different passages the dots drift from 0.5 to 1. Trp53 locus is very small compared to the length of the genomic region that underwent LOH, at least 41 mb. In addition, this is in agreement with our previous data that the mechanism that underlies this event is homologous recombination. Moreover, as can be noticed in Figure 16 upstream to p53 lays a fragile site that entails the location of the double strand break which led to HR repair that resulted in LOH. Notably, in the iPSC clones that underwent p53 LOH only Het4 shows p53 LOH in the entire population, whereas in L8Het7 and R24 only 50% of the population underwent p53 LOH, similarly to our quantitative genotyping results. This implies that in L8Het7 and R24 clones, the first cell that underwent p53 LOH was after the reprogramming process. Interestingly, the two ESCs isolates showed a unique phenomenon of multiple crossover in the same genomic area, nevertheless, p53 remind heterozygous in both isolates. This result may suggest that a robust genomic integrity machinery that preserves Trp53 in ESCs exists. However, an alternative explanation would be that these crossovers occurred during meiosis of the sperm and oocyte prior to the formation of these ESCs. In order to resolve this issue, prior and following LOH status is required, yet since no change was detected between our earliest and late time points, it remains an open question. It was of essence to explore whether this phenomenon is unique to these ESCs isolates and does this occurs in differentiated cells as well. For this purpose, we performed Sanger sequencing of three regions in six different ESCs isolates, in early and in late passage (Figure 17). In addition, we sequenced four different fibroblast batches, two MEF isolates generated at 13.5 dpc, one at 18.5 dpc and one adult mouse derived tail fibroblasts. Each region contains 2-4 SNPs. The first region is upstream to Trp53 (SNP1) and the second (SNP2) is proximal to Trp53. The third one (SNP3) is downstream to Trp53, where a difference between the two ESCs isolate was noticed. These results recapitulated our previous study, which showed that p53 LOH could not be detected in ESCs cells (Rivlin et al., 2014). In five out of six ESCs isolates a single cross-over event was detected (Figure 17), indicating double cross over is less common event in this region. In contrast, all differentiated fibroblasts underwent LOH across

this region regardless of their age of origin, indicating that a fragile site lays upstream to SNP1 and that differentiated cell have a less stringent genome stability.

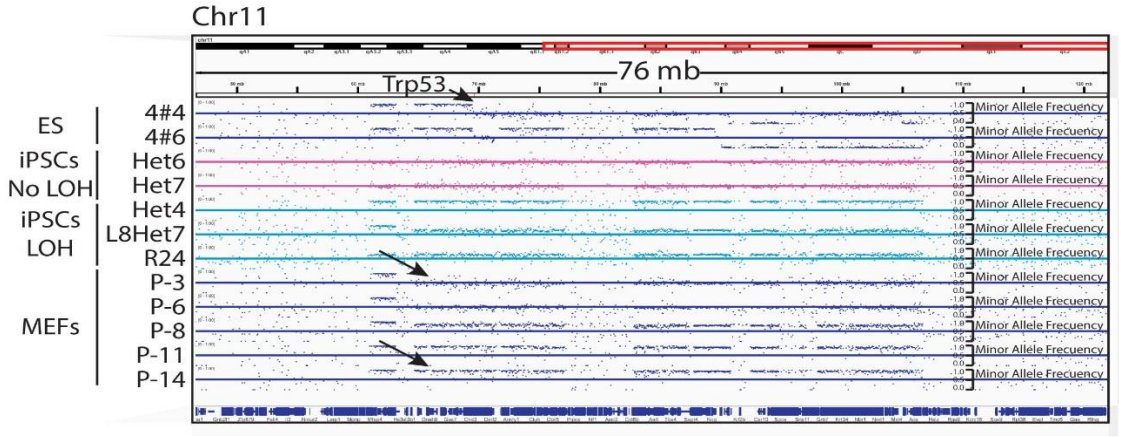


Figure 16. Genomic landscape of p53 and the surrounding chromosome 11 in ESCs, iPSCs and MEFs.

Two ESCs isolates, two iPSC clones that do not undergo p53 LOH, three iPSC clones that underwent p53 and MEFs in different passages (3, 6, 8, 11 and 14) were subjected to whole-genome sequencing. Integrative Genomic Viewer (IGV) image of a 76 mb section of chromosome 11 of the 12 samples is presented. Each SNP is represented by a dot. The horizontal line for each sample represent minor allele frequency. SNPs above the line represent common variant, while SNPs below it represent the rare variant. LOH events are indicated by an arrow. Trp53 is marked by an arrow head.

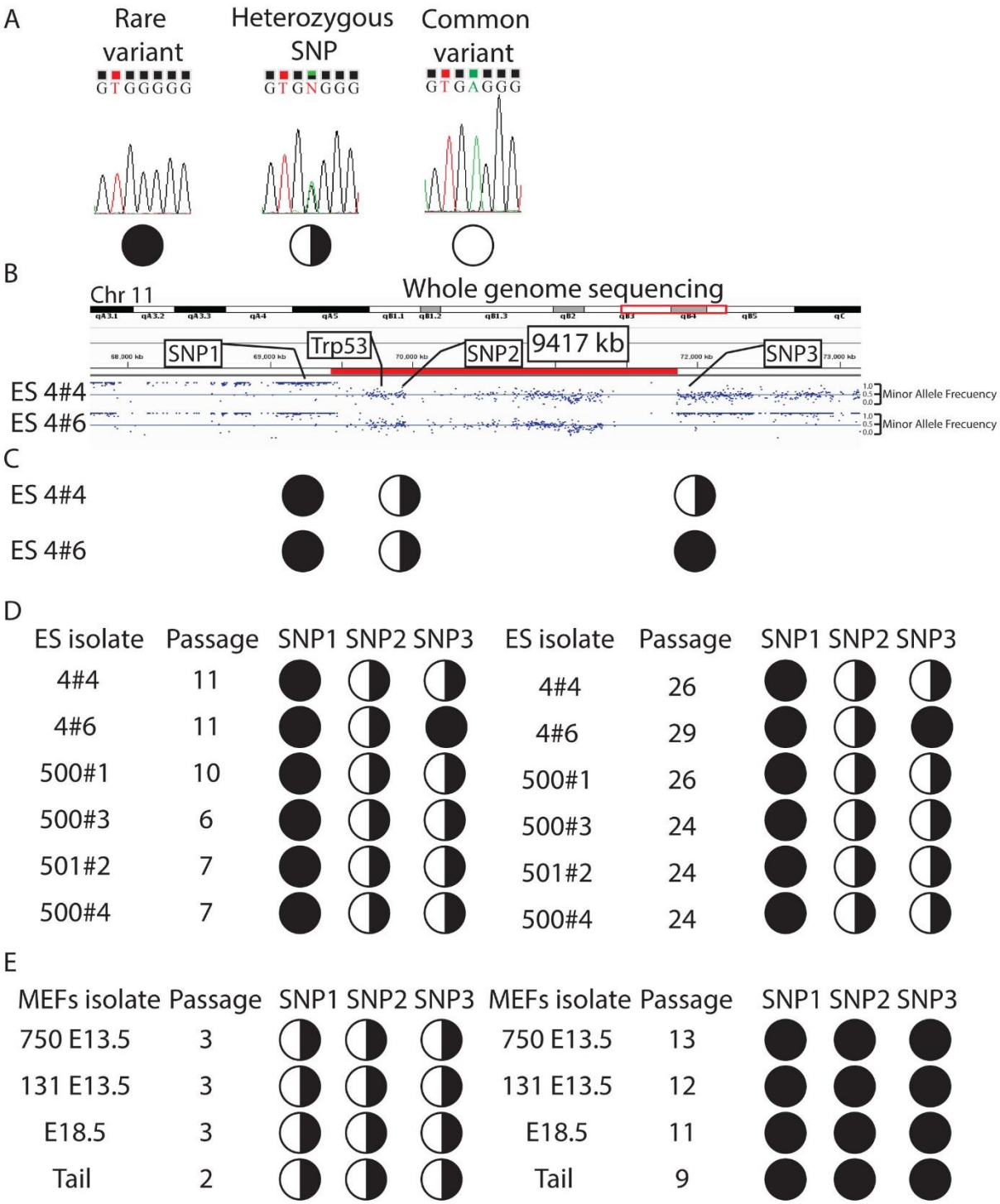


Figure 17. ESCs isolates retain heterozygosity around p53, while differentiated fibroblast undergo LOH.

Sanger sequencing of three regions in six different ESCs isolates, two MEFs isolates generated at day 13.5 dpc, one at 18.5 dpc and one adult mouse derived tail fibroblasts in early and in late passage. Each region (”SNP”) contains 2-4 SNPs. The first region, “SNP1”, upstream to Trp53, “SNP2” proximal to Trp53 and “SNP3” downstream to Trp53 as depicted in B. (A) Representative images of a rare variant, heterozygous SNP and common variant of

Sanger sequencing, graphical representation of the variant is aligned underneath. (B) IGV image of a 9417 kb section of chromosome 11 of the 2 ESCs isolates is presented. Each SNP is represented by a dot. The horizontal line for each sample represents minor allele frequency. SNPs above the line represent common variant, while SNPs below it represent the rare variant. (C) Graphical representation of the variant as indicated in A is presented. (D) Sanger sequencing of three regions in six different ESCs isolates in early and in late passage. (E) Sanger sequencing of two MEFs isolates generated at day 13.5 dpc, one at 18.5 dpc and one adult mouse derived tail fibroblasts in early and in late passage.

Large and multiple LOH events take place across the genome in MEFs

Next, we focused our genomic analysis on the MEFs system in which the LOH process can be followed. We examined whether LOH is restricted to chromosome 11 and if the frequency of this event is genome wide.

Allele change events in SNPs were aggregated across 1Mb windows. For each window, the number of allele gain/loss/maintained events was counted. A window event (allele loss/gain/maintained) was defined if the window contained at least 10 SNP allele change events and at least 75% of the SNP events were of the same type. Between passages 3 to 14 we detected 101 events of loss and one event of gain. Number of events per chromosomes is showed in table 1. This calculated number of events can reflect only genomic areas with high SNPs frequency, while in other areas it is not feasible to define LOH events or the lack of such. In addition, large genomic aberrations, such as in chromosome 11, are counted as multiple events, where, in fact, a single event is most likely responsible for it, however the lack of SNPs in middle regions results in multiple counts of LOH events by the automated algorithm.

Table 1: Called window events, by chromosome

Chr2	Chr4	Chr6	Chr10	Chr11	Chr12	Chr14	Chr17	Chr18
6	2	4	5	36	6	43	1	1

In Figures 16 and 18, graphical visualization of LOH events can be seen in chromosomes 6, 11, 12, 14, 17 and 18. Some of the events are extremely large, as in chromosome 11, a 57 mb region

in chromosome 14 is undergoing LOH with a small region of 1 mb in its middle that retained heterozygous, indicating a double cross over event. In other chromosomes the event seems to be between 2.7 – 7.5 mb. However, it should be noticed that this is the minimum length rather than its maximum, due to the lack of SNPs beyond this regions. In chromosomes 11, 12 and 14 one end of the recombination can be deduced, while in the rest of the chromosomes none can be inferred. The edge is where the break point occurred. This location might contain a fragile site, yet deciphering the exact location is not feasible due to lack of sufficient number of SNPs. In all, this implies that large and multiple LOH events take place across the genome, yet their effect remains largely unknown.

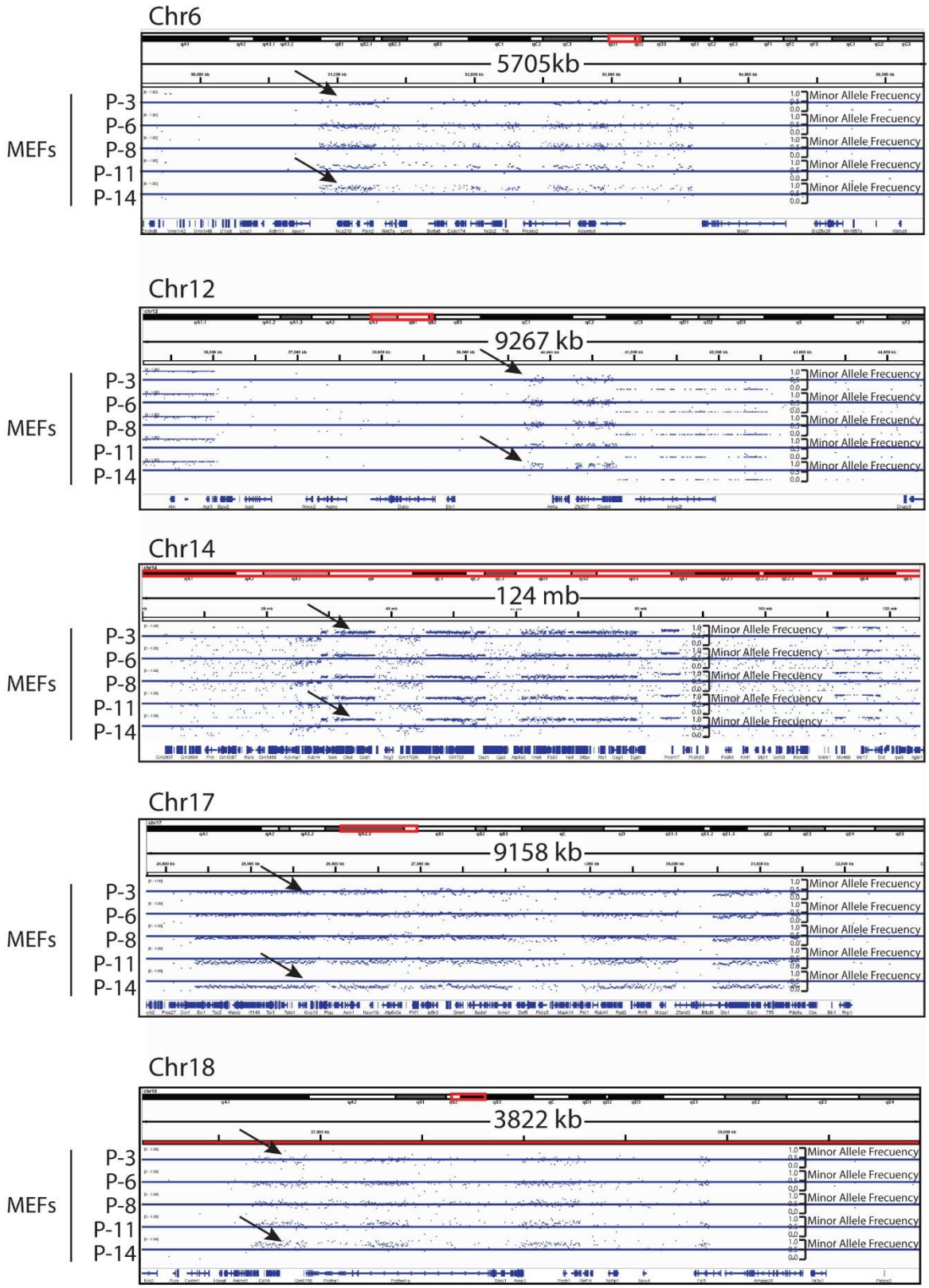


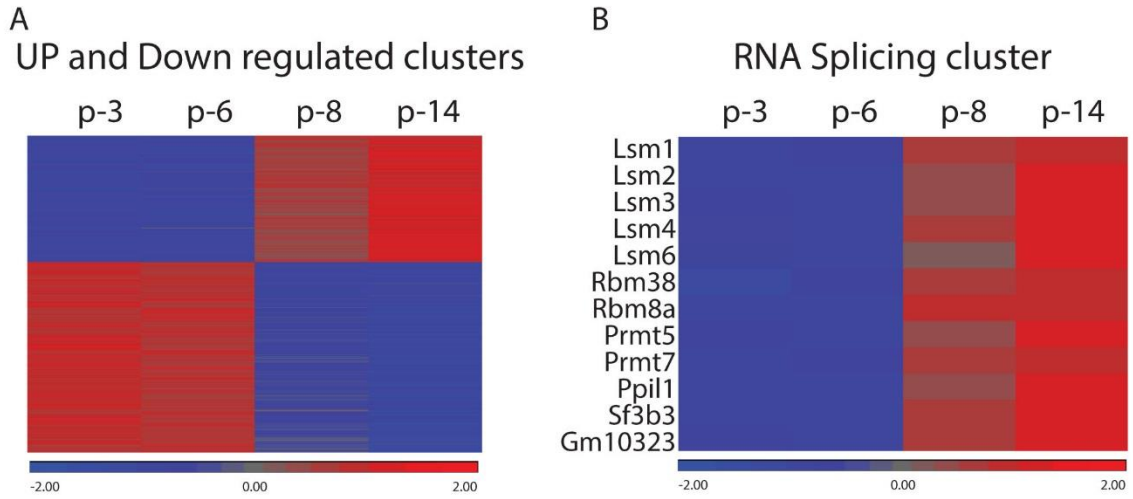
Figure 18. LOH events landscape in the genome

MEFs of different passages (3, 6, 8, 11 and 14) were subjected to whole-genome sequencing. After standard preprocessing steps, IGV image of SNPs in chromosome 6, 12, 14, 17 and 18 is presented. Each SNP is represented by a dot. The horizontal line for each sample represent minor allele frequency. SNPs above the line represent common variant, while SNPs below it represent the rare variant. LOH events are indicated by an arrow.

p53 LOH is associated with RNA splicing

To investigate the gene expression alterations associated with p53 LOH in MEFs, we carried out mRNA profiling utilizing next-generation mRNA sequencing of four time points, passages 3, 6, 8 and 14 of a single HZp53 MEFs batch. As seen in figure 2, MEFs robustly undergo p53 LOH in passage 7, therefore passage 3 and 6 were considered as LOH-free, whereas passage 8 and 14 were considered as post p53 LOH. Paired-end mRNA analysis was performed, following standard quality control protocols, reads were mapped to mm10 genome using TopHat. DESeq analysis was utilized to identify differentially expressed genes. Only up/down regulated genes with adj. P value <0.05 and absolute log2 ratio>0.9 were selected. In all, 351 genes were differentially expressed between p53 LOH free and post p53 LOH states. Of note, minor allelic frequency of p53 was almost identical to the one calculated by quantitative genotype approach, indicating that p53 is expressed in bi-allelic manner and that p53 LOH can be deduced from RNA samples. In figure 19, a heat-map of both the up and down regulated cluster upon p53 LOH is presented. The up-regulated cluster showed enrichment of 'cell cycle' genes, such as: Aurka, Mcm5, Ccna2, Cdc25b, Ccnf and E2F4 ($p=5x10^{-7}$). This indicates a higher proliferation rate following p53 LOH, as detected in figure 2. Surprisingly, we observed upregulation of Cdkn2a ($p16^{INK4A}/p19^{Arf}$) and Gadd45gip, which are involved in cell cycle arrest and senescence. This supposedly implies that differentiated cells induce senescence, nevertheless, in murine, unlike in humans, p53 is the main route of senescence and in its absence p16 is not sufficient to accomplish senescence (Ben-Porath and Weinberg, 2005; Kuilman et al., 2010). Indeed, we could not detect senescence associated β-gal staining in MEFs following p53 LOH (data not shown). In addition, DAVID functional annotation revealed novel pathways associated with p53 LOH, RNA splicing ($p=2.2x10^{-6}$) and ribonuclease complexes ($p=4.9x10^{-8}$). Figure 19B illustrates a 'heat map' of expression levels of all the upregulated genes involved in RNA splicing. Moreover, as demonstrated in figure 19D, STRING analysis showed multiple potential protein-protein interactions in both the cell cycle group and the RNA associated proteins, further

supporting the correlation between p53 LOH and RNA splicing. This novel finding suggests that upon p53 LOH hyper alternative splicing is taking place, which may contribute to mechanisms mediating LOH events and affect the tumorigenic potential of MEFs as demonstrated in figure 2.



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Category	Term	# of genes	% of genes	P value
SP_PIR_KEYWORDS	cell cycle	16	12.1	5.0E-7
GOTERM_BP_FAT	RNA splicing	11	8.3	2.2E-6

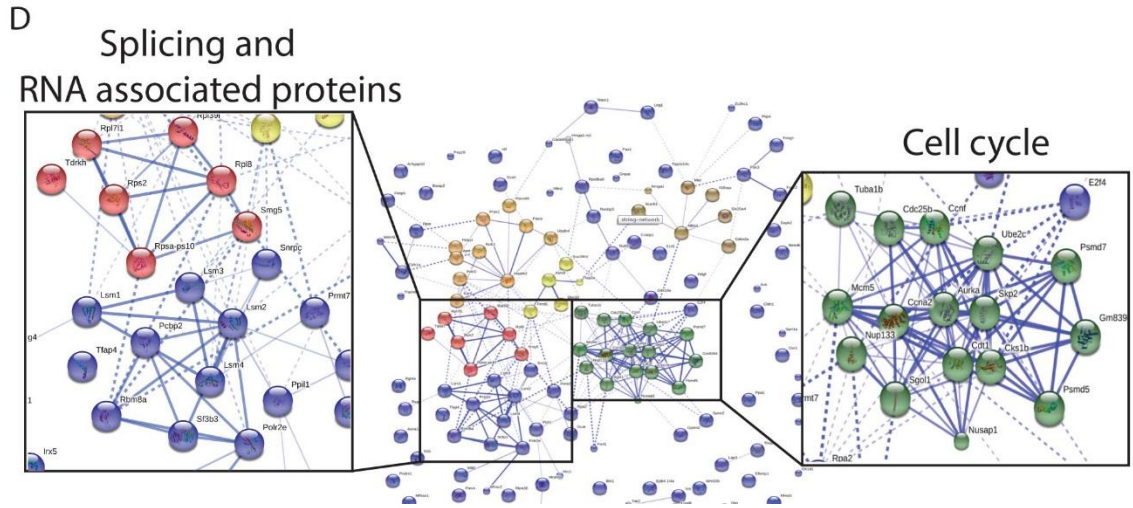

Figure 19. Upregulation of cell cycle and mRNA splicing genes coincide with p53 LOH in MEFs.

mRNA profiling utilizing next-generation mRNA sequencing of four time points, passages 3, 6, 8 and 14 of a single HZp53 MEFs isolate. Passage 3 and 6 were considered as LOH-free, whereas passage 8 and 14 were considered as post p53 LOH. Paired-end mRNA analysis was performed, following standard quality control procedures, reads were mapped to mm10 mouse genome using TopHat. DESeq analysis was utilized to identify differentially expressed genes. Only up/down regulated genes with adj. P value <0.05 and

absolute log2 ratio>0.9 were selected. In all, 351 genes were differentially expressed between p53 LOH free and post p53 LOH states. (A) A `heat-map` of up and down regulated clusters is presented. (B) A `heat-map` of RNA splicing genes is presented. (C) Two of the main enriched pathway by DAVID functional annotation of the up-regulated cluster. (D) STRING analysis of protein-protein interactions of the up-regulated cluster is depict. Blowup of interactions between cell cycle proteins on the right and blowup of interactions between RNA splicing and RNA associated proteins on the left.

Discussion

Although LFS patients seem to mature normally, they develop a wide spectrum of cancer types in childhood and adult life where about 40- 60% of tumors demonstrate p53 LOH (Varley et al., 1997a; Varley et al., 1997b). The possibility of a link between cancer development and deregulation of stem cells (Reya et al., 2001) challenged us to study the significance of p53 LOH in stem cells and tumorigenesis. This was possible due to the availability of the reprogramming technology that enables to recapitulate a transition from somatic cells into embryonic-like cells, as well as due to the generation of HZp53 mice that allowed us to address the above question, both *in vitro* and *in vivo*. Our data that in HZp53 cells, the reprogramming process kinetics are similar to cells harboring solely WTp53, indicates that WTp53 serves as a barrier to dedifferentiation even in the presence of Mutp53. Moreover, only upon p53 LOH these cells exhibit malignant phenotype when engrafted into mice, suggesting that the dedifferentiation and transformation are interwoven together (Shetzer et al., 2014a). Indeed, ample data suggest that the emergence of CSCs occurs in part as a result of EMT. Transformed mammary epithelial cells that were induced to undergo EMT gave rise to cells with breast CSCs markers and features such as the increased capacity to form mammospheres, soft agar colonies, and tumors (Mani et al., 2008). Recently, a landmark report stated that there is a significant correlation between lifetime risk to develop a specific type of cancer and the number of lifetime SC divisions in the host tissue. Using meta-analysis, this study showed correlative evidence that SCs are the origin of two-thirds of the human cancer types examined (Tomasetti and Vogelstein, 2015). Here, we showed that regardless of its cell of origin, p53 serves as a barrier to CSCs formation. p53 maintains a pool of normal SCs by controlling the quantity and quality of SCs. p53 restricts processes of *in vivo* dedifferentiation and *in vitro* reprogramming, preventing the transformation and dedifferentiation of differentiated cells into CSCs as shown by the reprogramming kinetics and the lack of malignant phenotype in HZp53 iPSCs tumors. SCs have the potential to undergo mutation in p53. In heterozygous p53 SCs LOH can occur as a DNA repair process, leading to the loss of the mutant allele and ensuring the quality of the SCs. In the case where the WT allele is lost CSCs will be formed (Figure 20).

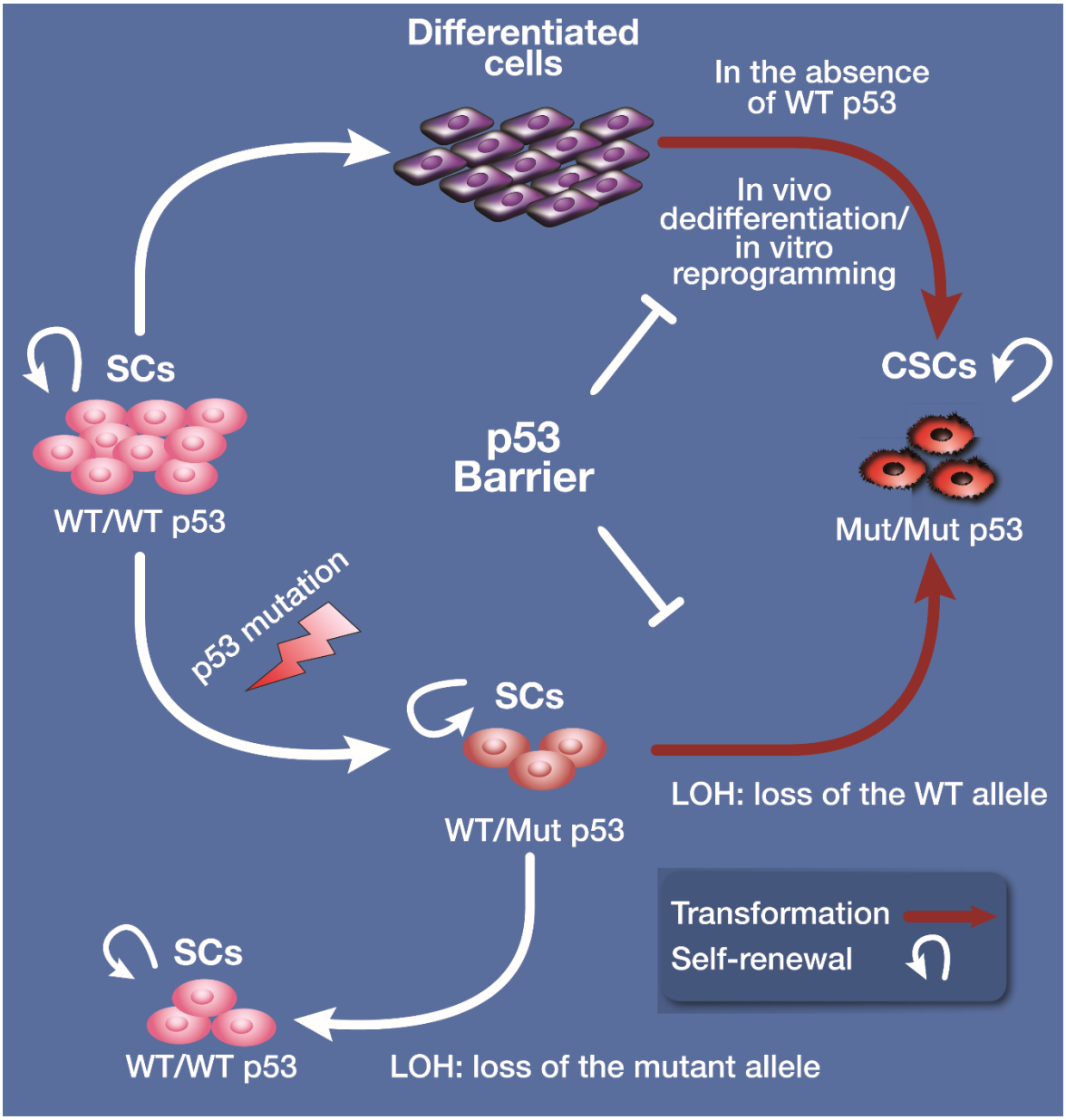

Figure 20. p53 the barrier to cancer stem cells formation.
p53 maintains a pool of normal SCs by controlling the quantity and quality of SCs. p53 restricts processes of *in vivo* dedifferentiation and *in vitro* reprogramming, preventing the transformation and dedifferentiation of differentiated cells into CSCs.
SCs have the potential to undergo mutation in p53. In heterozygous p53 SCs LOH can occur as a DNA repair process, leading to the loss of the mutant allele and ensuring the quality of the SCs. In the case where the WT allele is lost CSCs have a higher probability to be formed. Modified from (Aloni-Grinstein et al., 2014)

p53 LOH encompasses not only the loss of WTp53 function as a barrier, but extenuates its mutant role as an oncogene as well, because of its GOF features. The mutant p53 GOF

characteristics and mechanisms of action have been broadly described (Brosh and Rotter, 2009; Muller and Vousden, 2014). We have suggested that the complexity and heterogeneity of tumors, attributed to the sub-population CSCs within the tumors encompass Mutp53 GOF properties (Shetzer et al., 2016). Apparently, Mutp53 GOF and characteristics of CSCs seem to coincide (broadly described in (Shetzer et al., 2014b). CSCs display tolerance to chemotherapy and play a crucial role in cancer recurrence (Visvader and Lindeman, 2008), likewise p53 mutants exhibit GOF in conferring drug resistance in numerous tumor types. Furthermore, Mutp53 exhibits GOF by up-regulating MDR1 (Dittmer et al., 1993), while these efflux pumps are considered to be pivotal means to detect and isolate CSCs. Additionally, Mutp53 confers apoptosis resistance by affecting Bcl-2 family members (Brosh and Rotter, 2009; Huang et al., 2013). Similarly, CSCs exhibit abundant expression of pro-survival proteins of the Bcl-2 family members compared to normal adult stem cells (ASCs) and somatic cells allowing the former cells to sustain cellular stress (Mandal et al., 2011; Merritt et al., 1995). Another pathway that is shared by CSCs and Mutp53 GOF is the ability to induce angiogenesis by its main regulator VEGF (Bao et al., 2006; Calabrese et al., 2007). We and others have shown that various mutations in p53 promote the reprogramming process and concomitantly displaying an oncogenic GOF (Sarig et al., 2010; Shetzer et al., 2014a; Yi et al., 2012). Indeed, we have demonstrated that Mutp53 MEFs undergo the reprogramming process with shorter latency and higher efficiency compared to their p53-deficient counterparts (Sarig et al., 2010). Importantly, upon injection into immune-compromised mice Mutp53 iPSCs formed malignant and invasive tumors, instead of the benign teratomas generated by WTp53 iPSCs. This illustrates the oncogenic GOF of Mutp53 that alters both the quantity and quality of the reprogramming process, permitting generation of CSCs. Interestingly, we showed that HZp53 iPSCs were comparable to WTp53 iPSCs as manifested by similar reprogramming kinetics and formation of benign teratomas. Most of the clones retained heterozygosity for prolonged time in culture, however the small percentage of clones that underwent p53 LOH formed malignant tumors *in vivo*. This intriguing observation suggests that during the reprograming process of untransformed cells harboring endogenous Mutp53, WTp53 dominates over the Mutp53 and only upon p53 LOH were these cells able to induce malignant tumors in mice (Shetzer et al., 2014a).

Importantly, mutations in p53 are not a marker for CSCs. Nevertheless, p53 mutations augment the probability to generate CSCs, by either malignant transformation of normal SCs or dedifferentiation of somatic cells. The observation that CSCs and Mutp53 share common features makes it tempting to speculate that the ability to form CSCs comprise the essence of mutant p53 GOF features (Figure 21).

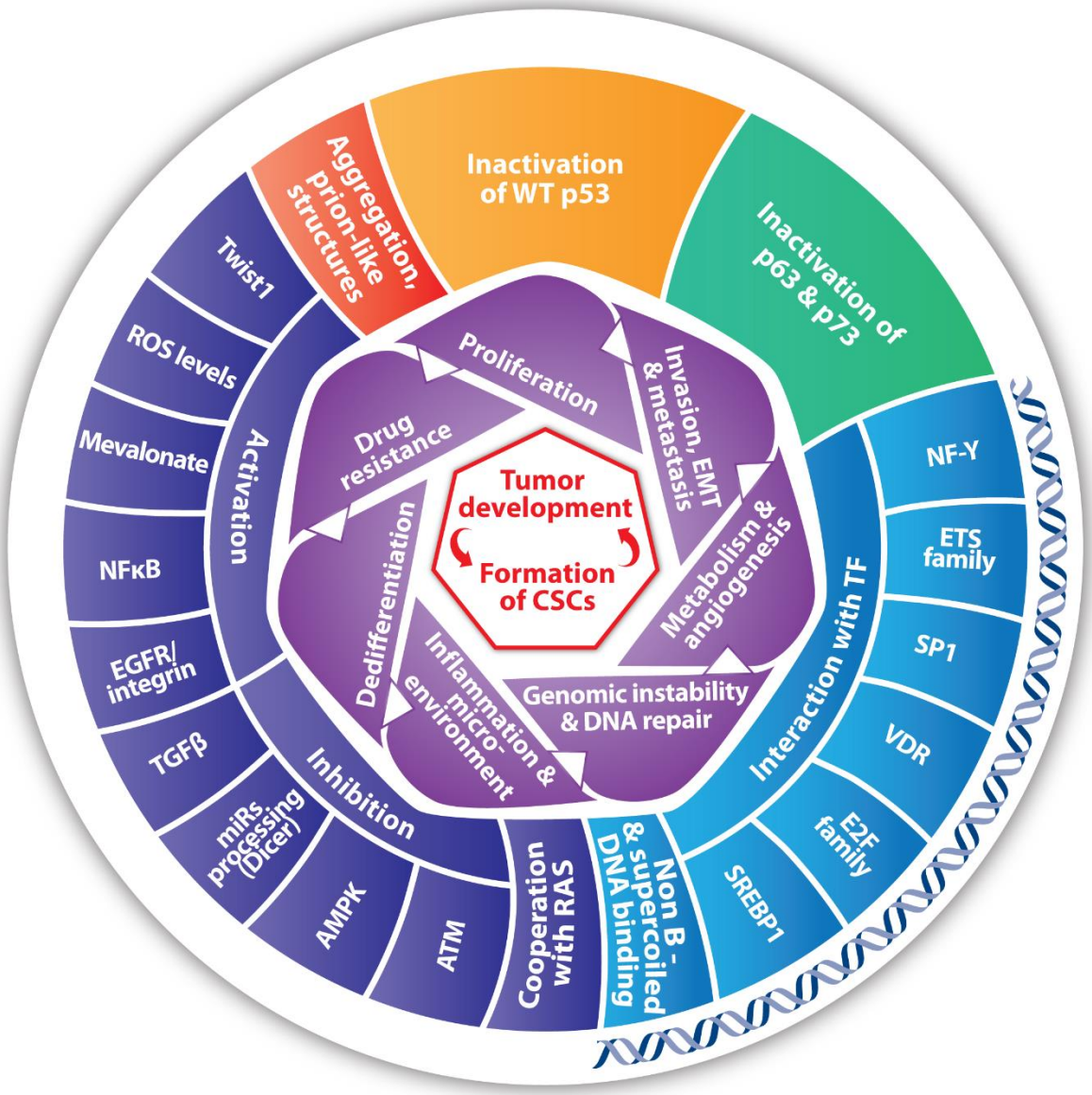

Figure 21. Selected phenotypes and mechanisms underlying oncogenic Mutp53 GOF. The inner level (purple) represents oncogenic GOF phenotypes associated with Mutp53. The outer level illustrates pivotal mechanisms of Mutp53 GOF. Importantly, each phenotype can be associated with several underlying mechanisms. Mechanisms of Mutp53 interactions with DNA are depicted near a double-strand DNA. Since CSCs are suggested to

be the cell of origin of many human cancers, their formation constitutes the cornerstone of Mutp53 GOF in tumor initiation and progression capabilities. AMPK, 5' AMP-activated protein kinase; ATM, Ataxia telangiectasia mutated; EGFR, epidermal growth factor receptor; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, Reactive oxygen species; SREBP-1, sterol regulatory element-binding protein; TGF-β, Transforming growth factor beta; VDR, vitamin D receptor. (Shetzer et al., 2016)

Importantly, p53 LOH was more pronounced in MSCs established from BM of adult mice compared with adolescent mice, reflecting either a higher incidence of p53 LOH *in vivo* or degeneration of protective mechanisms with age. Thus suggesting a link between p53 LOH, aging and tumorigenesis. It was previously reported that transformation of MSCs seemed to be strongly dependent on alterations in the p53/p21 pathway; mainly through inactivation of WTp53 and that MSCs might require few genetic alterations to undergo transformation (Rodriguez et al., 2009). Moreover, others have shown that only after long-term *in vitro* cultivation, p53 deficient MSCs were able to form tumors in mice (Armesilla-Diaz et al., 2009). We observed that HZp53 MSCs obtained from adolescent and adult mice, injected shortly after the completion of p53 LOH, did not give rise to tumors. This agrees with the notion that MSCs require several genetic alterations for transformation that can be acquired gradually after p53 restrain is released. Indeed, injection of HZp53 MSCs isolates 10 passages post p53 LOH gave rise to aggressive tumors similar to those induced by Mutp53 MSCs. These results suggest that loss of WTp53 in MSCs is an initiating step in sarcomagenesis. The link between p53 LOH, tumorigenesis and aging observed in our *in vitro* stem cell system was further examined *in vivo*. We measured p53 LOH in BM progenitors isolated from HZp53 mice at various ages and demonstrated, for the first time, that p53 LOH occurs *in vivo*. Surprisingly, the majority of cells that underwent p53 LOH lost the mutant allele. This phenomenon of Mutp53 LOH was also observed in single-cell sub-clones of our *in vitro* stem cell systems. Although this is the first report on the loss of the Mutp53 allele through LOH in an apparent healthy tissue, Mutp53 LOH was once noticed in two Li-Fraumeni tumors (Varley et al., 1997b) and in other TSGs with a role in DNA repair (Boettger et al., 2003; Clarke et al., 2006; Loveday et al., 2012; Sanchez de Abajo et al., 2006; Yan et al., 2007). It is tempting to speculate that LOH can be seen as a physiological genetic repair mechanism. An alternative explanation would be that the loss of either WT or Mutp53 allele is stochastic, while upon WTp53 loss the cell undergoes cellular

alterations that are recognized by the immune system that eliminates them, thus leaving higher percentage of cells that lost the Mutp53 allele. However, when we examined this hypothesis by using CFU-Fs derived HZp53 immune deficient mice, the results recapitulated p53 loss directionality as in immune-competent mice. This finding may indicate that the adaptive immune system does not play a role in the elimination of cells that lost WTp53. Still, it could also be that the NK cells, which are part of the innate immune system, despite being in reduced numbers, cleared those cells. Another possibility may be that cell autonomous death is triggered following the loss of WTp53, despite the presence of mutant p53. Further research is needed to resolve this question.

Another intriguing observation in LFS research is the strikingly high prevalence of soft-tissue and osteosarcomas in these patients, ~30% vs. ~1% of all adult solid malignant cancers (Burningham et al., 2012; Malkin, 2011). It was demonstrated that alternative genetic background of distinct mouse colonies knocked-in with Mutp53^{R172H} affects the spectrum of developing tumors types. For example, HZp53 C57BL/6 mice predominantly develop sarcomas and lymphomas (Lang *et al.*, 2004), whereas HZp53 129S/Sv develop a variety of carcinomas, soft tissue and bone sarcomas, leukemia, and even a glioblastoma multiforme (Olive *et al.*, 2004). Furthermore, backcrossing HZp53 C57BL/6 mice to BALB/C background gave rise to mostly osteosarcomas and mammary carcinomas (Xiong *et al.*, 2014). Herein, our specific established mice colony of ID/IC HZp53 generated by crossing C57BL/6 and NOD background exhibited similar tumor trait as their progeny, the C57BL/6 HZp53 genotype. Indeed, they share a similar tumor spectra and tumor-free survival. However the percentage of adeno- and squamous cell carcinomas was elevated, suggesting that C57BL/6 background has an intrinsic resistance to carcinomas, as suggested before (Kuperwasser *et al.*, 2000). The two colonies, the progeny C57BL/6 HZp53 and the novel C57BL/6 /NOD IC HZp53, showed similar incidence of p53 LOH and its directionality in BM progenitors, suggesting that this phenomenon is background independent.

Interestingly, HZp53 ID mice showed shorter tumor free survival and 100% p53 LOH in spontaneous tumors. Thus, it is tempting to speculate that in ID mice cells that lost WTp53 generate tumors in shorter latency compared to IC mice. In the later, the immune-surveillance eliminates cells that lost the WTp53, while other cells that evade the immune system, develop additional genetic aberrations over time, thus have a longer tumor free survival and lower

percentage of tumors that underwent p53 LOH (Figure 22). Nevertheless, no difference in the bi-directional LOH might imply that BM derived MSCs are not the cell of origin of these sarcomas. Indeed, there are accumulating reports suggesting that tissue mesenchymal progenitors rather than BM derived MSCs are the cell of origin of sarcomas (Choi et al., 2010), including in a model of LFS derived iPSCs (Lee et al., 2015).

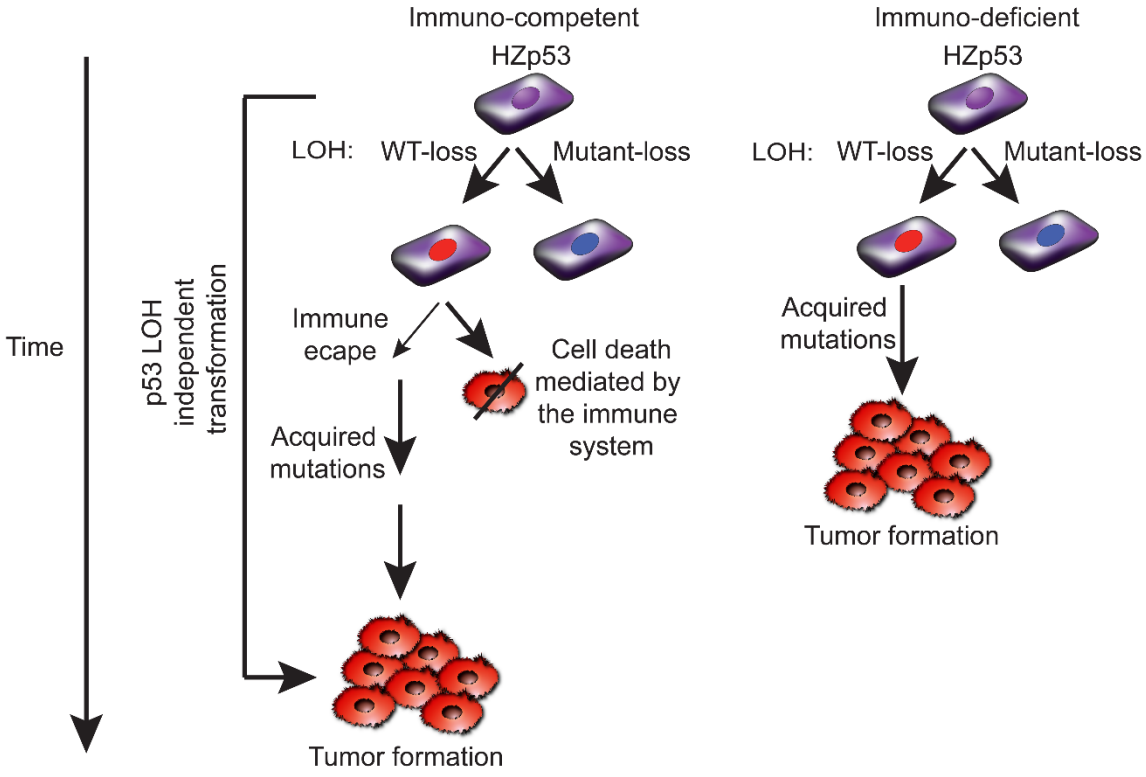

Figure 22. Proposed model: immune escape following WTp53 LOH require longer latency of tumor formation due to p53 LOH independent transformation.
The immune system can recognize and clear cells that underwent p53 LOH, whereas in its absence, those cells will form tumors in shorter latency. In an individual with a competent immune system p53 LOH independent pathways are utilized in the transformation process, which require longer period of time to form tumors (Shetzer et al., 2017).

Our data of gene expression, copy number and sequencing analyses point to the induction of HRDRP events as the mechanism, which underlies most cases of p53 LOH in MSCs, iPSCs and MEFs. Mutp53 seems to induce a state of chronic DNA insults, as cells harboring either Mutp53 or HZp53 exhibit a DNA repair gene expression signature. The observed gene-signature is manifested by the upregulation of specific genes involved in the dsDNA break response, which includes both HR and NHEJ. Although HR is a high-fidelity DNA repair mechanism, NHEJ is

highly error prone, and thus these two mechanisms may have significantly different consequences (Shrivastav et al., 2008). Studies in the Drosophila male germline have indicated that HR increases linearly with age. The authors speculated that in young individuals, selective pressures may favor usage of NHEJ and single-strand annealing, which are faster but more error prone. However, in old individuals, the fidelity obtained by HR is favorable (Preston et al., 2006). Recently, a link between HR, reprogramming and p53 was established, wherein cells defective in their HR pathway yield a smaller number of reprogrammed cells. This decrease is mediated by p53-dependent growth arrest and apoptosis, which is responsible for the elimination of cells with damaged DNA. In the absence of p53, the reprogramming process continues at the expense of accumulating genetic aberrations (Gonzalez et al., 2013). The LOH landscape across the genome indicates that p53 encompasses an extremely small region compared to the large region undergoing LOH in chromosome 11, and is part of many other LOH events. The question is what are the consequences of LOH at large and p53 LOH in particular? SNPs as regulatory elements of gene expression were demonstrated (Guo et al., 2014). The clonal takeover of cells that underwent LOH in chromosomes other than 11 indicates that they confer cells with proliferative or survival advantage, yet examination of their mRNA profile showed no enrichment of differentiated gene expression post LOH in region that underwent LOH. This could imply that either the advantage is post translational in the protein level, similarly to p53, or that a single catastrophic event such as chromothripsis (Rausch et al., 2012; Stephens et al., 2011) is responsible for the LOH events in the genome, but one that utilize HR as the repair mechanism instead of NHEJ. In this case, p53 LOH might be the event that facilitates these cells to takeover. Nevertheless, we cannot rule out additional changes that support tumorigenic ability as shown in a recent study that in some cases of p53 LOH, second allele deletions could have stronger tumorigenic properties due to loss of other genes in p53 proximity (Liu et al., 2016).

Our observation that LOH is attenuated in ESCs and iPSCs, while adult MSCs readily undergo LOH, coupled with the fact that p53 LOH yields cells lacking WTp53, can explain why LFS patients do not acquire tumors during development, yet develop tumors (sarcomas in particular) later in life. Our data indicated that genomic stability and fidelity is a function of cell-state. The finding that p53 locus in ESCs retains heterozygosity, while other regions might undergo LOH makes it tempting to speculate that a governing mechanism protects this region. Thus, the notion

that p53 plays a regulatory role in the life of SCs, coupled with the observations that p53 mutations may contribute to the evolvement of CSCs makes it challenging to speculate that drug resistance and cancer recurrence are mediated by CSCs that express mutant p53. Accordingly, it may suggest that efficient cancer therapy in mutant p53-expressing tumors should be based on a combination of chemotherapy and a p53-based therapy. The chemotherapy will target the tumor bulk, whereas only the conversion of mutant p53 protein into WTp53 form will allow CSC eradication (Figure 23). We speculate that reverting mutant p53 in CSCs into WTp53 will render their sensitivity to chemotherapy. Hopefully, these systems will allow to shed light on physiological methods to reeducate cancerous cells in order to establish tailor-made therapy in p53 harboring tumors.

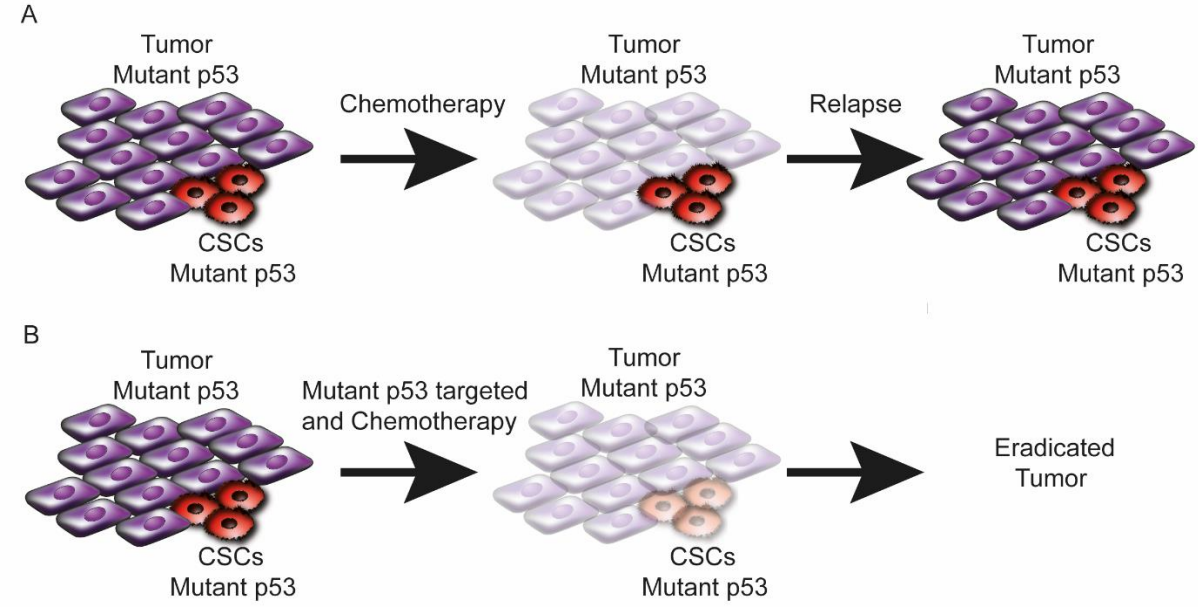

Figure 23. Suggested model for combining mutant p53-targeted cancer therapy and conventional chemotherapy.
(A) Tumor expressing mutant p53 when treated with chemotherapy will show regression due to elimination of the bulk tumor cells. However, the CSC compartment is resistant to chemotherapy-induced death, thus allowing tumor relapse. (B) Treatment with mutant p53-targeted therapy will convert the mutated p53 into intact p53 and sensitize CSCs to chemotherapy. Hence, both the bulk tumor cells and the CSCs will be eliminated and full eradication is expected. (Shetzer et al., 2014b)

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Review

p53: The barrier to cancer stem cell formation

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Cancer stem cells
Tumorigenesis

ABSTRACT

The role of p53 as the "guardian of the genome" in differentiated somatic cells, triggering various biological processes, is well established. Recent studies in the stem cell field have highlighted a profound role of p53 in stem cell biology as well. These studies, combined with basic data obtained 20 years ago, provide insight into how p53 governs the quantity and quality of various stem cells, ensuring a sufficient repertoire of normal stem cells to enable proper development, tissue regeneration and a cancer free life. In this review we address the role of p53 in genomically stable embryonic stem cells, a unique predisposed cancer stem cell model and adult stem cells, its role in the generation of induced pluripotent stem cells, as well as its role as the barrier to cancer stem cell formation.

1. Introduction

The balance between genome stability and plasticity is crucial in determining cell fate, yet this balance varies between somatic and stem cells (SCs). In a somatic cell, p53 has a major role in translating stress signals into classic processes such as apoptosis, cell cycle arrest, DNA repair and senescence, contributing to its main role as the "guardian of the genome" [1]. However, p53's function in SCs varies in a context-dependent manner. Imbalance between genome stability and plasticity may lead to intensive senescence or apoptosis, which can result in a severe depletion of the functional SC reservoir and to improper development or early aging. This dilemma emphasizes the important balance between the quantity and quality of SCs [2]. In recent years, p53 was found to have great impact in processes such as cellular differentiation [3–7], self-renewal [8,9] and plasticity [10,11], ensuring a balance between genome stability and plasticity in normal SCs.

SCs have a profound impact on embryonic development and are central for organ renewal during adult life [12]. As such, SC genomes must be guarded to minimize genetic lesions that may occur during their expansion and may lead to premature aging, failure to repair tissue injury and to cancer [13–15]. Genomic stability and fidelity are a hallmark of pluripotent Embryonic Stem Cells (ESCs). ESCs can differentiate into three lineages in the embryo, including germ cells [16]; thus genome stability is crucial for avoiding tumorigenesis as well as preventing mutations from being passed onto progeny. Indeed, ESCs have a low rate of spontaneous mutations compared to somatic cells [17]. Adult Stem Cells (ASCs), which reside in many tissues of the body, also hold the potential for self-renewal and differentiation into specific cell lineages – although they do not have the capacity to form an embryo. ASCs proliferate through asymmetric cell division, giving rise to one daughter SC and one transit-amplifying cell. Their activation occurs during particular developmental stages or after external injury, and their regulation is strictly controlled in their niches [18].

Dedifferentiation of somatic cells holds promise as a source for patient-specific transplantation therapies. Conversion of differentiated cells into a pluripotent state has been achieved by three methods: nuclear transfer – first achieved by transferring the nuclei of differentiated intestinal epithelium cells of feeding tadpoles into enucleated recipient eggs [19,20]. The second method used fusion of human amniocytes with differentiated mouse muscle cells, which provided valuable insights but not as a source of cells for regenerative medicine [21,22]. However, the major breakthrough in the field was provided by Takahashi and Yamanaka, who demonstrated the induction of pluripotent SCs from mouse embryonic fibroblasts (MEFs) by introducing four defined factors, Oct3/4, Sox2, Klf4 and c-Myc (OSKM) under Embryonic Stem (ES) cell culture conditions [23]. This development of induced pluripotent embryonic stem cells (iPSCs) provides insights into the biology of ESCs. Since then iPSCs have been generated from multiple tissues by various combinations of factors or techniques [24]. These iPSCs

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hold ES-like features, i.e. cells that retain the potential to differentiate into all three germ layers in vitro, form teratomas (a differentiated and non-malignant tumor) when injected into immunodeficient mice, and produce chimeric live pups when injected into blastocyst or germ cells. In fact, germ-line transmission is the most convincing demonstration of true pluripotency. Recently, it was shown that removing epigenetic barriers can improve reprogramming efficiency and induce pluripotency in nearly all the cells in a deterministic manner [25]. Yet the major concern in the use of iPSCs for therapeutic means – their tumorigenic potential – still remains. Thus, elucidation of the specific master regulators of pluripotency may enable efficient induction of safer cells to be used in regenerative medicine in numerous diseases. Indeed, studies by Buganim et al. have shed some light on the phases of transcriptional and epigenetic changes that occur during reprogramming and on the hierarchy of the regulators involved [26,27]. These studies may provide criteria that will allow assessment of iPSCs quality.

Much attention in the SCs field is drawn to the Cancer Stem Cell (CSC) theory. The CSC theory is based on the developmental hierarchy seen in normal tissue, wherein the undifferentiated SCs reside at the top, followed by a gradient of various degrees of differentiated cells. Similarly, tumors are organized in a hierarchical order that sustains a distinct subpopulation of CSCs. CSCs can divide asymmetrically, giving rise to a bulk tumor cell and a CSC, keeping the CSC reservoir small in numbers. Only the CSCs have the capability to initiate new tumors. These CSCs were found in a number of human hematological and solid tumors and have been defined experimentally by their ability to seed new tumors [28]. Just as normally proliferating tissues such as wounds are nourished and regenerated by SCs, so is a tumor – which may be considered as a "wound that never heals" [29] – nourished by tumor cells with an unlimited renewal potential. Indeed, CSCs and SCs share functions, such as self-renewal asymmetric cell division, the ability to generate a large number of differentiated cells, and the expression of specific markers [12,30,31]. Moreover, just as normal SCs have the ability to migrate to distinct parts of the body where they exert their functions, CSCs also seem to have the potential to migrate and establish metastasis [32]. Taken together, it is not surprising that SCs and CSCs share similar regulatory factors that modulate these biological functions [33]. However, SC function remains under physiological control, whereas the division and differentiation of CSCs are decidedly not [34,35]. These uncontrolled pathways include those regulated by WNT/β-catenin, PTEN, TGF-β, Hedgehog, Notch and Bmi-1 [36]. Moreover, CSCs are also resistant to chemotherapy and radiation and may be, as normal SCs are, protected against various insults, likely by mechanisms such as quiescence, expression of ATP binding cassette (ABC) pumps which may lead to multidrug resistance, high expression of anti-apoptotic proteins and resistance to DNA damage [37–39]. Unfortunately, CSC-rich tumors are associated with aggressive disease and poor prognosis [40] emphasizing the importance of unraveling their biology and the need to develop means to combat them.

CSC may arise from the transformation of a normal ASC or progenitor cell. Although the number of SCs is very small, they can undergo continuous division for a long time and are thus more likely to accumulate the molecular mutations that cause tumorigenesis. Indeed, Dick and colleges showed that only the transfer of a small population of human leukemia cells, displaying the cell surface markers of HSC, into immunodeficient mice, rather than more differentiated cells gave rise to new tumors. This suggests that normal primitive SCs, rather than committed progenitor cells, are the target of leukemic transformation [41,42].

Other studies favor the option that CSCs may have taken advantage of cellular plasticity and originate from differentiated cells through a process of dedifferentiation [43]. Regulated dedifferentiation may be regarded as a cellular homoeostasis mechanism through which tissues can regenerate after SCs are lost. For example, single secretory cells from the epithelium of the mouse trachea were able to dedifferentiate into multipotent SCs. This dedifferentiation process was triggered upon SC ablation and was prevented by direct contact of SCs with the committed cells, ensuring epithelial architecture. The authors suggest that the reciprocal interaction of stem and committed cells may have been designed to ensure robust self-organizing properties in diverse tissue types [44]. Recently, a role for p53 during salamander limb regeneration was published. It was shown that the activity of p53 initially decreases and then returns to baseline. The down-regulation is required for formation of the blastema and is critical for cell cycle reentry of post-mitotic differentiated cells, and the up-regulation is necessary for the redifferentiation phase to muscle. The authors suggest that the regulation of p53 activity is a pivotal mechanism that controls the plasticity of the differentiated state during regeneration [45]. These studies indicate that dedifferentiation is a regulated process in homeostasis and regeneration. Unfortunately, uncontrolled dedifferentiation may have cancerous consequences. Although much knowledge on CSCs has been obtained in the past years, how and when a CSC is formed in a particular tumor are still open questions. The two ways to obtain CSC do not exclude each other, but rather depend on the cancer type and context (Tables 1 and 2). Regardless of whether it is transformation of a progeni-

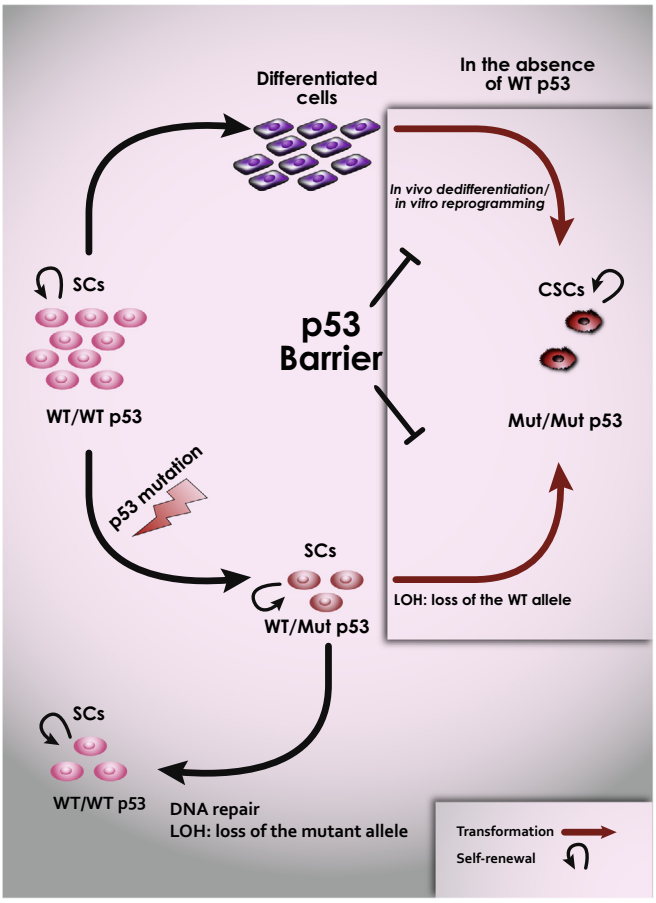

Fig. 1. p53 the barrier to cancer stem cells formation. p53 maintains a pool of normal SCs by controlling the quantity and quality of SCs. p53 restricts processes of in vivo dedifferentiation and in vitro reprogramming, preventing the transformation and dedifferentiation of differentiated cells into CSCs. SCs have the potential to undergo mutation in p53. In heterozygous p53 SCs LOH can occur as a DNA repair process, leading to the loss of the mutant allele and ensuring the quality of the SCs. In the case where the WT allele is lost CSCs will be formed.

Table 1
Cancer types in which p53 aberrant ASCs has been shown to be involved in their initiation and progression.

Cancer	Stem cell	Refs.
Multiple myeloma	Hematopoietic stem/progenitor cells	[136]
Leiomyosarcoma	Fat-derived MSCs	[92]
Fibrosarcoma (mouse model)	Aged MSC	[87]
Osteosarcoma	MSC of the limb bud	[137]
Glioma -glioblastoma	NSCs	[138]
Glioma- astrocytoma	NSCs	[94]
Ovarian cancer	Ovarian stem-like cells	[139]

tor/stem cell or dedifferentiation, p53 stands as a barrier to both routes of transformation.

During the last several years the stem cell field has expanded, providing more questions than answers. The findings that induced pluripotency and induced tumorigenesis are related processes, as judged by gene expression profiles [46], and that CSC hierarchy mimics normal SC hierarchy, emphasize the need for regulatory proteins that will guard and maintain a cancer-free repertoire of normal SCs. In this review we address the role of p53 in normal SCs as well as CSC prevention. The fate of an intermediate phase of SCs, namely those that harbor both wild type and mutant p53, presenting a state predisposed to CSCs, will also be discussed.

2. p53 in the life of a normal stem cell

2.1. The role of p53 in ESCs

Over 30 years ago, a set of studies described the expression of p53 in primary cell cultures obtained from embryos. High expression of p53 was observed in cell cultures of 12–14 day old mouse embryos, which declined in cells of 16 day old embryos [47–49]. These studies, among others, highlighted that although p53 is highly abundant in mouse ESCs [50,51], it was localized mainly in the cytoplasm [52,53] and was found to be inactive [54,55]. In contrast to mouse ESCs, in human ESCs p53 is localized in the nucleus, in a deacetylated inactive state and at low levels [56]. Indeed, whereas in somatic cells p53 classical response to DNA damage is G1/S cell cycle arrest, apoptosis or cellular senescence, this is not the case in mouse ESCs [55]. Although these observations are in line with the requirement of ESCs for rapid cell division and self-renewal, they also present a paradox; how do ESCs manage to maintain a stable genome without the classical functions of the "guardian of the genome"? Does p53 exert its guardian functions through other biological pathways? Moreover, the observations from the early nineties that p53 knockout mouse embryos developed normally, suggesting that p53 is redundant in embryogenesis [57], prompted more questions on the role of p53 in embryogenesis. Since then many studies have shed light on the important roles played by p53 in embryonic development. Indeed, a role in regulating suppression of self-renewal and induction of differentiation after DNA damage was assigned to p53. p53 binds and suppress the promoter of the master transcription factor Nanog and the pluripotency factor Oct4, which are highly abundant in mouse ESCs and drive self-renewal and the maintenance of an undifferentiated state [58,59]. Thus, suppression of these two genes in DNA damaged mouse ESCs will force differentiation [6] into cell types that can be subjected to classical p53 processes such as cell-cycle arrest or apoptosis. Recently, it was reported that silencing of Oct4 in human ESCs leads to the activation of p53, through the reduction in the expression of Sirt1, a deacetylase known to inhibit p53 activity, leading to increased acetylation of p53 at lysine 120 and 164 and promotion of differentiation [60]. Moreover, p53 was found to activate the expression of miR-34a and miR-145, which in turn repress stem cell factors Oct4, KLF4, LIN28A and Sox2 and prevent backsliding to pluripotency [56]. Furthermore, it was reported that a single aurora kinase A (Aurka)-mediated phosphorylation event is largely responsible for inactivating p53 and that in the absence of Aurka, increased p53 signaling promotes mouse ESC differentiation [61]. Recently an in-depth study of the genes regulated by p53 in human ESCs in response to early differentiation, induced by retinoic acid, revealed that p53 promotes differentiation of human ESCs by activating expression of developmental transcription factor genes involved in patterning, morphogenesis and organ development. Differentiation-specific p53 gene targets in human ESCs include several members of the homeodomain family (HOX, LHX, DLX, PAX), the forkhead family of FOX genes, the SOX gene family, and members of the TBX family of genes, all of which regulate a wide variety of developmental processes [62]. In addition, p53 targets members of the CBX family, specifically CBX2 and CBX4, which are part of the Polycomb complex and are crucial for cell-fate determination [63]. Moreover it was found that several p53 gene targets are down regulated during RA-mediated differentiation, including genes that direct mesodermal differentiation (FOXO3, KLF6, HDAC5, HDAC6) and telomere repeat binding factor TERF1, associated with pluripotency [64]. In all, in ESCs p53 seems to be a homeostatic protein ensuring proper development by governing pluripotency potential. In ESCs with damaged DNA p53 will force differentiation by harnessing many developmental pathways.

2.2. The role of p53 in iPSCs

Many studies have addressed the role of p53 in the biology of iPSCs. p53 was found to have a major role in the generation of iPSCs both in attenuating reprogramming as well as in quality control of the reprogramming. Indeed, in agreement with others, we found that WT p53 constrains iPSC generation in vitro [65–73]. It was

Table 2
Cancers and tumor lines in which p53 aberrations resulted in dedifferentiated phenotype.

Cancer	Phenotype	Refs.
Chondrosarcoma	High grade/dedifferentiated zones of chondrosarcoma	[140,95]
Liposarcoma	Dedifferentiated liposarcoma	[141]
Adenoid Cystic Carcinoma (AdCC)	Dedifferentiated AdCC	[142,143]
Thyroid carcinoma	Poorly differentiated and undifferentiated thyroid tumors	[144,145,97]
Carcinoma	Carcinomas from the p53 null and hemizygotes are more frequently undifferentiated than those from wild-type mice	[96]
Glioma	Dedifferentiation of astrocyte during tumorigenesis	[138]
Wilms tumor	Strong association between the appearance of anaplastic clones and TP53 mutations	[146]
Undifferentiated-Gastric Carcinoma (UGC)	The inactivation of wild-type TP53 is an earlier event before dedifferentiation to mixed-type UGC	[147]
Medulloblastoma	TP53-ARF pathway is disrupt in anaplastic medulloblastoma	[148,149]
Hepatocellular carcinoma	Mutant p53 may have contributed to dedifferentiation during the development of HCC	[150,151]

found that fibroblasts with compromised p53 exhibit a higher frequency of iPSC generation. Furthermore, it was suggested that p53 may induce cell cycle arrest and apoptosis and thus function as a barrier to select exclusively perfect reprogrammed SCs [74]. A p53 mediated DNA damage response was shown to limit reprograming to ensure iPSC genomic integrity [70]. An additional role of p53 during reprogramming may be an indirect effect on cell proliferation [75]. One scenario suggests that p53 up regulates miR-199a-3p, which imposes G1 cell cycle arrest [76]. Another study demonstrated that p53 exerts its suppression of iPSC generation through the axis of p53-upregulated modulator of apoptosis (PUMA) [77]. We showed that p53 restricts mesenchymal-to-epithelial transition (MET) during the early phases of reprogramming and that this effect is primarily mediated by the ability of p53 to inhibit Klf4-dependent activation of epithelial genes [11]. Recently we have reported that iPSCs generated from homozygous mutant p53 MEFs, using only 2 transcription factors (Oct4 and Sox2), exhibited fully reprogrammed iPSC phenotype in vitro yet formed malignant terato-carcinomas in vivo, instead of the benign teratomas induced by the WT p53 iPSCs [73]. It is conceivable that these are pre-iPSCs [78] that may represent cancer iPSCs. Latest studies in the field suggest that the reprogramming process is comprised of an early stochastic phase and a late hierarchical one [26]. Reactivation of p53 at any of the stages hampers the formation of iPSC clones [79]. This suggests that p53 is not a transient roadblock, but rather a full-time monitoring agent. Recently, homologous recombination (HR) pathway genes were found to be necessary for the reprogramming process. Interestingly, in the absence of p53, cells with a defective HR pathway could undergo reprogramming, allowing the generation of iPSCs with genetic aberrations, emphasizing the role of p53 in the quality control of this process [80]. In all, this suggests that in addition to the rate-limiting role p53 plays in reprogramming it also has a quality control role, ensuring the generation of proper cancer-free iPSCs.

2.3. The role of p53 in ASCs

Under physiological conditions, an optimal balance exists between the maintenance of a sufficient ASC pool for tissue regeneration and the elimination of severely damaged SCs, thus ensuring maximal longevity. However, when encountering severe DNA damage programmed cell death or, alternatively, temporary or permanent cell cycle arrest is induced. The latter, which prevents cancer development, may tilt this fine balance and by the same token cause depletion in the SC reservoirs leading to long-term negative effects [81]. Although damage can be repaired in cells through one or more of the many sophisticated genome maintenance pathways, DNA repair and incomplete restoration of chromatin after substantiate damage may produce sequence mutations and epi-mutations, both of which have been shown to accumulate with age. The accumulation of faulty DNA containing mutations and/or epi-mutations in aged tissues increases cancer risk [2]. As p53 is regarded as the "guardian of the genome" [1] it is not surprising that dysfunction of p53 will affect processes critically dependent on genomic fidelity such as proliferation, differentiation and transformation of various ASCs.

The term ASCs includes many types of SCs, the more familiar of which are mammary gland SCs, neural SCs, hematopoietic SCs and mesenchymal SCs (MSCs). In this review we will address only the role of p53 in MSCs. MSCs represent a population of adult heterogeneous multipotent stem cells, which can be isolated from many adult tissues throughout the body and are able to self-renew and differentiate into various cell types of mesodermal origin [82,83]. p53 was shown to control differentiation of MSCs [4,84]. We and others have demonstrated that the absence of WT p53 [85] or the presence of a mutant p53 (unpublished results) confers selective advantages in the acquisition of typical MSC markers along with an increased proliferation of BM-derived MSC progenitors. Both knockout p53 [85] and mutant p53 mice (unpublished result) contained a larger number of colony forming precursors compared to WT progenitors. Furthermore, knockout p53 MSCs presented genomic instability with an increased expression of c-MYC [85]. MSC strains derived from mutant p53 also exhibited genome instability as judged by spectral karyotyping analysis (unpublished results). Interestingly, chromosome 11, where the p53 gene resides, exhibited major alterations that increased with age. A role for p53 in MSC aging may be suggested by the specific decrease in p53 RNA and protein in MSCs during the aging process, which does not occur in heart or spleen and may explain how MSCs avoid age-related senescence [86]. Moreover, aged MSCs were shown to exhibit spontaneous expression of embryonic factors and p53 point mutations, suggesting that mesenchymal tumors may have originated from aged MSCs [87]. Interestingly, MSCs also have a tumor promoting effect as supportive cells. p53 status in tumor stromal cells has a key role in tumor development by modulating immune responses. The tumor-promoting effect of p53-deficient MSCs was not observed in immune-compromised mice, indicating that the immune response has a critical role [88]. Altogether, p53 plays an essential role in MSC proliferation, maintaining their quantity as well as assuring their quality by preventing their transformation. The decrease in p53 levels upon aging or the acquisition of a mutation in the p53 gene may contribute to the high risk of MSC sarcomagenesis and to the role of MSCs in supporting carcinogenesis.

3. p53 as the barrier to formation of CSCs

CSCs could arise from accumulation of genetic insults in normal stem or progenitor cells or by dedifferentiation of existing differentiated cells. One example of the transformation of stem/progenitor cells into CSCs is provided by MSCs, which were proposed as candidate cells of origin for several sarcoma types [89]. Increasing evidence suggests that MSCs that acquire mutations in oncogenes or tumor suppressors may function as tumor initiating cells (TICs) leading to *de novo* tumor formation. In this regard MSCs might be the TICs capable of initiating sarcomagensis [90] as was shown for hematopoietic SCs, which may serve as TICs for hematopoietic malignancies [41]. Several studies in mouse models have indicated that p53 deficient MSCs may lead to sarcomagenesis. Transformation of MSCs seems to be highly dependent on alterations in the p21/p53 pathway, mainly by the abolishment of WT p53, but not on the retinoblastoma pathway [90–93]. Moreover, analysis of fibrosarcomas derived from aged mice showed that these tumors may have originated from MSCs harboring mutated p53. Furthermore, MSCs isolated from young mice and then aged in culture revealed the acquisition of clinically significant p53 mutations [87]. Another example of tumors originating from SCs was provided by mouse models based on conditional inactivation of p53, NF1 and Pten. This study showed that brain tumors originate from neural stem/progenitor cells while more mature cells cannot form tumors [94], identifying SCs as the cell of origin of CSCs. Table 1 provides examples of cancer types in which p53 aberration in ASCs has been shown to promote initiation and progression.

Reports on the link between p53 loss and the differentiation state of tumors were first published about 20 years ago [95–98]. Those studies showed that the high grade/de-differentiated phenotype of some sarcomas and carcinomas correlates with p53 loss and increased malignancy. Although these reports were consistent, they received little attention. Only after the burst of the reprogramming era came the understanding that all cell types have the potential to

dedifferentiate. In addition, reprogramming only occurs in a very small percentage of the transfected cells, suggesting the existence of reprogramming barriers. Indeed, we and others showed that down regulation of p53 enhances the efficiency of iPSC generation, whereas re-expression of p53 in p53 null MEFs markedly impedes this [65–73]. In addition, we have shown a new gain-of-function property of mutant p53 that enhances reprograming efficiency beyond that of p53 null MEFs. However, homozygous mutant p53 iPSCs formed malignant terato-carcinomas in vivo, perhaps recapitulating the transition of a differentiated p53 mutant cell to a dedifferentiated CSC. Others have extended our observation, demonstrating that the Myc pathway cooperates with the p53-R175H human mutant protein to disrupt the efficiency of reprogramming and that different mutant alleles of p53 have diverse efficiencies in enhancing iPSC colonies formation [79]. Thus, it is conceivable that a differentiated cell in the body gains mutations that drive the first phase of the cancer phenotype. Following a second hit of a p53 mutation, the barrier of dedifferentiation and formation of CSCs is removed. Indeed, an analysis of human tumors revealed that poorly differentiated aggressive tumor express an ESC transcription signature as observed in SCs [99]. Interestingly, breast, lung and prostate tumors with an ESC signature were found to contain a p53 mutation. In contrast, well-differentiated tumors contained a WT p53 [100,101]. One mechanism by which p53 prevents dedifferentiation is by binding to the promoter of CD44, one of the better known CSC markers, repressing its expression. Interestingly, constitutive expression of CD44 blocks p53 dependent apoptosis leading to cells resistant to doxorubicin [102]. Moreover, loss of p53 may lead to increased expression of the multidrug-resistance genes (ABCB1 or MDR1) and to chemotherapy resistance. Table 2 provides examples of cancers and tumor lines in which p53 aberrations resulted in a dedifferentiated phenotype.

4. Facing a chronic DNA insult – the story of the p53 heterozygous stem cells

At the junction between normal SCs and CSCs lay the heterozygous p53 SCs, namely SCs which concomitantly express a functional WT p53 and a mutant p53. Such a genotype is presented in Li-Fraumeni syndrome (LFS) patients. LFS is a rare type of cancer predisposition syndrome associated with germ line p53 mutations [103]. It appears that in LFS patients, as well as corresponding mouse models [104], the WT p53 is dominant over the mutated p53 allele, and they apparently develop normally. Only later in adult life do they acquire a wide spectrum of tumors, including bone and soft-tissue sarcomas, acute leukemia, early onset of breast cancer, brain cancers such as glioblastoma, and adrenocortical tumors occurring over a wide age range [105]. Approximately 60% of the initially analyzed tumors exhibited loss of heterozygosity (LOH) in the p53 locus. The remaining 40% bypass the suppressive effect of the WT allele by diverse mechanisms such as promoter hypermethylation [106], increased activity of Mdm2, the E3 ligase responsible for p53 ubiquitination [107], by impairing other components of the p53 pathway [108] or by the enhanced oncogenic potential of missense p53 mutations that are common in both LFS and sporadically mutation somatic cells [109]. Gain of function mutants or those showing dominant negative features may be sufficient to induce tumor formation in the presence of the WT gene, especially in context of other genetic or environmental insults [105,110,111].

The mouse model of LFS (R172H which is homologous to human R175H hot-spot mutation) holds great promise to unravel questions regarding the role of p53 in SCs of various origins and functions. As SCs harboring exclusively either WT or mutant p53 represent an end-point of either a normal or a mutated SC, the p53 heterozygous SC may give a "snap shot" on the process of tumorigenesis in SCs, as manifested by the LOH process. Importantly, this mouse model reflects the majority of p53 aberrations in human malignancies, which are missense mutations (75%) [112]. Moreover, it is tempting to speculate that the presence of the mutant p53 in these heterozygous SCs endows them with CSC characteristics. This speculation is based on the fact that although $p53^{+/-}$ and $p53^{+/R172H}$ tumors show similarities, only osteosarcomas and carcinomas from $p53^{+/R172H}$ mice metastasize to various organs [104].

We have established ESCs and MSCs derived from heterozygous p53 LFS mice and generated iPSCs from MEFs of these mice. This panel of cells enables us to evaluate the impact of p53 LOH on tumorigenesis as a function of cell origin. Heterozygous p53 MEFs, an example of somatic cells, undergo in vitro p53 LOH in a robust manner. In contrast, the frequency of p53 LOH varied among the various SCs as a function of their genome stability. It is well accepted that ESCs have a high genome stability and fidelity mainly due specialized mechanisms aimed at preserving their genome [17]. Indeed, no p53 LOH was observed in heterozygous p53 ES cells that exhibited stemness characteristics typical of WT p53 ESCs (unpublished results). With iPSCs heterozygous for p53 the situation is less defined. iPSCs, on the one hand, resemble ESCs and are considered fairly genomically stable. On the other hand, iPSCs are generated from MEFs, which were shown to be less stable. Although both WT and mutant p53 iPSCs present normal SC markers, mutant p53 iPSCs appear earlier with greater reprogramming efficiency. Moreover, when injected in vivo the mutant iPSCs give rise to malignant tumors [73]. Heterozygous p53 iPSCs resemble WT p53 iPSCs- both exhibit similar rates of iPSC formation. However, about 20% of the heterozygous p53 iPSC clones did undergo LOH, giving rise to iPSCs that resemble p53 mutant iPSCs, which induce malignant tumors in mice. The observation that all heterozygous p53 MEFs undergo p53 LOH but the majority of heterozygous p53 iPSCs do not, suggests that reprograming from a less stable somatic cell into a more stable SC triggers mechanisms that guard genome fidelity. It seems that in ESCs and iPSCs the presence of a functional WT p53 is sufficient to maintain genome stability. Thus, ESCs and iPSCs employ mechanisms, yet to be defined, to prevent p53 LOH. Moreover, an in-depth examination of single cell sub-clones of iPSCs revealed that a small fraction of cells lose their mutant allele rather than the WT p53 allele (unpublished results). This phenomenon of bi-directional p53 LOH emphasizes the great efforts made by iPSCs to maintain a stable genome. Since emerging data suggests that dedifferentiation is a natural homeostasis process [44], it is conceivable that p53, as a first line of defense, regulates and controls the processes of dedifferentiation in vivo and reprogramming in vitro. In the event that this control checkpoint is compromised, a second line of defense will be triggered. This line of defense includes the attenuation of p53 LOH, which may otherwise lead to the loss of the WT p53, or the activation of a DNA repair LOH process leading to the loss of the mutant p53 allele. Taken together, it appears that p53 functions to maintain a balance between somatic cells and SCs. Moreover, great efforts are made to sustain a functional WT p53 in SCs and to ablate the mutant p53, ensuring genome stability.

LFS patients and LFS mouse models predominantly develop sarcoma of mesenchymal origin [103,104]. As mentioned above, sarcomas may arise from damaged MSCs. Although sarcomas are one of the most dominant tumor types in LFS patients, as well as in the mouse and rat LFS models [103,104,113], no data so far has pointed to a p53 LOH process occurring in SCs of mesenchymal origin. The availability of heterozygous p53 mice at various ages makes it possible to address the above question, both in vitro and in vivo, with regard to aging. Interestingly, the in vitro p53 LOH process is more pronounced in MSC isolates established from bone marrow of adult

mice than adolescent mice, reflecting the higher p53 LOH rates as a function of aging. Only the heterozygous p53 MSC isolates which were established from adult mice induced sarcomas upon injection into immunocompromised mice, suggesting that while p53 may be a barrier to sarcomagenesis, its removal is not sufficient to induce cancer and further mutations are needed. Genotyping of single cell clones revealed that, as in iPSCs, an attempt to lose the mutant allele also occurs in MSCs but to a lesser extent. In contrast to heterozygous p53 iPSCs, in heterozygous p53 MSCs most p53 LOH events involved the loss of the WT allele, as expected from a less stable SC. Similarly, *ex-vivo* examination of bone marrow progenitors has revealed that p53 LOH is non-existent or very rare in bone marrow of adolescent mice, reflecting the normal development and the lack of tumors in patients and mice. However, the p53 LOH process was accelerated with age, reaching up to 10% of the progenitor SCs in adult mice, pointing to a tight connection between p53 LOH and aging in vivo (unpublished results). This observation raises the question of whether LOH, as a marker of genomic instability, leads to aging or whether aging leads to increased LOH. In agreement with these results, studies in yeast have revealed an increase in LOH as the mother cell ages [114]. Analysis of the colony forming units derived from adult mouse bone marrow indicated that in addition to the well-documented WT p53 LOH, which endows cells with growth advantage, loss of the mutant allele may also take place (unpublished results). It seems that in cells that are assumed to be genomically stable, such as BM progenitors and iPSCs, the loss of the mutant p53 allele is detected more frequently than the loss of the WT allele. Thus it is tempting to speculate that p53 LOH can be a physiological DNA repair mechanism that helps maintain genomic integrity. Unfortunately, when this DNA repair mechanism fails and the WT allele is lost, the final outcome will be takeover by the homozygous mutant p53 cells, leading to accumulation of other mutations and tumor formation.

5. Facing the future – eliminating CSCs using p53

Conventional anti-cancer therapies kill proliferating cells and often lead to shrinkage of the tumor. These therapies do not eliminate quiescent tumor stem cells that may, with time, arise and cause relapse of the disease. Thus, while targeting the proliferating tumor cells is the first step in combating cancer, targeting CSCs may be crucial to finally eradicating various tumor types. This goal may be achieved by either differentiation therapy or elimination therapy. Differentiation therapy is based on the induction of differentiation of CSCs. This process will lead to the loss of their self-renewal properties and to susceptibility to DNA damage responses. A proof of concept was achieved and adopted in clinical practice with the treatment of acute promyleocytic leukaemia (APL) patients with all-trans retinoic acid (ATRA). The amazing effect of ATRA as a differentiation inducer has flipped APL from the most-difficult-to-treat into the most-easy-to-treat acute leukemia [115]. Similarly, the differentiating agent 13-cis-retinoic acid (RA) is used as a standard treatment for high-risk neuroblastoma, improving survival by 35% in children with metastatic neuroblastoma [116]. In glioblastomas, induction of astrocytic differentiation with bone morphogenetic proteins (BMPs) reduces the frequency of $CD133^+$ CSCs [117]. Recently, data has been published providing proof-of-concept that inhibitors targeting mutant isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2) could have potential applications as a differentiation therapy for cancer. Treatment with such an inhibitor (AGI-6780) induced differentiation of TF-1 erythroleukemia and primary human acute myelogenous leukemia cells in vitro [118]. Another inhibitor of mutant IDH1 was shown to delay growth and promote differentiation of glioma cells [119]. Other approaches towards differentiation therapy are based on mediating gene expression through histone deacetylases [120] and miRNAs [121]. For example, in glioblastoma, miR-34a targets Notch1 and Notch2 mRNAs, resulting in CSCs differentiation [122], while medulloblastoma CSCs undergo neural differentiation by virtue of miR-34a targeting the Notch ligand Delta-like ligand 1 (DLL1) [123]. Transfection of either miR-124 or miR-137 into glioblastoma multiforme CSCs ($CD133^+$) also induces cell cycle arrest and differentiation [124]. The profound role of p53 as a differentiation inducer in various cell types, together with its restricting activity in processes of dedifferentiation and reprogramming, places p53 as an attractive candidate for differentiation therapy. Initial data supporting this notion was obtained twenty years ago. Stable and regulated expression of WT p53 in a pancreatic carcinoma tumor model was shown to have multiple phenotypic consequences: the majority of the tumor cells (60–70%) underwent G1 growth arrest and apoptosis while the rest of the cells exhibited irreversible growth-arrest with morphologic and antigenic properties of a differentiated neuroendocrine-like phenotype in vitro [125]. Injection of lung metastases of human osteogenic sarcoma cells with WT p53 is associated with in vivo induction of terminal differentiation and apoptosis, inhibiting progressive growth of metastases [126]. SCs with target mutation in p53 possess the same self-renewal properties as CSCs and their number increases progressively in p53 null premalignant mammary glands [127]. Pharmacological reactivation of p53 correlates with restoration of asymmetric division of CSCs and tumor growth reduction [127]. In a model of squamous cell carcinomas (SCCs), one of the most aggressive and heterogeneous skin cancers, p53 restoration induces skin tumor cell differentiation and suppression with no apparent effect on apoptosis, proliferation, or senescence [128].

Another way to combat CSCs is to eliminate them. This could be achieved by targeting signaling pathway of self-renewal. For example, Hedgehog pathway inhibition is emerging as a feasible and promising therapeutic approach in several cancers and some inhibitors that directly target the positive Hedgehog signal transducer Smoothened (SMO) have entered clinical trials [129]. Attempts to target CSCs via surface markers were also suggested, although the expression of these surface markers may vary in different stages of the disease and may even vary between patients with the same disease [130]. Another strategy takes advantage of old chemotherapy drugs and combines them with a CSC targeting strategy. For example, treating gastric tumor cells, which express CD90, with trastuzumab (humanized anti-ERBB2 antibody) combined with traditional chemotherapy reduced the CD90+ population in tumor mass and suppressed tumor growth [131]. The same strategy has provided encouraging data in primary ovarian cancer cell lines and patient-derived xenograft models [132], non-small cell lung cancer cells [133] and primary colon cancer cells [134]. Similarly, it was shown that combining a p53 pathway-restoring agent such as ellipticine with a classical chemotherapy agent (5-fluoruracil) is associated with depletion of putative colon CSCs [135]. The mechanism leading to this phenomenon has yet to be defined, but it is conceivable that restoration of a functional WT p53 might reduce the expression of the ABC transporters, leading to an increase in the concentration and efficacy of some anticancer drugs.

6. Concluding remarks

SCs are essential for normal development and are crucial for organ regeneration. Damaged SCs may result in improper development, early aging and tumorigenesis. Thus, it is not surprising that p53 plays a major role in various processes ensuring that SCs will remain in sufficient quantity and quality. p53 serves as a barrier between normal SCs and CSCs by preventing processes such

as dedifferentiation and the formation of damaged SCs. Furthermore, p53 LOH is under tight control in genomically stable SCs. Moreover, in these SCs, the p53 LOH process is targeted towards the loss of the mutant allele, ensuring quality-controlled functional SCs (Fig. 1). Further studies aimed at understanding the mechanisms ensuring genomically stabled SCs and the pathways that lead to CSC formation may contribute to the development of means to combat cancer.

Conflict of interest statement

We state no competing interest.

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The onset of p53 loss of heterozygosity is differentially induced in various stem cell types and may involve the loss of either allele

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p53 loss of heterozygosity (p53LOH) is frequently observed in Li-Fraumeni syndrome (LFS) patients who carry a mutant (Mut) p53 germ-line mutation. Here, we focused on elucidating the link between p53LOH and tumor development in stem cells (SCs). Although adult mesenchymal stem cells (MSCs) robustly underwent p53LOH, p53LOH in induced embryonic pluripotent stem cells (iPSCs) was significantly attenuated. Only SCs that underwent p53LOH induced malignant tumors in mice. These results may explain why LFS patients develop normally, yet acquire tumors in adulthood. Surprisingly, an analysis of single-cell sub-clones of iPSCs, MSCs and *ex vivo* bone marrow (BM) progenitors revealed that p53LOH is a bi-directional process, which may result in either the loss of wild-type (WT) or Mut p53 allele. Interestingly, most BM progenitors underwent Mutp53LOH. Our results suggest that the bi-directional p53LOH process may function as a cell-fate checkpoint. The loss of Mutp53 may be regarded as a DNA repair event leading to genome stability. Indeed, gene expression analysis of the p53LOH process revealed upregulation of a specific chromatin remodeler and a burst of DNA repair genes. However, in the case of loss of WTp53, cells are endowed with uncontrolled growth that promotes cancer.
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Heterozygosity, caused by a mutation in a single allele of a tumor suppressor gene (TSG), is one of the first steps in malignant transformation.[1] Often, TSGs undergo loss of the wild-type (WT) allele, designated as loss of heterozygosity (LOH).[2–4] Patients with the rare cancer predisposition Li-Fraumeni syndrome (LFS), carrying germ-line heterozygous p53 mutations,[5] apparently exhibit normal development yet later in adult life develop a wide spectrum of tumors; predominantly sarcomas,[6–8] where 40–60% of tumors exhibit WT p53 loss of heterozygosity (p53LOH).[8]

Giving that cancer development could be associated with stemness deregulation challenges, the notion that the occurrence of p53LOH in stem cells (SCs) may contribute to the emergence of cancer SCs. Genomic fidelity is a hallmark of SCs.[9] The genome of embryonic stem cells (ESCs) is extremely stable, whereas adult stem cells (ASCs) exhibit a less stable genome.[10] Genetic deregulation in ASCs was shown to be associated with tumor development.[11–13] Mesenchymal stem cells (MSCs) that acquire mutations in oncogenes/TSGs such as p53 may function as tumor-initiating cells leading to *de-novo* sarcomagensis.[14–17] Furthermore, MSCs isolated from young mice, aged in culture acquired clinically relevant p53 mutations.[18] In all, these findings suggest a link between p53 inactivation in SCs and tumorigenesis.

Although induced pluripotent stem cells (iPSCs) seemed to represent ESCs,[19,20] several studies questioned the assumption that iPSCs are as genomically stable as ESCs.[21–24] p53 was found to have a major role in the generation of iPSCs both in attenuating reprogramming and controlling the quality of the reprogrammed cells.[25,26] An additional role of p53 during reprogramming may be an indirect effect on cell proliferation[27] and on the restriction of mesenchymal–epithelial transition during the early phases of reprogramming.[28] Importantly, Mutp53 cells exhibiting a fully reprogrammed iPSC phenotype *in vitro*, form malignant tumors *in vivo*, instead of the benign teratomas induced by the WTp53-iPSCs.[25] As p53 is the guardian of the genome, it is important to investigate how p53LOH would affect genome stability and tumorigenicity of iPSCs.

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Abbreviations: LOH, loss of heterozygosity; LFS, Li-Fraumeni syndrome; SCs, stem cells; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem cells; BM, bone marrow; WT, wild type; Mut, mutant; TSG, tumor suppressor gene; ESCs, embryonic stem cells; ASCs, adult stem cells; HZ, heterozygous; MEFs, mouse embryonic fibroblasts; SKY, spectral karyotyping; SNV, single-nucleotide variant; HRDRP, homologous recombination (HR) DNA repair pathway; NHEJ, non-homologous end joining; CFU-Fs, colony-forming units-fibroblast; p53LOH, p53 loss of heterozygosity; H&E, hematoxylin and eosin; QRT-PCR, quantitative real-time PCR

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The availability of *in vitro* SC p53LOH models (iPSCs, MSCs) can help decipher the role of p53LOH in cancer initiation. Indeed, the incidence of p53LOH was found to be extremely different between these SCs. Surprisingly, we found that reprograming of heterozygous p53 (HZp53) fibroblasts, which frequently undergo p53LOH, gave rise to iPSC clones, most of which retained their HZp53 status and exhibited features of normal WTp53-iPSCs. However, p53LOH process is robust in MSCs. Interestingly, single-cell sub-cloning of iPSCs, MSCs and *ex vivo* bone marrow (BM) progenitors revealed that, in addition to the loss of the WTp53, loss of the Mutp53 allele also takes place. Of note, this bi-directional p53LOH occurred in an age-dependent manner linking LOH to aging and tumorigenesis. Surprisingly, most of the p53LOH events in BM progenitors preferred the loss of the Mutp53 allele. Taken together, our results of a bi-directional p53LOH process, accompanied by a burst of DNA repair pathways, may suggest that p53LOH can be regarded as a DNA repair event. In the case of a DNA repair-orientated productive LOH process, where the Mutp53 allele is lost, cells are rescued of tumorigenesis. However, when the WTp53 allele is lost, cells become prone to tumor initiation.

Results

Mouse embryonic fibroblasts (MEFs) undergo p53LOH *in vitro*. p53LOH may lead to cancer, yet little is known about this process in SCs. Our study focused on SCs (iPSCs and MSCs) generated or derived from mice heterozygous for the p53R172H mutation (Supplementary Figure 1).[6] iPSCs are generated from MEFs, therefore we first examined whether HZp53-MEFs undergo p53LOH *in vitro* and found that WTp53-LOH occurred in 100% of examined MEFs at day 12 (passage 7). This correlated with a distinct shift in their proliferation capacity (Figures 1a and b) and with the decrease of p21 mRNA and protein levels (Figures 1c and d), indicating loss of WTp53 function. Our results suggest that in MEFs with one copy of WTp53 exhibited controlled cell growth, yet Mutp53 facilitates cell proliferation only upon the completion of WTp53-LOH.

Reprogramming attenuates LOH in HZp53-iPSCs. p53 status affects the oncogenic potential of iPSCs.[25] Nevertheless, the oncogenic potential of HZp53-iPSCs is still unknown. To that end, WTp53, HZp53 and Mutp53-MEFs were reprogrammed into iPSCs. Interestingly, the early reprogramming kinetics of the HZp53 cells were comparable to those of WTp53 cells, as shown by the early reprogramming marker alkaline phosphatase (Figure 2a) and the rate of appearance of Nanog-expressing colonies (Figure 2b). The p53 status did not influence proliferation or apoptosis (Supplementary Figures 2A and B). Suggesting that in the early steps of reprogramming of HZp53-iPSCs, the WTp53 dominates over the Mutp53. In contrast to MEFs, only 23% of HZp53-iPSC clones (6/26) underwent p53LOH (Figure 2c, Supplementary Table 1). This suggests that the reprogramming process attenuated p53LOH in iPSCs.

To study the effect of p53LOH on the genomic stability of the generated iPSC clones, we performed spectral karyotyping (SKY).[29] iPSCs that did not undergo p53LOH largely exhibited a normal karyotype (Supplementary Figure 3A), whereas those that underwent p53LOH showed translocations and irregular chromosome numbers (Supplementary Figure 3B). Interestingly, we observed that HZp53 ESCs did not undergo p53LOH under prolonged culturing (submitted Rivlin *et al.*), suggesting that p53LOH is a rare event in genomically stable SCs.

To unravel the mechanism underlying LOH, we examined the genotypic status by a Taqman quantitative real-time PCR (QRT-PCR) copy number assay (Figure 2d) and by sequencing. Interestingly, clones that lost the WTp53 allele duplicated the Mut allele and became homozygous rather than hemizygous (null/Mut), while the majority of clones retained their heterozygosity (Figure 2e). Furthermore, MEFs,

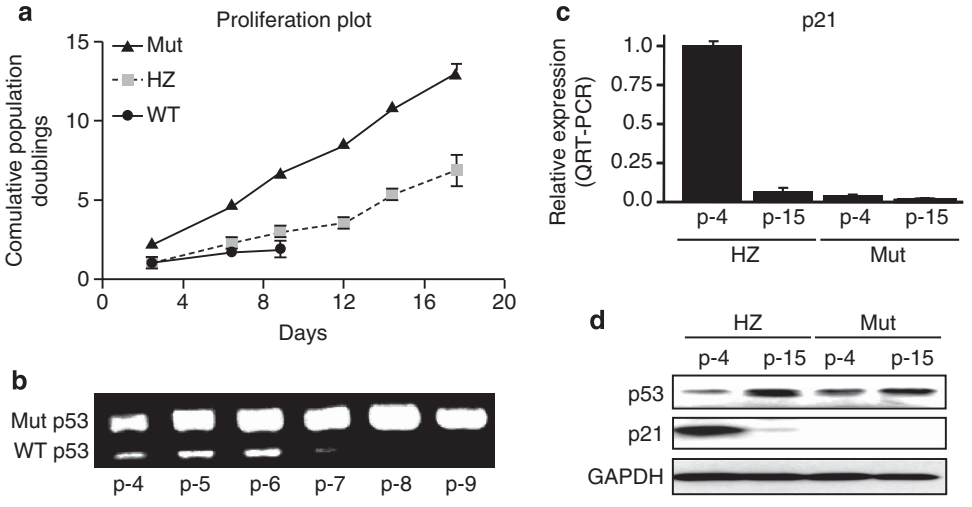


Figure 1 MEFs undergo p53LOH. MEFs derived from mice heterozygous for the murine R172H hot spot p53 mutation (HZp53) analogous to the human p53R175H hot spot mutation, as well as MEFs obtained from the corresponding WTp53 and mutant p53 (Mutp53) controls, were cultured and propagated *in vitro*. (**a**) Cumulative population doublings of WTp53, HZp53 and Mutp53-MEFs were calculated and plotted. (**b**) Genotyping analysis of p53 at various passages. (**c**) Relative mRNA expression of p21 in HZ and Mutp53-MEFs at early and late passages as measured by QRT-PCR. (**d**) Western blot analysis of p21 protein levels

which were heterozygous at passage 3, underwent duplication of the Mut allele at passage 9 (Figure 2f), as in iPSCs. These findings suggest that mitotic recombination or non-disjunction with duplication is the molecular mechanism involved in the LOH process. Whole-exome sequencing of four HZp53-iPSC clones and two HZp53-MEFs batches confirmed that three of the iPSCs clones retained their p53 heterozygosity, whereas one underwent p53LOH and all MEFs displayed a p53LOH pattern. Interestingly, a single-nucleotide variant (SNV) in Efnb3, an adjacent gene upstream of Trp53 remained heterozygous in all examined samples regardless whether p53LOH occurred or not (Figure 2g). We concluded that homologous recombination (HR) was the mechanism involved in the p53LOH process. Moreover, it is intriguing to speculate that a fragile site lies in between Efnb3 and Trp53. To examine whether MEF cultures contain a small

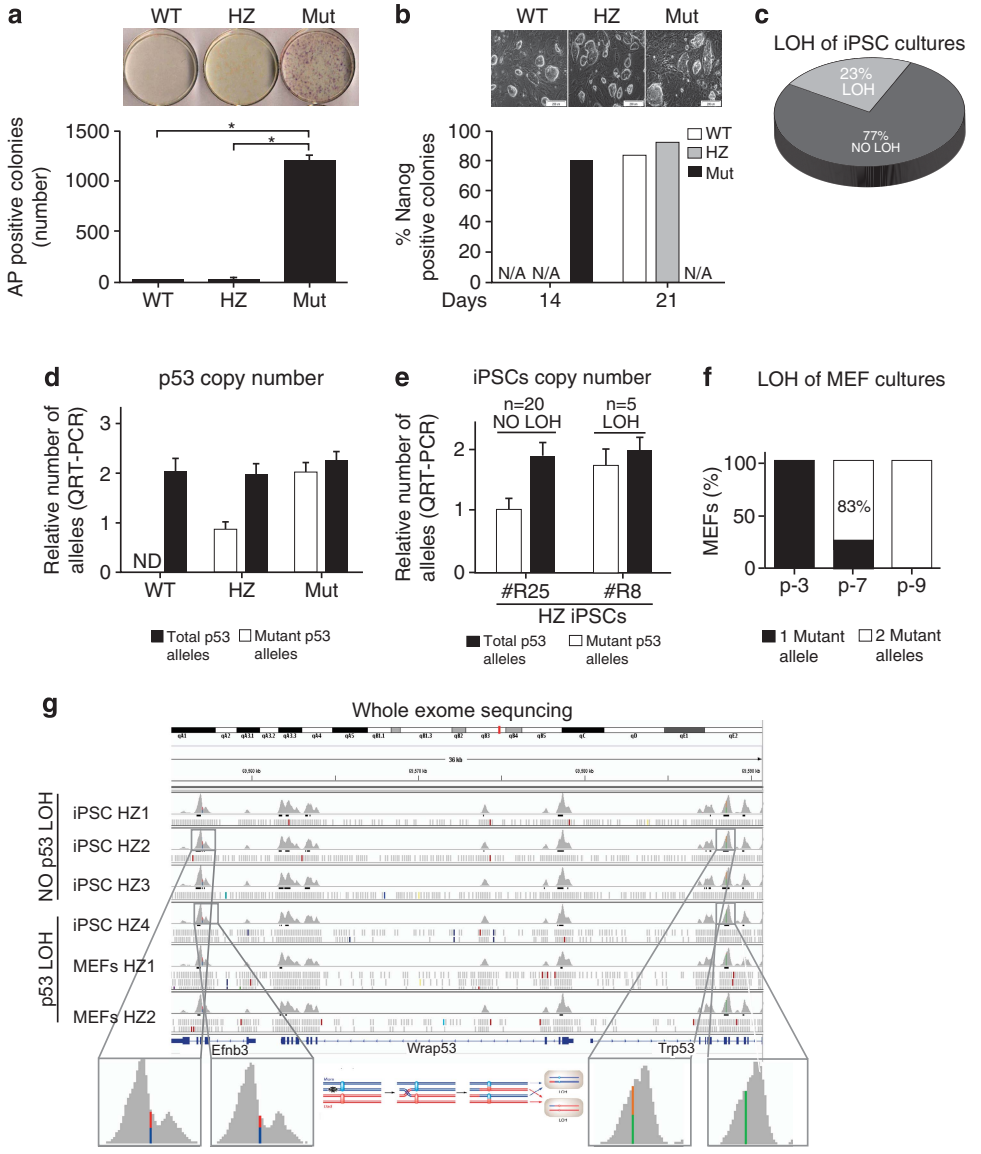


Figure 2 Reprogramming kinetics of HZp53-MEFs. (**a**) WTp53, HZp53 and Mutp53-MEFs were infected with lentiviruses encoding *Oct4*, *Sox2* and *Klf4*. Cells were plated and assayed for alkaline phosphatase (AP) activity, 2 weeks post infection. A representative image (top) of the plates and quantification (bottom) are presented. *$P = 0.0001$ one-way analysis of variance. (**b**) Light microscopy images obtained from the corresponding iPSC clones, depicting typical ES morphology. Lower panel: percentage of *Nanog*-positive colonies of WTp53, HZp53 and Mutp53 as measured by QRT-PCR, 14 and 21 days post infection. *Nanog* positivity was determined when Nanog mRNA expression was at least 50% that of ES cells. (**c**) p53 PCR genotype and sequencing of 26 HZp53-iPSC clones followed until p-40. Summary of the data from three independent experiments is presented in a pie chart. (**d**) Number of genomic p53 DNA copies was measured by Taqman QRT-PCR in WTp53, HZp53 and Mutp53-MEFs. Each well is normalized to the TFRC control gene. Total p53 and Mutp53 probes are designed to exon1 and loxP site, respectively. (**e**) A representative Taqman QRT-PCR of p53 copy number in HZ p53 iPSCs clones at p-12, ~70 days post infection (#R25 and #R8). Probes and normalization were as in **d**. $n = 25$. (**f**) A plot summarizing copy number of the mutant alleles of HZp53-MEFs in culture. (**g**) Four HZp53-iPSC clones at p-11 and two HZp53 MEF preparations at p-10 were subjected to whole-exome sequencing. Integrative Genomic Viewer image of a 36-kb section of chromosome 11of the six samples is presented. Insets depict heterozygous SNVs in Efnb3 found in iPSC HZ2 and HZ4, whereas these same cells are either heterozygous at a SNV in Trp53 (iPSC HZ2, two colors) or homozygous (iPSC HZ4, single color). At the bottom is shown a schematic representation of p53LOH through homologous recombination

sub-population of cells that retain their heterozygosity in culture, we re-examined the sequencing data of the two MEF samples. Indeed, between 1 and 11% of the reads were of the WTp53 allele. Hence, between 2 and 22% of the cells remained heterozygous for p53. One may speculate that the reprogramming itself is biased toward these cells, accounting for the low rates of p53LOH in iPSCs. However, the fact that homozygous Mutp53 facilitates reprogramming[25] (Figures 2a and b), argues against this explanation. Hypothetically, chromosomal mis-pairing, as a result of the *LoxP* insertion, could cause recombination-related effects. To address this point, analysis of minor allele frequency in the examined genomes revealed a few LOH events in distinct regions in similar frequencies as chromosome 11 that entails Trp53[30] (Supplementary Figure 4). Hence, p53LOH is not due to the *LoxP* site. Altogether, reprogramming attenuates p53LOH, which occurs by duplication of the mutation via HR.

LOH in HZp53-iPSCs leads to malignancy. Despite the low frequency of p53LOH, it is important to evaluate the outcome of p53LOH on the malignant capacity of HZp53-iPSCs. Injection of HZp53-iPSCs induced typical teratomas with well-differentiated regions of all three germ layers (Figure 3a), similar to that observed with the WTp53-iPSCs. However, injection of iPSC clones that underwent WTp53-LOH *in vitro* (before their injection) induced malignant tumors (Figure 3c), like those induced by Mutp53-iPSCs (Figure 3d). This reiterates the idea that WTp53 is essential for proper differentiation of iPSCs and that loss of WTp53, giving rise to iPSCs exclusively expressing Mutp53, leads to the development of malignant tumors.

Interestingly, some of the short-term *in vitro* cultivated HZp53-iPSCs, initially characterized as heterozygous cells, generated tumors when injected into mice, exhibiting heterogeneous phenotypes of fully malignant tumor cells with

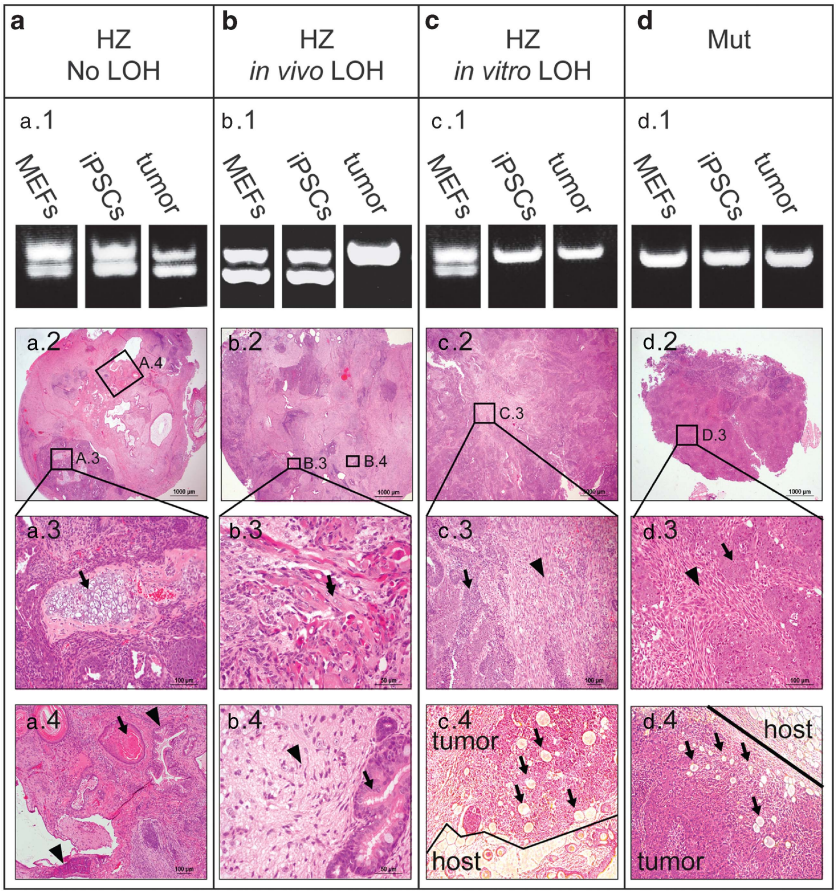


Figure 3 Histological sections of HZp53-iPSC-derived tumors. WTp53 and HZp53 that did not undergo p53LOH, HZp53 that underwent p53LOH *in vivo*, HZp53 that underwent p53LOH *in vitro* and Mutp53 iPSC clones were injected sub-cutaneously into nude mice. (**a**) Representative sections of no-LOH HZp53 clones, $n = 10$. (a.1) PCR analysis of the original MEFs, injected iPSCs and the removed tumor. (a.2) A highly differentiated teratoma. (a.3) Arrow indicating cartilage (mesoderm). (a.4) Arrow indicating keratin (ectoderm) and an upper arrowhead indicating respiratory epithelium (endoderm). The lower arrowhead identifies exocrine pancreatic glands (endoderm). (**b**) Representative sections of HZp53 clones that underwent LOH *in vivo*, $n = 4$. (b.1) As in a.1. (b.2) Predominantly well-differentiated teratoma. (b.3) Arrow indicating skeletal muscle fibers (mesoderm). (b.4) Arrow indicating respiratory epithelium (endoderm) and an arrowhead indicating well-differentiated nervous tissue (ectoderm). (**c**) Representative sections of HZp53 clones that underwent LOH *in vitro*, $n = 5$. (c.1) As in a.1. (c.2) Mostly poorly differentiated tumor. (c.3) Arrow indicating nervous tissue (ectoderm) and an arrowhead indicating poorly differentiated stroma (mesenchyme), with features of sarcoma. (c.4) Tumor with invasive fronts. Line indicates the border between host tissue and the tumor. Arrows indicate host adipocytes engrafted by the tumor. (**d**) Representative sections of Mutp53 clones, $n = 8$. (d.1) As in a.1. (d.2) A poorly differentiated tumor. (d.3) Arrow indicating nervous tissue (ectoderm) and an arrowhead indicating poorly differentiated stroma (mesenchyme), with features of sarcoma. (d.4) As in c.4. All sections were stained by H&E

an occasional incidence of differentiated teratoma (Figure 3b). This observation may suggest that these HZp53-iPSCs contain minor sub-populations that underwent WTp53-LOH *in vitro.* These cells may eventually give rise to malignant tumor cells *in vivo*. Indeed, prolonged *in vitro* cultivation of these clones revealed p53LOH.

Our *in vivo* results support the conclusion that the reprogramming process attenuates the loss of the WTp53 allele both *in vitro* and *in vivo*. In addition, it appears that in iPSCs p53LOH is sufficient to give rise to a malignant phenotype, although we cannot completely exclude the possibility that other driver mutations, occurring post p53LOH, may also contribute to malignancy.

MSCs are prone to undergo p53LOH. We next focused our study on ASCs, represented by HZp53-MSCs, which are known to be less genomically stable than ESCs and iPSCs. Moreover, familial sarcoma, which may arise from defective MSCs,[15] is one of the diagnostic criteria of LFS.[31] To that end, we established MSC isolates from adolescent and adult mice of various p53 genotypes (Supplementary Table 2). Comparison of cell proliferation indicated that the WTp53-MSC isolates exhibited the slowest growth rate, whereas Mutp53-MSCs exhibited the most rapid. HZp53-MSCs exhibited a bi-phasic growth pattern, with the first phase similar to WTp53 and the second similar to Mutp53 isolates, suggesting a switch in the p53 status of these cells (Figure 4a). Indeed, genotyping of individual HZp53 MSC isolates as a function of *in vitro* culturing time revealed that MSCs derived from adolescent mice lost the WTp53 allele at around passage 12, corresponding to the shift in the proliferation rates. MSCs isolated from adult mice lost WTp53 by passage 5, suggesting either a more rapid p53LOH process in MSCs originating from older mice or a higher fraction of cells, which underwent p53LOH before their isolation (Figures 4b and c).

Using antibodies that can distinguish between Mutp53 and WTp53 protein conformation, we found that, in agreement with the genotype profiling (Figure 4c), both proteins were detected at an early passage before LOH completion, whereas only the Mutp53 protein was present (Figure 4d) post LOH (passage 18).

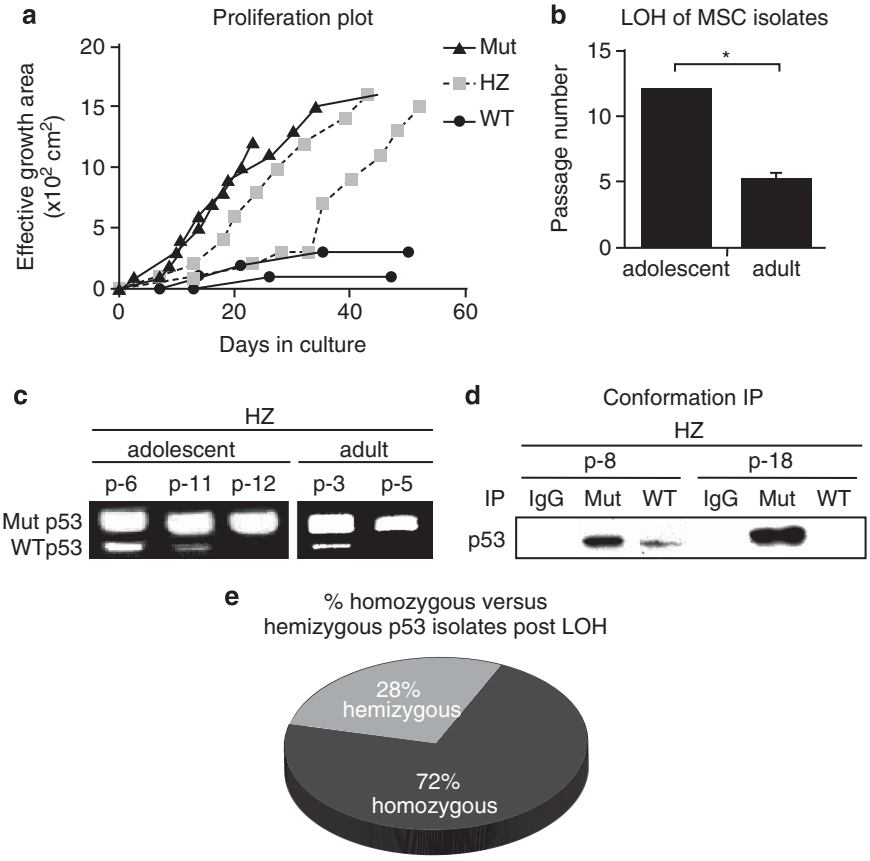


Figure 4 MSC isolates are prone to undergo p53LOH. MSC isolates derived from WTp53, HZp53 and Mutp53 adolescent (6–12 weeks) and adult (13–60 weeks) mice were established. Isolated clones were characterized for specific surface markers, to ensure they were free of contaminating hematopoietic and endothelial cells (Supplementary Figures 5 and 6). The MSC isolates were further characterized by their ability to differentiate into adipocytes and osteocytes (Supplementary Figures 7 and 8). (**a**) Proliferation plot presenting effective growth area over time in culture. Each curve represents MSC isolate derived from two mice. Two isolates were prepared for each mouse genotype. (**b**) p53 status of MSC isolates derived from two adolescent and four adult mice was determined by PCR genotyping every time the cells were passaged. The plot represents the mean passage number at which p53LOH completion was detected. (**c**) A representative figure of p53 PCR genotype analysis of MSC isolates derived from adolescent and adult mice. (**d**) Western blot of immune-precipitated (IP) p53 from adolescent HZp53 MSC isolate probed with Mutp53 (PAb-240), WTp53 (PAb-246) and IgG (control). (**e**) Percentage of homozygous and hemizygous HZp53 MSC isolates examined post LOH ($n=7$) using Taqman QRT-PCR as described in the legend of Figure 2d. *$P<0.05$ one-tail Student's *t*-test

Characterization of the genetic mechanism that underlies p53LOH in MSCs revealed that 72% of examined clones duplicated the mutant (Mut) allele (Figure 4e). In two out of seven isolates, we observed a single Mutp53 copy after LOH, while the Taqman probe recognizing exon 1 indicated that two copies were present. Thus, MSC isolates undergo WTp53-LOH in an age-dependent manner, mainly through a duplication of the mutated allele (see Figures 2e and f).

WTp53-LOH in HZp53-MSCs may lead to sarcomagenesis. Next, the tumorigenic potential of the various MSC isolates as a function of p53 status was examined. WTp53, HZp53 and Mutp53 MSC isolates derived from adolescent mice were subcutaneously injected into immune-compromised mice. All 10 mice injected with Mutp53-MSCs developed tumors (Figure 5A), whereas none of mice injected with WTp53-MSCs did (Figure 5A). HZp53 isolates were injected shortly after the completion of p53LOH. Despite the completion of the p53LOH process, no tumors were detected in mice injected with HZp53 MSC isolates derived from adolescent mice. In contrast, HZp53 MSC isolates derived from adult mice, which underwent WTp53-LOH 10 passages before injection, induced tumors (Figure 5A). These tumors exhibited histological characteristics of sarcoma with invasive edges, similar to tumors induced by Mutp53-MSCs (Figures 5B and C). Thus, the mere WTp53-LOH is not sufficient to promote tumorigenesis, but rather permits other tumor-promoting events to occur.

SKY analysis of the various HZp53 MSCs indicated a facilitated incidences of chromosome 11 translocation correlating with the age of mice the isolates were derived of (Supplementary Figure 6D). The highest number of chromosome 11 translocations was noticed in a tumor line derived from a MSC isolate obtained from aged mice (Supplementary Figures 3C and D). As indicated above, only HZp53-MSCs isolated from adult mice were tumorigenic (Figure 5a). Furthermore, MSCs from adult mice showed higher rate of chromosomal aberrations by SKY. Together these findings imply that p53LOH permits other oncogenic events to occur, together leading to tumorigenic transformation at an advanced age.

Alterations in the expression pattern of DNA repair genes coinciding with WTp53-LOH. To unravel the gene expression patterns associated with the LOH process, we performed mRNA profiling by cDNA microarray of MSC isolates derived from adolescent mice at passage 9, where the p53LOH process has already begun but was not yet completed (Figure 4c) and HZp53 isolate at passage 2, with no detectable LOH. Our analysis yielded 11 clusters. Cluster 1, the 'downregulated cluster' (Figure 6a) was enriched for known 'p53 signaling' genes, such as *Fas, Ccng1, Cdkn1a,*

A

MSC strain	Mice age (weeks)	Tumor take	Days to tumor detection (mean)
WT	8-10	0/10	ND
HZ		0/10	ND
Mut		10/10	65-99 (83)
HZ	59	10/10	53-104 (72.5)

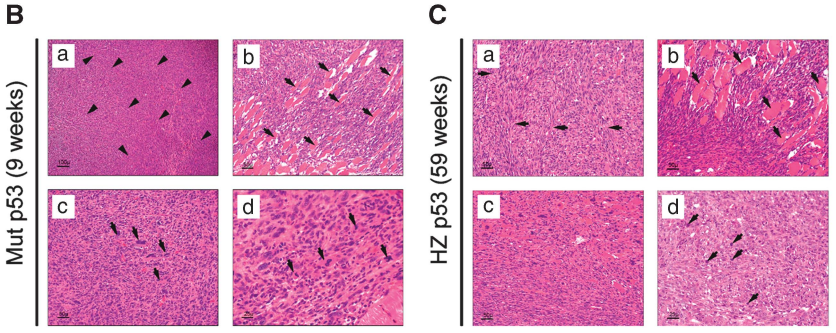


Figure 5 Aged adult mouse-derived HZp53 MSC isolates that underwent LOH form malignant sarcoma upon injection into immune-deficient mice. Cells of two MSC isolates each from WTp53, HZp53, Mutp53 adolescent mice and HZp53 adult mice were injected subcutaneously into NOD-SCID mice (5 per group, total of 10 mice for each p53 genotype) at passages 13–15. Tumors were removed and stained by H&E. (**A**) Table summarizing the results of tumor take and days until detection of the tumors of the different MSC isolates. (**B** and **C**) Representative sections of tumors formed by Mutp53 (**B**) and HZp53 (**C**) MSC isolates. The cells injected are from the MSC isolates derived from 9-week-old Mutp53 mice and 59-week-old HZp53 mice. Tumors exhibited histological features typical of sarcoma. (a) Arrowheads indicate neoplastic cells arranged in interlacing fascicles, in an arrangement similar to fibrosarcoma. (b) A typical invasive edge is presented. The neoplastic cells engulfed pre-existing tissue elements; arrowheads indicate skeletal myofibers. (c) Marked difference in the overall size (anisocytosis) and nuclear size (anisokaryosis) of neoplastic cells. Several larger cells were identified (**A**, arrowheads). (d) Neoplastic cells exhibit a high mitotic rate (arrows indicate mitotic figures)

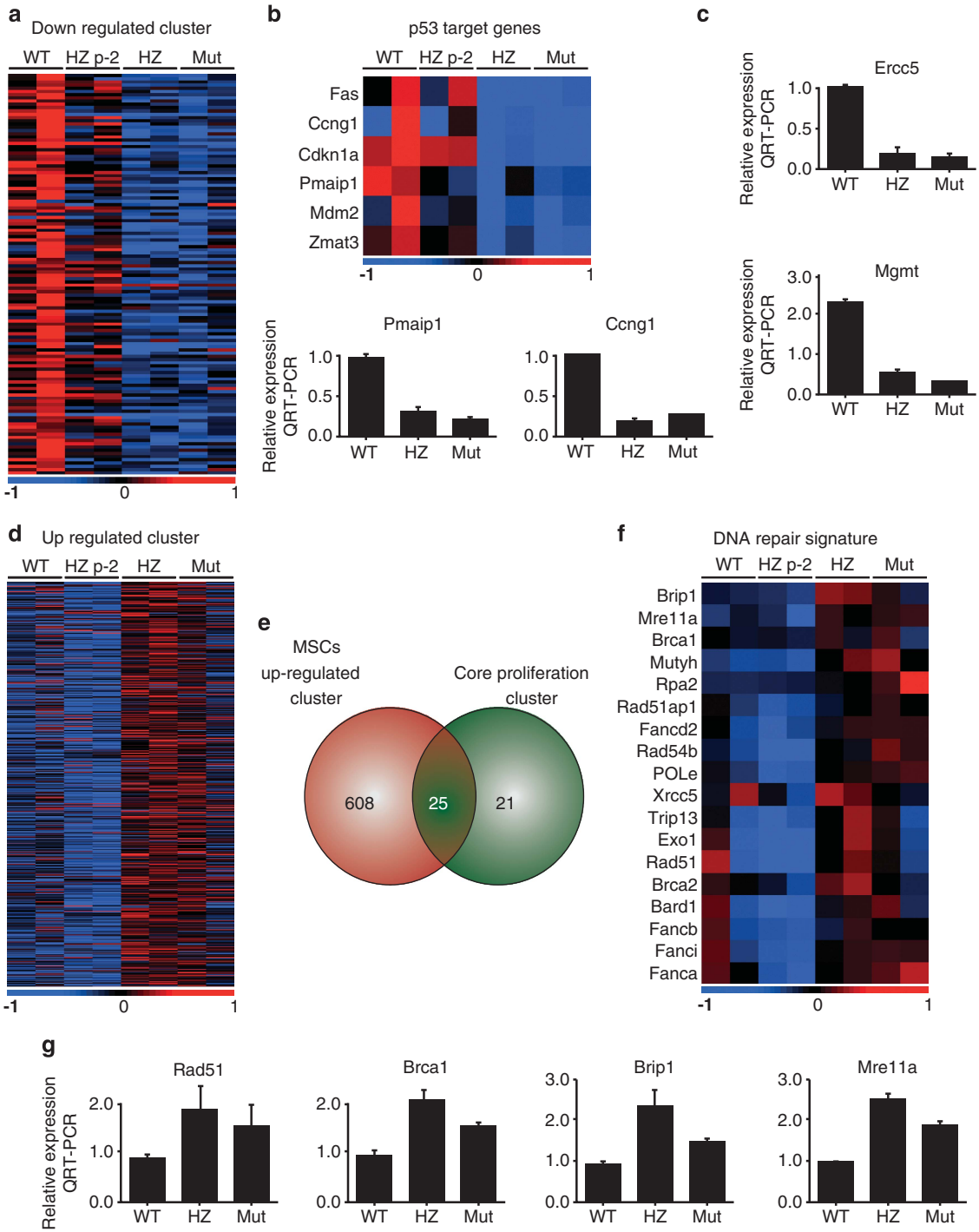


Figure 6 Downregulation of p53 target genes and upregulation of HR DNA repair genes coincide with p53LOH in MSC isolates. Genome-wide expression screen to identify changes associated with p53LOH. Samples originated from MSC isolates duplicates of WTp53 p-9, HZp53 p-2, HZp53 p-9 and Mutp53 p-9. The various samples were hybridized to the Agilent mouse Genome Array and the relative mRNA abundance of 55 681 mRNA species was monitored. After standard preprocessing steps (see Materials and Methods), $>400\,000$ expression values were collected from eight microarrays. Of these, 4524 genes exhibited a greater than twofold change between any two conditions, and were clustered according to their Pearson correlations. (**a**) Heat-map representation of Agilent microarray data depicting the downregulation of 133 genes between WTp53 (WT) and HZp53 p-2 (HZ p-2) to HZp53 p-9 (HZ) and Mutp53 (Mut) (cluster 1). (**b**) Heat-map representation of known p53 targets from cluster 1 and validation of gene expression of *Pmaip1* and *Ccng1* by QRT-PCR. (**c**) Validation of the expression changes in DNA repair genes from cluster 1 (*Ercc5* and *Mgmt)* by QRT-PCR. (**d**) Upregulation of 633 genes between WTp53 (WT) and HZp53 p-2 (HZ p-2) to HZp53 p-9 (HZ) and Mutp53 (Mut) (cluster 2). (**e**) 'Upregulated cluster' was compared with the 'core proliferation cluster' (right hand). $^*P<0.001$. A Fisher exact test was used to compare this overlap. (**f**) Heat-map representation of genes that were implicated as homologous recombination (HR) DNA repair pathway genes ($P=9.14\times10^{-4}$) using IPA functional annotation, genes associated with non-homologous end joining (NHEJ), and genes associated with Fanconi Anemia crosslinked DNA repair known to engage HR. (**g**) QRT-PCR validation of expression changes in *Rad51*, *Brca1*, *Brip1* and *Mre11a*. QRT-PCR results of each gene were normalized to *Hprt*. All samples were collected at p-9 unless indicated otherwise. Bars represent mean ± S.D.

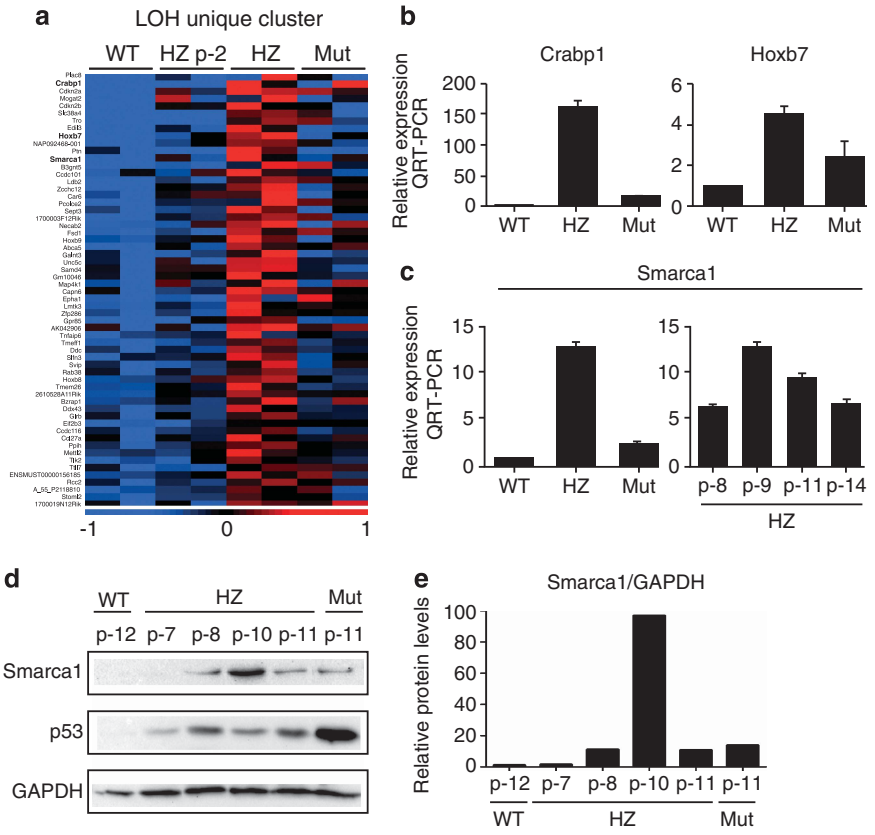


Figure 7 Elevated *Smarca1* expression is associated with p53LOH in MSC isolates. A list of 'LOH unique' genes with highest expression in HZp53 p-9 was generated out of cluster 2. Only the genes with expression values at least 1.5-fold higher in HZp53 p-9 cells than in Mutp53 p-9 cells, and expression values at least 2-fold higher in HZp53 p-9 cells than in WTp53 p-9 and HZp53 p-2 cells, were selected. (**a**) A p53LOH-associated gene signature is presented as a heat-map of WTp53 p-9 (WT), HZp53 p-2 (HZ p-2), HZp53 p-9 (HZ) and Mutp53 p-9 (Mut) MSC isolates. (**b** and **c**) Validation of *Crabp1*, *Hoxb7* and *Smarca1* expression by QRT-PCR. The bars represent mean ± s.d. (**d**) Western blot analysis of *Smarca1* and p53. GAPDH was used as a loading control. (**e**) Quantities of *Smarca1* protein levels were determined by measuring band intensity using the Lab Image 4.1 software

Pmaip1, Mdm2 and *Zmat3* (Figure 6b), $P = 2.76 \times 10^{-7}$ as well as for the p53-dependent DNA repair genes *Ercc5* and *Mgmt*. Reduced expression of p53 target genes and the above DNA repair genes in HZp53 p-9 isolates confirm a progressive loss of functional WTp53 in these cells.

Cluster 2 represents a group of 633 upregulated genes (Figure 6d). We evaluated whether this 'upregulated cluster' contains genes previously described to be proliferation-related genes[32,33] and found an overlap of 25 genes (Figure 6e; Supplementary Table 3). Functional annotation revealed that the 'upregulated' cluster is enriched for genes of the HR DNA repair pathway (HRDRP; $P = 9.14*10^{-04}$). Notably, the HRDRP genes do not overlap with the 'proliferation cluster' genes, with one exception (Trip13). We validated the elevated expression of *Rad51, Brca1, Brip1* and *Mre11a* in p-9 HZp53-MSCs (Figure 6g). Fanconi Anemia proteins (*Fanci, Fanca, Fancb* and *Fancd2*) that execute cross-linked DNA repair, known to engage HR,[34] also appeared in the upregulated cluster (Figure 6f) as well as *Xrcc5* (Ku80) and *Mre11a,* known to have a role in non-homologous end joining (NHEJ; Figure 6f). Figure 6f illustrates a 'heat map' of expression levels of all the upregulated genes involved in HRDRP and additional DNA repair pathways, suggesting that HRDRP is one of the major mechanisms leading to LOH.[35]

Expression of the chromatin remodeler *Smarca1* is altered along the p53LOH process in MSC isolates. As LOH involves chromosomal alterations, we also searched for genes controlling chromosomal integrity. A 'LOH unique' list, with the highest expression level in HZp53 p-9 was derived of the 'upregulated' cluster (Figure 7a). This identified the chromatin remodeler *Smarca1*.[36] We found that the expression of *Smarca1* mRNA and protein peaked at passages 8-9, followed by a decline after p53LOH was attained in the majority of the population (Figures 7c–e). No change in the expression patterns of other major chromatin remodelers,[36] such as *Smarca2, Smarca4* and *Smarca5,* was observed (Supplementary Figure 9). This indicates a unique correlation between *Smarca1* expression and p53LOH.

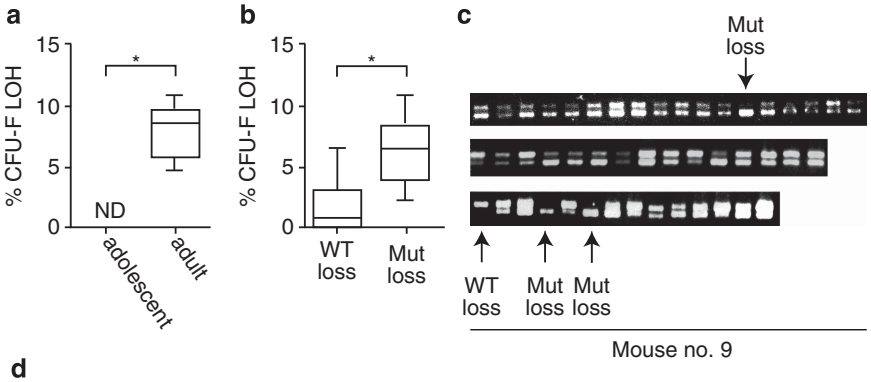
Bi-directional p53LOH. As both iPSCs and MSC isolates represent *in vitro* SC models, it was important to examine whether p53LOH may occur *in vivo*. BM progenitors were isolated and tested *ex vivo* for their p53 status. As we observed that p53LOH in MSCs is age dependent, we evaluated the *in vivo* p53LOH process as a function of age in mice.

We could not detect any p53LOH in colony-forming units-fibroblast (CFU-Fs) derived from adolescent HZp53

mice. However, CFU-Fs derived from BM of adult HZp53 mice exhibited p53LOH at varying frequencies (Figures 8a and c). Surprisingly, the majority of p53LOH (average of 6.2% *versus* 1.7%) events led to the loss of the Mutp53 allele (Figures 8b–d). This suggests that a specific mechanism dictates a preference to retain the WT allele, assuring genome integrity *in vivo*.

Surprisingly, genomic analysis of 156 iPSC single-cell sub-clones of an iPSC clone that retained its heterozygosity indicated that the majority kept their HZp53 genotype, however three of them have lost their Mut allele (Figure 8e). In order to ensure that it is not the loss of only the *LoxP* gene sequences, masquerading as the loss of the Mutp53 allele, we sequenced the area around the R172H mutation (Supplementary Figure 10) and found solely WTp53 DNA sequences. Similarly, genotyping of 220 single-cell sub-clones of early passage MSC isolates also identified the loss of the Mutp53 allele (1.4%; Figure 8f). All together our results suggest that bi-directional LOH is not restricted to a specific type of SC.

In sum, we observed p53LOH in somatic cells, iPSCs and MSCs. However, the p53LOH frequency was different between the systems and correlates with their genome stability. The highest frequency was observed in somatic cells; it was lower in ASC, in an age-dependent manner and lowest in embryonic-like iPSCs. Only cells that underwent p53LOH gave rise to malignant tumors. In most cases, p53LOH resulted of HRDRP. Interestingly, in BM progenitors



d

Mouse number	Mice age	Number of screened CFU-F	Total LOH (%)	Loss of WT allele (%)	Loss of mutant allele (%)
1	Adolescent (5-12 weeks)	74	0	0	0
2		70	0	0	0
3		39	0	0	0
4		82	0	0	0
5	Adult (13-60 weeks)	94	6.4	0	6.4
6		120	9.1	1.7	7.5
7		46	10.8	0	10.8
8		88	4.5	0	4.5
9		47	8.4	2.1	6.3
10		45	8.8	6.6	2.2

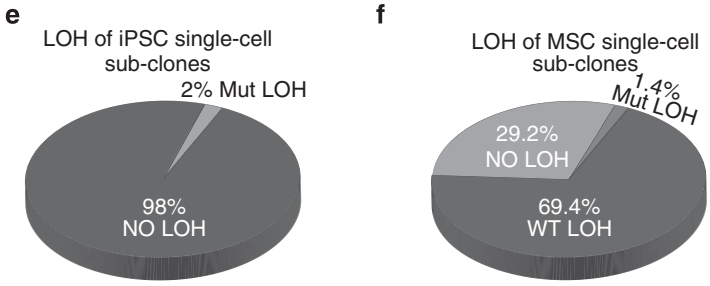


Figure 8 WTp53 and Mutp53-LOH in BM mesenchymal progenitors, iPSCs and MSCs. BM was isolated from HZp53 adolescent and adult mice and CFU-F formation was assessed. Two weeks later, colonies were genotyped by PCR. (**a**) Percentage of BM-derived CFU-F colonies that underwent p53LOH, derived from adolescent and adult mice. (**b**) Percentage of CFU-F colonies derived from BM of adult mice that lost their either WTp53 or Mutp53 allele. Box plots represent median, 25th percentile, 75th percentile and extreme values. (**c**) Raw unprocessed data of PCR-genotyped CFU-F colonies derived from mouse no. 9. WT or Mut LOH is marked accordingly. (**d**) Table summarizing the results obtained from genotyped CFU-Fs presented in panels **b** and **c**. (**e**) Two HZp53-iPSC clones were single-cell sub-cloned in 96 well-plates. After 2–3 weeks, plates were genotyped by PCR. A summary of 156 single-cell sub-clones is presented in a pie chart. (**f**) Two HZp53 MSC isolates derived from adolescent mice were sub-cloned at a density of either one or five cells per well in 96-well plates, and PCR-genotyped. Summary of the data from three independent experiments of 220 single-cell sub-clones is presented in a pie chart. The diagram summarizes the percentage of sub-clones that did not undergo LOH (NO LOH), sub-clones that lost the WTp53 (WT LOH) and sub-clones that lost the Mutp53 (Mut LOH). ND, not detected. *$P<0.05$ one-tail Student's *t*-test

of adult mice, p53LOH was surprisingly frequent. Strikingly, the loss of the Mutp53 allele was four times more frequent than the loss of the WT allele in these cells. Our findings highlight that bi-directional p53LOH can determine whether a cell lose its predisposition to cancer or initiate events leading to malignant transformation.

Discussion

Although LFS patients seem to mature normally, they develop a wide spectrum of cancer types in childhood and adult life where about 60% tumors demonstrate p53LOH.[8] Sarcomas, which are of mesenchymal origin, are prevalent tumors in LFS patients and murine models.[6–8,37] The possibility of a link between cancer development and deregulation of SCs[11–13] challenged us to study the significance of p53LOH in SCs and tumorigenesis. The availability of the reprogramming process that enables to recapitulate a transition from adult somatic cells into embryonic-like cells and HZp53 mice allowed us to address the above question, both *in vitro* and *in vivo*, as a function of age. Indeed, we observed that although p53LOH is attenuated with the onset of reprogramming, the *in vitro* p53LOH process is active in MSCs. Moreover, p53LOH was more pronounced in MSCs established from BM of adult mice compared with adolescent mice most likely reflecting a higher incidence of p53LOH *in vivo*. Furthermore, our data suggest a link between p53LOH, aging and tumorigenesis. It was previously reported that transformation of MSCs seemed to be highly dependent on alterations in the p53/p21 pathway; mainly through inactivation of WTp53[14] and that MSCs might require few genetic alterations to undergo transformation.[14] Moreover, others have shown that only after long-term *in vitro* cultivation, p53 knockout MSCs were able to form tumors in mice.[38] We observed that HZp53-MSCs obtained from adolescent mice, injected shortly after the completion of p53LOH, did not give rise to tumors. This agrees with the notion that MSCs require several genetic alterations for transformation that can be acquired gradually after p53 restrain is released. Indeed, injection of HZp53-MSCs isolates obtained from older mice, injected at the same passage as all other isolates but having undergone p53LOH 10 passages earlier, gave rise to aggressive tumors similar to those induced by Mutp53-MSCs. Interestingly, a specific reduction in p53 levels in MSCs but not in the spleen of old mice was reported.[39] These results suggest that loss of WTp53 in MSCs is an initiating step in sarcomagenesis.

The link between p53LOH, tumorigenesis and aging observed in our *in vitro* SC system led us to examine this link *in vivo*. We examined BM progenitors from HZp53 mice at various ages and demonstrated, for the first time, that p53LOH occur *in vivo*. This process is accelerated with age, reaching up to 10% of the progenitor SCs in adult mice. Studies in yeast have revealed an increase in LOH as the mother cell ages.[40] Surprisingly, the majority of cells that underwent p53LOH lost the Mut allele. This phenomenon of Mutp53-LOH was also observed in single-cell sub-clones of our *in vitro* SC systems. Although this is the first report on the loss of the Mutp53 allele through LOH in an apparent healthy tissue, Mut-LOH was noticed for other TSGs with a role in DNA repair.[41–45] It is tempting to speculate that LOH can be seen as a physiological genetic repair mechanism. Gene expression, copy number and sequencing analyses (Figures 6f, 2e, f and h) point to the induction of HRDRP events as the mechanism underlie most cases of LOH. Mutp53 seems to induce a state of chronic DNA insults, as cells harboring either Mutp53 or HZp53 exhibit a DNA repair gene expression signature. The observed gene-signature is manifested by the upregulation of specific genes involved in the dsDNA break-response, which includes both HR and NHEJ. Although HR is a high-fidelity DNA repair mechanism, NHEJ is highly error prone, and thus these two mechanisms may have significantly different consequences. Studies in the *Drosophila* male germline have indicated that HR increases linearly with age. The authors speculated that in young individuals, selective pressures may favor usage of NHEJ and single-strand annealing, which are faster but more error prone. However, in old individuals, the fidelity obtained by HR is favorable.[46] Recently, a link between HR, reprogramming and p53 was established, wherein cells defective in their HR pathway yield a smaller number of reprogrammed cells. This decrease is mediated by p53-dependent growth arrest and apoptosis, which is responsible for the elimination of cells with damaged DNA. In the absence of p53, the reprogramming process continues at the expense of accumulating genetic aberrations.[47]

The accessibility of DNA repair enzymes to damaged DNA is blocked by nucleosomes, thus chromatin remodeling must occur during the detection and repair of damaged DNA.[48,49] Indeed, we found that the 'unique p53LOH' list contains the chromatin remodeler *Smarca1,* which belongs to the ISWI ATP remodeling family.[36]

In summary, we present for the first time the evidence for a physiological bi-directional p53LOH process, which may serve as a cell fate checkpoint in SCs. At large, p53HZ-SCs, although carrying a Mutp53 allele, manage to suppress its activity and exhibit a normal phenotype. However, with age, cells become less stable and may activate LOH as a second line of defense in an attempt to lose the mutated allele. LOH can be regarded as an event that helps maintain genomic stability. Indeed, this LOH DNA repair mechanism was restricted to less genomically stable cells. For example, BM progenitors of adolescent mice, which are genomically more stable than BM of adult mice, did not undergo p53LOH. Similarly, reprogramming of HZp53-MEFs, which robustly undergo p53LOH, mostly restrained the p53LOH process. However, in cases where the WT allele is lost, cells acquire properties leading to tumor formation. Our observation that LOH is attenuated in embryonic iPSCs while adult MSCs readily undergo LOH, coupled with the fact that p53LOH yields cells lacking WTp53, can explain why LFS patients do not acquire tumors during development, yet would develop tumors (sarcomas in particular) later in life.

Materials and Methods

Mice strains. The following mice strains were used in this study: C57BL/6 containing WTp53, HZp53 or Mutp53 alleles (kindly provided by Professor G Lozano) *Hfh11nu* Nude mice and NOD.CB17-prkdc-SCID/NCrHsd (Harlan, Rehovot, Israel). Animal protocols were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Cell cultures. MEFs prepared as previously described[25] and were maintained in DMEM (Biological Industries, Bet-Haemek, Israel) supplemented with 10% FCS and antibiotics.

MSCs were grown in MSC medium, containing murine MesenCult Basal Media (StemCell Technologies, Vancouver, BC, Canada) supplemented with 20% murine mesenchymal supplement (StemCell Technologies), 60 μg/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml kanamycin. Cells were incubated at 37 °C in a humidified atmosphere of 10% CO_2. Fresh medium was added twice a week. iPSCs were maintained on irradiated MEFs in ES medium: DMEM (Biological Industries) containing 15% FCS, 5 mg recombinant human LIF (Millipore, Bellerica, MA, USA LIF1005), 1 mM glutamine (Biological Industries), 1% nonessential amino acids (Biological Industries), 0.1 mM β-mercaptoethanol (Invitrogen), 60 μg/ml penicillin and 100 μg/ml streptomycin (Biological Industries).

Generation and characterization of iPSCs. The EF1a–STEMCCA lentiviral vector, a kind gift of Dr. Mostoslavsky G,[50] allows for constitutive expression of the four proteins *Oct4, Klf4, Sox2* and *mCherry* from a single polycistronic transcript. Lentiviruses were produced in 293T packaging cells as previously described.[51] Forty-eight hours post infection, 3.5×10^5 cells were plated in 10 cm plates, on top of a feeder layer of irradiated MEFs (irradiated with 60 gray gamma irradiation), and medium was replaced to ES medium. At this stage, the medium was supplemented with 2I: small-molecule inhibitors CHIR99021 (GSK-3binhibitor, 3 mM; Axon Medchem, Groningen, Netherlands) and PD0325901 (ERK1/2 inhibitor, 1 mM; TOCRIS Bristol, UK). Emerging colonies were selected by morphology. Each colony was isolated either mechanically or by incubating for 15 min in a trypsin filled glass cylinder on the emerging clone. The colonies were then transferred to 12-well plates containing ES + 2I medium and separately passaged. A few passages later, 2I was removed gradually from the medium. Alkaline phosphatase activity was performed as previously described.[52] Colony number was determine using Image-Pro Plus analysis software (Media Cybernetics, Rockville, MD, USA). Further characterization and verification of the nature of the reprogrammed clones were performed by QRT-PCR as described below in detail.

Preparation of BM cells suspension. BM nucleated cells were obtained from femurs and tibias of 6–8 weeks old, 4–5 months old and 13 months old WTp53, HZp53 and Mutp53 mice. The bone was flushed with PBS containing 2% FCS (Biological Industries LTD). The cells were dissociated to single-cell suspension and were centrifuged at 300 g for 5 min at room temperature. Red blood cells (RBCs) were removed by RBC lysis buffer (R7757, Sigma, St. Louis, MO, USA).

Production and characterization of MSC isolates. For the standard MSC production, the pellet BM cells were re-suspended to single-cell suspension and seeded in six-well plates containing MSC medium (ratio of one mice to one well). The medium was replaced every 3 days to remove the non-adherent cells. Once the adherent cells had reached confluence, the cells were trypsinized using Trypsin B solution (0.05% EDTA, 0.25% trypsin), centrifuged for 5 min at 300 × *g*, 4 °C, re-suspended in their medium and split 1 : 2. Expression of MSC isolates surface markers was analyzed using the following antibodies: anti-CD11b-PE, anti-CD45.2-PE, anti-CD31-PE, anti-CD34-PE, anti-Ter119-PE, anti-Sca1-PE, Rat IgG2b isotype control–PE, Rat IgG2a isotype control-PE and Mouse IgG2a isotype control (eBioscience, San Diego, CA, USA). 10^6 MSC cells were harvested and incubated for 10 min on ice with the Fc blocker antibody of anti-CD16/CD32, following 1 h incubation on ice with the specific antibodies listed above. Cells were subjected to flow cytometry analysis using a LSRII flow cytometer (BD, Franklin Lakes, NJ, USA, Immunocytometry Systems).

Evaluation of MSC differentiation potential

Adipogenesis: Cells were seeded at a concentration of 2×10^4cells/well in a 24-well plate. The next day, adipogenic medium containing 10 μg/ml insulin (Sigma), 0.5 mM IBMX (Sigma) and 1×10^{-5} M dexamethasone (Sigma) was added. The cells were grown for 1–3 weeks, with medium replacement twice a week. Adipogenesis was detected by Oil red O staining. For Oil red O quantification, 4% IGEPAL CA 630 (Sigma) in isopropanol was added to each well. Light absorbance was measured in 492 nm.

Osteogenesis: Cells were seeded at a concentration of 2×10^4cells/well in a 24-well plate. The next day, osteogenic medium containing 50 μg/ml L-ascorbic acid-2 phosphate, 10 mM glycerol 2-phosphate disodium salt and 1×10^{-7} M dexamethasone (all from Sigma) was added. The cells were grown for 1–3 weeks with medium being replaced twice a week. Osteogenic differentiation was detected by Alizarin red staining. For Alizarin red quantification, 0.5 N hydrochloric acid (HCl) and 5% SDS were added to each well. Light absorbance was measured in 405 nm.

Population doubling time and growth area measurement: Proliferation rates of the various MEFs were evaluated by calculating population doubling time. Cells (5×10^5) were plated in 6 cm plates in duplicates. The cells were counted every 3 or 4 days and re-plated at the same density. This procedure was repeated five times. MSCs proliferation rates were evaluated by defining the number of times that the cells were transferred to a larger growth area at defined time points. For instance, if the growth area doubled itself then the effective growth area increased by one unit of 6 cm plate (28.3 cm^2).

Single-cell cloning: iPSC and MSC isolates were serially diluted to reach 1–5 cells per well. The cells were plated on gelatin-coated 96-well plates. The colonies were examined by microscope to ensure that they originated form a single cell. Two to three weeks later, colonies were subjected to genomic DNA genotyping.

CFU-Fs assay: BM nucleated cells of HZp53 adolescent (four mice at the age of 5–12 weeks old) and adult (six mice at the age of 13–60 weeks old) were plated at cell densities of 20×10^6–30×10^6 in 10 cm BD falcon plates (BD). The cells were grown in MSC medium as described above and re-fed once a week without further treatment. At day 14, un-fixed colonies were subjected to genomic DNA genotyping.

In vivo tumorigenesis assays: iPSC clones were trypsinized and re-plated with ES medium for 15 min. The non-adherent cells were collected, resuspended in PBS and injected sub-coutaneously into 6–8 weeks old *Hfh11nu* Nude mice (10^6 cells/100 μl, with Matrigel matrix at a ratio of 1 : 1; Becton Dickenson FAL354232). The tumors were removed 2–16 weeks post injection, fixed in 4% paraformaldehyde, decalcified and embedded in paraffin. Selected sections, derived from three distinct tumor levels were stained with hematoxylin and eosin (H&E).

Seven- to eight-week-old age NOD.CB17-prkdc-SCID/NCrHsd mice were injected subcutaneously with 3×10^6 MSC cells expressing WTp53, HZp53 or Mutp53. Mice were killed when their tumors reached a diameter of 10 mm or after 120 days after inoculation. Upon tumor removal, half the tumor was mechanically disaggregated on mesh to establish MSC-transformed cell lines. The remaining portion of the tumor was used for histological analysis by H&E staining.

Western blot and immunoprecipitation analysis: Cells were lysed in 1 × passive lysis buffer (Promega, Madison, WI, USA), 1 mM Tris-HCl (pH 7.5), 5 Mm $MgCl_2$, 0.13 mM $CaCl_2$, 25 μg/μl DNase (Sigma), incubated for 1 h at 37 °C and 0.5 × TLB buffer was added (50 mmol/l Tris-HCl, 100 mmol/l NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails I and II (Sigma) for 15 min on ice, followed by centrifugation. BCA reagent (Pierce, Rockford, IL, USA) was used to determine Protein concentration. Fifty micrograms protein of each sample were separated by SDS-gel electrophoresis, and transferred to nitrocellulose membranes. The following primary antibodies were used: anti-mouse *p53* monoclonal 1c12 (Cell Signaling Technology, Danver, MA, USA), anti-Smarca1 polyclonal (Biorbyt, Cambridge, UK) and anti-GAPDH mab374 (ChemiconTemecula, CA, USA). The protein–antibody complexes were detected using horseradish peroxidase-conjugated secondary antibodies and the Amersham ECL western blotting detection reagents (GE Healthcare, Wauwatosa, WI, USA). For immunoprecipitation, PAb240, a monoclonal anti-Mutp53 antibody (a kind gift from Dr. D Lane), PAb246, a monoclonal anti-WTp53 antibody (a kind gift from Dr. D Lane) or control IgG antibody (Sigma) were incubated overnight at 4 °C with the lysate followed by the addition of 30 μl protein A beads for 2 h at 4 °C. The immunoprecipitated material was washed and pellets were resuspended in SDS sample buffer and subjected to western blot analysis.

Genomic DNA extraction: Cells were resuspended in lysis buffer (100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% SDS, Sigma) and incubated at 65 °C for 30 min, following by incubation in 4 °C for 15 min with LiCl/KAc solution (Sigma). DNA was precipitated with isopropanol and washed with 70% ethanol. The genomic DNA was re-suspended in DDW.

Genomic sequencing: Prior sequencing, a PCR analysis was performed using 5 ng genomic DNA and primers surrounding the p53 R172H mutation site. Forward primer: 5′-TCCCAGTCCTCTCTTTGCTG-3′. Reverse primer: 5′-CTCGGGTGGCTCATAAGGTA-3′. PCR reactions consisted of 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s, purified with HiYield

gel/PCR DNA fragment extraction kit (RBCBioscience, Xindian, Taiwan). Twenty nanograms of DNA was sequenced at the sequencing unit of Weizmann Institute of Science with either forward or reverse primer above.

Quantifying copy numbers in genomic DNA using the TaqMan copy number assay: Copy number genotyping was performed using RT-PCR–based copy number analysis (TaqMan Copy Number Assays, Applied Biosystems, Foster City, CA, USA) for *p53* (exon 1- intron 1 Chr.11:69394017, Applied Biosystems) and custom *loxP* site Mut *p53* (intron 4, Applied Biosystems). Analyses were initially performed on a subset of mouse tip fibroblasts of known genotypic origin. For each single-well reaction using 20 ng genomic DNA and 1 × TaqMan Universal PCR Master Mix, a 1 × TaqMan Copy Number Assay, which contained forward primer, reverse primer and FAM dye-labeled MGB probe specific for the gene of interest, was run simultaneously with a 1 × TaqMan Copy Number Reference Assay, which contained forward primer, reverse primer and a VIC dye-labeled TAMRA probe specific for transferrin receptor (TFRC) 17 according to the manufacturer's instructions. PCR was performed in 96-well plates using a PCR system (7300 Real-Time PCR System, Applied Biosystems). Samples were assayed using triplicate wells for each gene of interest. Copy numbers were estimated (CopyCaller Software version 2.0, Applied Biosystems) using the ΔCt relative quantification method. A maximum likelihood algorithm was used to estimate the mean ΔCt expected for copy number 1 (CN = 1) based on the probability density distribution across all samples, and this parameter was used in subsequent copy number calculations for each given gene. This analytical method was used to calculate the relative copy number of a target gene normalized to *TFRC*, a reference of known copy number (CN = 2).

Genomic DNA genotyping: Genotyping was performed by PCR analysis using 50 ng of genomic DNA and primers surrounding the *lox*P site (see Supplementary Figure 1). Forward primer: 5′-ACCTGTAGCTCCAGCAC TGG-3′. Reverse primer: 5′-ACAAGCCGAGTAACGATCAGG-3′. PCR reactions consisted of 35 cycles of 95 °C for 60 s, 60 °C for 60 s and 72 °C for 180 s and run on 2% agarose electrophoresis gel.

Reverse transcription and QRT-PCR: Total RNA was isolated using the Nucleospin II kit (Macherey Nagel, Duren, Germany) according to the manufacturer's protocol. An aliquot of 2 μg of total RNA was reverse-transcribed using Bio-RT (BioLab, Jerusalem, Israel) and random hexamer plus oligo-dTprimers (NEB, Ipswich, MA, USA). QRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 instrument (Applied Biosystems). The values for the specific genes were normalized to HPRT housekeeping gene control. Specific primers were designed for the following genes: Pmaip1: forward: 5′-GCAGAGCTACCACCTGAGTTC-3′, reverse: 5′-CTTT TGCGACTTCCCAGGCA-3′, Cong1: forward: 5′-ACAACTGACTCTCAGAAACT GC-3′, reverse: 5′-CATTATCATGGGCCGACTCAAT-3′, Ercc5: forward: 5′-TG CTGGCCGTGGATATTAGC-3′, reverse: 5′-GCCGGTGGAATAATGTGAGAAGA-3′, Mgmt: forward: 5′-TGCTCTCCATCACCCTGTGTT-3′, reverse: 5′-AACACCTGT CTGGTGAATGAATCTT-3′, Rad51: forward: 5′-AAGTTTTGGTCCACAGCCTA TTT-3′, reverse: 5′-CGGTGCATAAGCAACAGCC-3′, Brca1: forward: 5′-CGAATCT GAGTCCCCTAAAGAGC-3′, reverse: 5′-AAGCAACTTGACCTTGGGGTA-3′, Brip1: forward: 5′-TACTCTGGCTGCAAAGTTATCTG-3′, reverse: 5′-TCGTGCATCTACA TGGTGGAC-3′, Mre11a: forward: 5′-CCTCTTATCCGACTACGGGTG-3′, reverse: 5′-ACTGCTTTACGAGGTCTTCTACT-3′, Crabp1: forward: 5′-CAGCAGCGAGAATT TCGACGA-3′, reverse: 5′-CGCACAGTAGTGGATGTCTTGA-3′, Hoxb7: forward: 5′-AAGTTCGGTTTTCGCTCCAGG-3′, reverse: 5′-ACACCCCGGAGAGGTTCTG-3′, Smarca1: forward: 5′-TGCTACAAATGATCCGTCATGG-3′, reverse: 5′-GCGTTCT CGTTTAGGAGGTTCA-3′, Smarca2: forward: 5′-AGCCAGATGAGTGACCTGC-3′, reverse: 5′-TGCTTGGCATCCTTTTCGGAA-3′, smarca4: forward: 5′-CAAAGACA AGCATATCCTAGCCA-3′, reverse: 5′-CACGTAGTGTGTGTTAAGGACC-3′, smarca5: forward: 5′-GACACCGAGATGGAGGAAGTA-3′, reverse: 5′-CGAACAGCTCTGT CTGCTTTA-3′.

Flow cytometric determination of apoptosis by annexin V/propidium iodide double staining: Cells were analyzed for phosphatidylserine exposure by an Annexin-V FITC/propidium iodide Annexin using V FLUOS staining kit (Roche, Basel, Switzerland) according to the manufacturer's protocol.

DNA Library Preparation and Sequencing: Exome capturing was carried out with Agilent SureSelect V4 All Exon Mus musculus kit (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. In brief, 2–5 μg of gDNA were fragmented to ~170 bp (PE) insert-size with a Covaris S2 device (Covaris, Woburn, MA, USA). 500 ng of Illumina adapter-containing libraries (Illumina, San Diego, CA, USA) were hybridized with the exome baits at 65 °C for 24 h. Each enriched final paired-end library was sequenced using multiplexing of six samples on two Illumina Hiseq2000 lanes (Illumina).

Mapping and analysis: Illumina sequence data were aligned to the mm10 mouse reference genome assembly using BWA (0.5.9,[53]) duplicate and non-uniquely mapping reads were excluded. We subsequently detected SNVs and InDels as described in references Rausch *et al.*[54] and Jones *et al.*[55] adjusting the pipeline by using mouse genome annotations for Mapability, simple tandem repeats, repeat masker, segmental duplications, dbSNP137 as well as mm10 SNVs by ENSEMBL.

cDNA microarray: Total RNA was extracted using Tri-Reagent (MRC Global Inc., Houston, TX, USA) according to the manufacturer's protocol, and submitted for analysis to the Micro-Array unit of Weizmann Institute of Science, Rehovot, Israel. Agilent chips were used as a platform for RNA loading. The limma package[56] was used for microarray processing. Background was subtracted using the function backgroundCorrect and normalization within and between arrays was performed using the functions normalizeWithinArrays and normalizeBetweenArrays, respectively. Spots with the same probes were averaged. Analysis of variance including contrasts was applied to the data set using Partek Genomic Suite 6.5 (Partek Inc., St. Charles, MO, USA).

SKY analysis: Half a million cells were plated in a 10-cm plate and were cultivated for 48 h following replacement of medium and additional incubation period of 24 h. Colcemid (0.1 μg/ml) was added to the culture for 4 h. Cells were trypsinized and lysed with hypotonic buffer following fixation in glacial acetic acid/methanol (1 : 4). The chromosomes were simultaneously hybridized with 24 combinatorially labeled chromosome painting probes and analyzed using the SD200 spectral bioimaging system (Applied Spectral Imaging Ltd, Carlsbad, CA, USA).

Statistical analysis. Unless specified otherwise, all statistical analyses were performed using Graphpad Prism Software Inc (La Jolla, CA, USA).

Conflict of Interest

The authors declare no conflict of interest.

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REVIEW

The paradigm of mutant p53-expressing cancer stem cells and drug resistance

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It is well accepted that expression of mutant p53 involves the gain of oncogenic-specific activities accentuating the malignant phenotype. Depending on the specific cancer type, mutant p53 can contribute to either the early or the late events of the multiphase process underlying the transformation of a normal cell into a cancerous one. This multifactorial system is evident in ~50% of human cancers. Mutant p53 was shown to interfere with a variety of cellular functions that lead to augmented cell survival, cellular plasticity, aberration of DNA repair machinery and other effects. All these effects culminate in the acquisition of drug resistance often seen in cancer cells. Interestingly, drug resistance has also been suggested to be associated with cancer stem cells (CSCs), which reside within growing tumors. The notion that p53 plays a regulatory role in the life of stem cells, coupled with the observations that p53 mutations may contribute to the evolvement of CSCs makes it challenging to speculate that drug resistance and cancer recurrence are mediated by CSCs expressing mutant p53.

Introduction

Years of intensive research have yielded important clues regarding the nature of cancer. Various experimental models have shown that a normal cell undergoes malignant transformation following deregulation of major cellular signaling pathways (1). This usually occurs by accumulation of mutations in pivotal genes, epigenetic changes and environmental insults. Both acquired mutations and genetic predisposition have been shown to account for the onset and progression of cancer. Currently, full recovery from most cancer types is still an unsolved enigma. Indeed, frequently following therapy, where an apparent regression of tumor is observed, tumors often relapse and acquire a drug-resistant phenotype. Considering this observation, the development of efficient cancer therapy is closely dependent on the unraveling of drug resistance mechanisms operating in cancer cells. Conventional cancer therapy strategy aims to eliminate transformed somatic cells; however, the possibility of converting transformed cancer cells into normal ones should also be considered because it might restore cells with drug sensitivity.

It is well accepted today that cancer development is a multistep process that involves the accumulation of mutations in a given cell (2).

Abbreviations: ABC, adenosine triphosphate (ATP)-binding cassette; ALDH, aldehyde dehydrogenase; ASC, adult stem cell; BM, bone marrow; CSC, cancer stem cell; EMT, epithelial-to-mesenchymal transition; ESC, embryonic stem cell; GOF, gain of function; GSH, glutathione; IFNβ, interferon β; iPSC, induced pluripotent stem cell; KO, knockout; miR, microRNA; MSC, mesenchymal stem cell; NF-κB, nuclear factor-kappa B; ROS, reactive oxygen species; SC, stem cell; TNFα, tumor necrosis factor α; WT, wild-type.

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Most of the acquired mutations are silent and do not affect the normal homeostasis of the cell. However, it is well established that modifications in oncogenes and tumor suppressor genes are central for tumor development. Both have profound effects on pivotal pathways, such as the cell cycle, programmed cell death, DNA repair, cellular energy metabolism, angiogenesis, cell attachment, immune surveillance and replicative mortality (1). Although oncogenes have been shown to be overactivated in cancer cells, tumor suppressor genes are inactivated, leading to the loss of their normal function.

A frequent event in human cancer development is the impairment of the wild-type (WT) p53 tumor suppressor pathway, most frequently due to a point missense mutation in the *TP53* gene. It is well accepted that mutant p53 exhibits oncogenic gain of function (GOF) that, among others, confers cancer cells with drug resistance. Recent studies suggest that the cancer stem cell (CSC) subpopulation within tumors accounts for the drug resistance of cancer cells. Given the fact that compromised p53 expression may lead to the generation of CSCs, it is of interest to study the mutant p53-expressing CSCs and drug resistance paradigm.

The guardian of the genome and beyond—normal functions of WT p53

The WT p53 is a pivotal tumor suppressor, termed 'guardian of the genome', because it ensures genomic stability and thus prevents cancer onset (3). Under normal conditions, WT p53 is maintained at low levels due to its constant proteasomal degradation, mediated mainly by the E3 ubiquitin ligase, MDM2 (4). Subsequent to cellular insults such as DNA damage, oncogene activation, telomere erosion, hypoxia and ribosomal stress, WT p53 is stabilized and activated. Following its activation, WT p53 may induce a variety of processes, depending on damage severity and specific cell type. These include cell cycle arrest, programmed cell death (apoptosis), DNA repair, differentiation, autophagy, senescence and other processes (4,5).

The role of p53 in animal development. In addition to its tumor suppressive activity, p53 has also been found to be associated with normal development. One of the major obstacles in resolving the question of whether p53 is indeed involved in development was the initial observation that p53 knockout (KO) in mice was not lethal, which initially suggested that p53 is dispensable for proper development. Nevertheless, and in agreement with the notion that p53 is a tumor suppressor, p53 KO mice frequently develop tumors later in life (6–8). Moreover, an in-depth analysis indicated that p53 KO mice exhibit a lower fertility and that some of the newborns display a variety of developmental defects (reviewed in refs 9–11). These include exencephaly, impaired early neural crest development, ocular abnormalities, polydactyly of the hind limbs and defects in upper incisor tooth formation (12–14). Further examination of p53 null mice revealed abnormalities in reproduction. This is manifested by both defects in spermatogenesis in males (15–17) and impaired embryonic implantation in females (18,19), due to abrogated leukemic inhibitory factor activation, which is required for implantation of blastocysts (18). Additionally, we have recently demonstrated that p53 is required for proper brown fat development and function (20). These findings indicate the critical role of p53 during various developmental processes. The existence of viable p53-deficient mice might suggest that there is an incomplete penetrance of the p53 null phenotype, indicating a compensatory mechanism that may involve the interaction between alternative genetic and environmental factors.

The notion that p53 plays a role in development is substantiated by more directed studies demonstrating, in both mouse and chicken models, that the transcription of p53 is tightly regulated during embryonic development (reviewed in refs 21,22). Analysis of early-stage mouse embryos revealed that the expression of p53 mRNA in all tissues declines during the process of organogenesis and is barely detected in terminally differentiated tissues (23).

The role of p53 in differentiation. A growing body of evidence derived from *in vitro* models suggests that p53 plays a major regulatory role in cell differentiation. Interestingly, it was noticed that p53 seems to be a specific regulator in a variety of differentiation programs. Although

it facilitates some differentiation programs, others are attenuated (10,24,25). Initial studies have shown that reconstitution of WT p53 in a pre-B cell line, deficient in p53, accelerated cell differentiation and reduced the capacity to form tumors following injection into syngeneic mice (26,27). p53 has also been suggested to exert a positive effect on neural cell differentiation (28–30). Indeed, neural differentiation-relevant target genes are transactivated by p53 in the process of PC12 cell differentiation (31). During myogenic differentiation, p53 upregulates transcription of pRb, which is essential for the induction of the muscle differentiation program (32–34). Interestingly, it has been demonstrated that p53 plays contradictory molecular roles in osteogenic differentiation during normal development and tumorigenesis. Whereas p53 decreases differentiation of early osteogenic precursors (35–37), it facilitates terminal differentiation of tumor-forming osteogenic cells and thereby attenuates the cancerous outcome (38). Additionally, p53 was found to differentially regulate adipogenic differentiation. Although it inhibits white adipogenic differentiation (37,39,40), p53 is crucial for proper brown adipogenesis (20). Thus, p53 functions as a homeostatic protein, which promotes proper differentiation in accordance with a given cellular state, thereby avoiding malignant transformation. This is mediated either via its well-established role as an inducer of cell cycle arrest and apoptosis or by regulating the expression of specific differentiation-related factors required for various differentiation programs. In all, the well-established role of p53 in development and differentiation challenges the notion that p53 plays a role in the life of stem cells (SCs).

p53 and SCs. Proper embryonic development and adult tissue homeostasis rely on the capacity of SCs for self-renewal and differentiation into various cell types. Increasing evidence supports the notion that deregulation of the functions of embryonic stem cells (ESCs) and adult stem cells (ASCs) may lead to developmental aberrations, alterations in adult tissue maintenance and generation of CSCs, which may lead to tumor development.

It is well accepted that there is a wide repertoire of SC types. ESCs are pluripotent cells that are able to self-renew and maintain their cellular identity and they can differentiate into the endoderm, mesoderm and ectoderm cell lineages (41). The tissue-specific multipotent ASCs residing within adult organisms are capable of self-renewal and differentiation into the tissue-specific cells. The ASCs are necessary for normal homoeostasis of tissues and are vital for regeneration after damage (42,43). WT p53 has been implicated in the proper regulation of self-renewal and differentiation of ESCs (11,44,45). p53 is also implicated as a major regulator of the ASC compartment through control of cell differentiation (37,39,46), quiescence and asymmetrical division (47). Interestingly, compromised p53 expression in both ESCs and ASCs seems to confer SCs with accentuated oncogenic activity (46,48–50).

The reprogramming technology, which allows the generation of induced pluripotent stem cells (iPSCs) by dedifferentiation of somatic cells (51,52), opened an interesting platform to study the potential contribution of various factors central to the establishment of SCs *in vitro* (53). Of note, iPSCs and cancer cells have similarities between them with respect to overall gene expression patterns and epigenetic status (54,55), which suggests that tumorigenesis and reprogramming processes may share overlapping pathways. Thus, one of the risks of using iPSCs in cell transplantation therapy is cancer development from iPSC-derived cells (56). Numerous studies have implicated p53 as an important regulator of the reprogramming process. In agreement with others, we found that p53-compromised cells exhibit an accelerated rate of iPSC generation (50,57–64). Thus, p53 has an important role in the maintenance of a fine balance among SC generation, self-renewal and differentiation capacity. Interestingly, we found that reprogramming of mouse embryonic fibroblasts harboring a mutated p53 gene led to generation of CSCs that were capable of forming aggressive tumors in mice (50). This suggests that disruption of p53 function may lead to a burst of accentuated levels of SC proliferation in addition to diversion of SCs toward CSCs.

p53 and aging. Aging is defined as the process whereby life span is reduced with age, accompanied by common changes in phenotype occurring over time (65). It is accepted that as a tumor suppressor, activated p53 prevents cancer development, thus increasing longevity; however, overactivated p53 has been shown to promote premature aging (66,67). The most compelling evidences are obtained from mouse models displaying p53-dependent accelerated aging. For example, mice deficient in the DNA-repair-related gene Ku80 (68), telomerase-deficient mice (69) or mice lacking functional lamin A (70) showed enhanced aging phenotypes that could be rescued by reducing the levels of p53. In addition to the well-accepted mechanisms that mediate p53-dependent aging (71,72), it was shown recently that different p53 isoforms and the balance in their expression regulate aging and life span (66,73). One of the hallmarks of aging is the exhaustion of the ASC reservoir within the tissue (74). Although the role of WT p53 in regulating SCs is still to be completely elucidated, it seems that WT p53 prevents cellular transformation of damaged SCs by inducing either differentiation or cell death (46,50,75). As a result, the renewable cells of the tissue might be depleted, leading to premature aging.

p53 in human cancer

In most human tumors, the p53 pathway is altered, with high incidence of missense mutations, reaching to ~50% of all human tumors (76–78). Unlike other tumor suppressor genes, p53 not only loses its tumor-suppressive function (loss of function or LOF) but also gains novel oncogenic features in some of its mutated forms, independently of normal WT p53 roles, a phenomenon that was termed GOF. Furthermore, p53 is initially mutated in a single allele, leading to the concomitant expression of both WT and mutant proteins. Interestingly, in a heterozygous state, it was shown that some of the mutated forms can override the WT p53 in a dominant-negative manner. Mutant p53 GOF notion was first demonstrated in 1984, whereby introduction of mutant p53 was shown to transform cells lacking p53 (79), and this was followed up by vast research in the field (80). The most compelling evidence for mutant p53 GOF was shown in a mutant p53 knockin mouse model, which exhibited high incidence of metastatic tumors compared with KO mice (81,82). In addition to p53 mutations in somatic cells, p53 germ-line mutations were found to be highly associated (~95% of cases) (83) with a rare cancer predisposition called Li–Fraumeni syndrome (84), which is associated with the development of distinct tumor types, including sarcoma, breast cancer, brain tumor and adrenocortical tumors (85).

The mutation patterns of p53. More than 2000 different mutations have been reported in *TP53*, with several hot-spot mutations being frequently found in human cancers (86). p53 mutations can be categorized into two subgroups, according to their effect on p53 stability: 'DNA-contact mutations', which include mutations in residues essential for DNA binding, and 'conformational mutations', which include mutations that affect the conformational folding of the DNA-binding domain. The expression 'mutant p53' is frequently misused because the variety in both mutations and genotype–phenotype relations is a complex issue. Recent studies comparing the function of the different p53 hot-spot mutations suggest that the various p53 mutations exert different activities (87). When different p53 mutations were introduced into immortalized human fibroblast cells, in conjunction with the H-Ras oncogene, we found that different mutations regulated different signaling pathways [e.g. nuclear factor-kappaB (NF-κB) and H-Ras) to induce the expression of cancer-related genes and to promote transformation. Interestingly, the mutant $p53^{G245S}$ barely induced cellular transformation and expression of the cancer-related gene signature (88,89). This observation was further supported by two recent studies that analyzed different mutant p53 knockin mice models. Examination of two different humanized mutant p53 knockin mice revealed that although $p53^{R248Q/-}$ mice showed accelerated tumorigenesis with expended hematopoietic and mesenchymal stem cells (MSCs), compared with KO mice, $p53^{G245S/-}$ mice did not exert oncogenic GOF activities (90). An additional study suggested that unlike mutant $p53^{R172H}$ (human mutant $p53^{R175H}$ equivalent), mutant $p53^{R246S}$ (human mutant $p53^{R249S}$ equivalent) did not show higher levels of the transformed phenotype and did not promote tumorigenesis (91). With the rise of personalized

medicine in cancer therapy, understanding the exact p53 mutation type expressed in a patient's tumors is of great interest.

Normal WT p53 exerts its function in two main ways: activating/repressing transcription through binding to the promoter of a target gene at a specific sequence, namely, the responsive element; and via protein–protein interactions. In the past 2 decades, accumulating data have shed light on the effect of each p53 mutation on the possible properties of p53. Several mechanisms have been implicated in mutant p53 GOF. A key aspect by which mutant p53 exerts its GOF involves accumulation of the protein in the cell (92). Despite the observation of exceptionally high protein levels in tumor tissue, it was initially considered to be merely a side effect. Nevertheless, in both mouse models and Li–Fraumeni syndrome patients, the protein levels of mutant p53 in normal cells are kept at low levels (80,93). This indicates that p53 mutations by themselves are not sufficient for the high expression levels of p53 found in tumors and that mutant p53 accumulation is required for its GOF properties (78).

Mutant p53 GOF activities

The fact that p53 shows a wide range of hot-spot mutations that generate a highly accumulated aberrant p53 protein level in tumor cells suggests that cells expressing mutant p53 acquire selective growth advantage and tumorigenic potential. Indeed, mutant p53 was found to promote most of the events involved in the malignancy process (1), as discussed below.

Mutant p53 disrupts cell cycle control and enhances proliferation. One of the early observations pertaining to the function of WT p53 was that this tumor suppressor is a cell cycle regulator. Following genotoxic stress, intact WT p53 prevents damaged cells from undergoing malignant transformation by promoting either cell cycle arrest or cell death (4). However, when p53 is mutated, this important cell cycle control is disrupted, leading to enhanced proliferation, one of the typical hallmarks of cancer cells. In agreement with this observation, it was shown that expression of mutant p53 in conjunction with nuclear transcription factor Y (NF-Y) augments the expression of cell cycle-promoting genes and increases DNA synthesis (94). Interestingly, these highly expressed genes are clustered with other cell cycle-controlling genes in a gene signature annotated as the 'proliferation cluster'. This cluster of genes is upregulated in various tumor cells and was found to be positively correlated with high-grade breast tumor and with the expression of mutant p53 (95). Accordingly, mutant p53 was found to facilitate the transcription of genes that underlie the increased proliferation of cancer cells (80,86). In addition to regulation of gene expression, mutant p53 was found to regulate the expression of various microRNAs (miRs) that mediate several mutant p53 GOF activities. For example, mutant p53 was found to suppress the expression of miR-27a, which leads to enhanced epidermal growth factor signaling and extracellular signal-regulated kinase activation, which in turn were shown to enhance cell proliferation and augment the tumorigenic phenotype of cells (96).

Mutant p53 mediates genomic instability. By losing their 'guardian of genome' nature, mutant p53 proteins eventually lead to the collapse of the mechanism dominating genome stability and integrity. Cells that have lost their WT p53 and express instead mutant p53 exhibit extensive chromosomal aberrations and high mutation rates. This phenotype was observed in humanized mutant p53 knockin mice that express the chimeric human/murine mutant p53 gene. Genomic analysis of these mice indicated interchromosomal translocations, which were rarely observed in p53 KO cells, and these were accompanied by impaired G_2–M checkpoint, caused by inactivation of ataxia telangiectasia mutated gene (97). These phenomena, initially observed in embryonic fibroblasts and thymocytes, were further confirmed in mammary epithelial cells, in which it was suggested that the impairment of ataxia telangiectasia mutated gene by mutant p53 leads to the expansion of mammary CSCs and to tumor development (98). These observations are in line with chromosomal instability and aneuploidy demonstrated in mutant p53 transgenic mice (99–101). Furthermore, it was recently shown that mutant p53 expression correlated with massive chromosomal rearrangements observed in Sonic-Hedgehog medulloblastoma of Li–Fraumeni syndrome patients and in patients with acute myeloid leukemia. This phenomenon is manifested in cells as chromothripsis, a one-step catastrophic event, further reiterating the notion that mutant p53 exerts an oncogenic GOF activity in deteriorating genomic stability in cells (102).

Mutant p53 drives epithelial-to-mesenchymal transition, cell motility, tumor invasion and metastasis abilities. The abilities of tumor cells to invade the surrounding tissue and to metastasize are crucial for local carcinoma to evolve to a higher grade of malignancy. Interestingly, when mutant p53 knockin mice were initially generated and examined, the spectrum of spontaneously developing tumors was similar to that of p53 KO mice; however, a more in-depth analysis indicated that mutant p53 knockin mice also showed a high incidence of metastases that were not found in their p53 KO counterpart mice (81,82). This points to yet another defined tumor-promoting activity that underlies invasion and metastasis that are solely attributed to the oncogenic GOF activity mediated by mutant p53. Changes in the expression of cell adhesion molecules such as E-cadherin and N-cadherin are central to EMT, a process that allows cell detachment and migration (1). Recently, we found that mutant p53 enhances EMT in prostate tumor cells by elevating the expression of Twist1, a key regulator of EMT (103). This notion is further supported by a recent study suggesting that mutant p53 enhances EMT by modulating the miR-130b–Zeb1 (zinc finger E-box binding homeobox 1) axis in endometrial cancer (104). Additional processes that are enhanced by mutant p53 include cell motility (105) and cell migration that is mediated by overactivation of epidermal growth factor receptor/integrin signaling pathway (106) and by augmented chemokine expression (107). These findings agree with our previous studies showing that mutant p53 cooperates with oncogenic Ras to highly induce cancer-related genes, including chemokines, cytokines and extracellular matrix modulators (88,89).

Mutant p53 regulates nutrient supply by modulating angiogenesis and glycolysis. In order to support the continually accelerated growth, tumors acquire abilities to supply nutrients and oxygen to cells. This is manifested by enhancement of blood supply in the tumors by the generation of blood vessels through angiogenesis. This would occur when endothelial cells are reprogrammed to construct new blood vessels within the tumor mass (1). By binding to E2F1, mutant p53 was found to induce the expression of ID4, which promotes angiogenesis by stabilizing the proangiogenic factors IL8 and GROα (108). Additionally, mutant p53 expression was demonstrated to correlate with the expression of the key angiogenesis factor, vascular endothelial growth factor (109–111). Another mechanism that tumor cells adopt to control nutrient supply is to turn on aerobic glycolysis, whereby cells undergo glycolysis even under normal oxygen conditions, coined in the past as the 'Warburg effect'. This mechanism, although restricting adenosine triphosphate molecules, allows cells to gain metabolites that are incorporated into biosynthetic pathways, including generation of nucleotides and amino acids that are essential for growth (1,112). In accordance with the mutant p53 oncogenic GOF notion, presence of mutant p53 in cells was also associated with the 'Warburg effect'. In this case, mutant p53 was suggested to be involved in the translocation of the glucose transporter, GLUT1, to the plasma membrane, which is essential for high glucose uptake, by enhancing Rho/Rock signaling pathway (113).

Mutant p53 promotes inflammation. The notion that inflammation plays a critical role in promoting cancer is already well established (114) and thus was recently included as a bona fide oncogenic characteristic of cancer (1). The general notion is that inflammation serves as an important factor in tumor microenvironment that provides the developing tumor with growth and survival factors that limit cell death. Furthermore, inflammation involves proangiogenic factors, extracellular matrix-modifying enzymes that facilitate invasion and metastasis, and reactive oxygen species (ROS). Several studies have indicated that mutant p53 GOF supports processes associated with inflammation. We have previously found that mutant p53 enhances the response of cancer cells to the proinflammatory cytokine tumor

necrosis factor α (TNFα) (115). This was further reinforced by a study using a mouse model for chronic inflammation of the colon, in which mutant p53 was found to promote chronic inflammation associated with colorectal cancer (116). Although the mechanisms underlying the oncogenicity-enhancing inflammatory response are not entirely elucidated, several studies suggest possible molecular links. For example, we showed that mutant p53 enhances the expression of proinflammatory genes by activating Ras oncogene, ERK-MAPK, phosphoinositide 3-kinase and NF-κB signaling (88,89). This is supported by the observations that the proinflammatory genes signature that includes chemokines, cytokines and extracellular matrix modulators was also found to be synergistically elevated by mutant p53 and Ras oncogene in adult murine colon cells (117). These factors were shown to be highly elevated by mutant p53 in tumor-derived cell lines, mediating tumor cell migration (107). Mutant p53 was found to enhance the activity of the NF-κB effector, p65 (also termed RelA), as observed by higher p65 nuclear localization (115), and mutant p53 was suggested to promote the transcriptional activation of NF-κB by facilitating its binding to chromatin (116). Additionally, mutant p53 was found to induce the expression of another NF-κB family member, NF-κB2, which leads to chemoresistance (118). We have recently found an interesting cross talk between interferon β (IFNβ) and mutant p53. When cancer-associated fibroblasts and mutant p53-expressing tumor cells are cocultured, cancer-associated fibroblasts significantly promote the IFNβ pathway, which attenuates the migration of tumor cells and reduces mutant p53 mRNA levels. In turn, mutant p53 moderates the response to IFNβ in cancer cells by inhibiting the IFNβ downstream effector, signal transducers and activators of transcription 1 (STAT1), in a negative feedback loop (119).

Mutant p53 attenuates cell death and mediates drug resistance. The observation that mutant p53 confers cells with drug resistance and thus avoids apoptosis can be seen as the first milestone in the suggested mutant p53 GOF notion. Indeed, early studies have shown that M1/2 cells, expressing temperature-sensitive mutant p53, under conditions allowing mutant p53 conformation, avoid apoptosis induced by serum starvation (120). Attenuation of apoptosis in these cells was also observed following γ-irradiation and chemotherapy treatment (e.g. doxorubicin and cisplatin) (121), in addition to being seen in other tumor-derived cellular systems (122). Apparently, the attenuation of cell death conferred by mutant p53 is not restricted to chemotherapy. Indeed, over the years, independent studies have indicated that mutant p53 protects cells from additional death inducers such as 12-*O*-tetradecanoylphorbol 13-acetate (123), TNFα (115) and vitamin D (124). Figure 1 presents an example for mutant p53-dependent attenuation of TNFα-induced cell death. Although H1299 cells that were treated with high doses of TNFα underwent cell death, mutant p53-overexpressing cells were barely affected (Figure 1, unpublished data). Finally, it was recently suggested that mutant $p53^{P151S}$ displays Anoikis resistance (125), which was found to be essential for survival of metastatic cells (126). Several mechanisms can be attributed to the resistance to death effected by mutant p53, as illustrated in Figure 2. For example, it was suggested that the integrity of N′ terminus of p53, containing the transactivation domain, is essential for mutant p53-dependent chemoresistance, suggesting that mutant p53 attenuates drug-dependent death by transactivation activity (127). Indeed, mutant p53 was found to transcriptionally induce the expression of the multidrug resistance gene MDR1 by stimulating its promoter. As an adenosine triphosphate-dependent efflux pump, MDR1 transports foreign substances out of cells and clears drug accumulation in cells. Thus, by elevating its expression, mutant p53 confers tumor cells with drug resistance (128). In addition, mutant p53 was found to modulate the expression of genes involved in cell death regulation, such as the elevation in the antiapoptotic gene Bcl-xL (129), augmentation in EGR1 expression (130) and the downregulation of the proapoptotic gene Fas (131). More information regarding the modulation of expression of other death-related genes is reviewed in references 80,86. A recent study has suggested that tumors with high levels of p53, as observed in mutant p53-expressing cells, may evade apoptosis through the inhibition of caspase 9 activity (132). A well-studied mechanism for mutant p53 GOF is its interaction with other p53 family members, e.g. p63 and p73 (133). These interactions may explain mutant p53-mediated chemoresistance in colon adenocarcinoma-derived cell line, SW480 (134). Finally, regulation of miRs by mutant p53 is an additional mechanism explaining mutant p53-dependent drug resistance and death protection. Indeed, mutant p53 was found to induce the expression of miR-128 in lung cancer (135) and to downregulate the expression of miR-223 in breast and colon cancers (136), conferring resistance to chemotherapy.

To conclude, the various oncogenic GOF activities mediated by the mutant p53 protein greatly support tumor cell survival and can be attributed to the common cancer recurrence frequently seen following the standard therapy accepted today. In the past decades, relevant research was mostly focused on understanding the role of mutant p53 GOF in somatic cells because these cells were seen as the main target for cancer therapy. However, ample data accumulated recently indicate that CSCs residing within tumors are of great significance in conferring tumor aggressiveness. Accordingly, the role of mutant p53 in conferring CSCs drug resistance becomes a central issue in tumor recurrence at large.

Cancer stem cells

It appears that as in normal tissues, tumors show population hierarchy, whereby a subpopulation of CSCs has the most pronounced tumorigenic potential compared with the general cancer cell population (137). CSCs are characterized as rare, chemotherapy-resistant, malignant cells within the tumor, which are able to self-renew and differentiate and thus can recreate a tumor with the entire original complex cell pool when injected into immunosuppressed mice (138). Human and mouse CSCs were first isolated from hematological malignancies (139,140) and later have been identified in a wide range of solid human cancers, such as cancers of the breast (141), brain (142), pancreas (143), colon (144,145), ovaries (146–148) and other organs. To date, the accepted method for CSC isolation from tumors takes advantage of cell sorting by specific surface markers. For example, CD44 and CD133 are common for a variety of tumors; however, tissue-specific markers have also been reported (149). Other strategies for CSC isolation and enrichment include spheroid formation assay, which is based on accentuated self-renewal capacity of the CSCs (150), side-population assay that relies on the capacity of CSCs to cause the efflux of certain chemicals (151) and an assay based on selection of cells displaying high aldehyde dehydrogenase (ALDH) activity (48). Although the notion that CSCs are central for tumor development is quite accepted, exceptions exist. For example, melanoma was proposed to obey the CSC model (152); however, an in-depth analysis of a permissive *in vivo* model indicated that most of the cancerous cells are capable of initiating new tumors, thus arguing against the CSC model in this tumor type (153).

Evolvement of CSCs. One of the important unsolved issues pertains to the understanding of the evolution of CSCs. The cells of origin for CSCs are assumed to be normal SCs that underwent oncogenic genetic modifications—inherent as germ-line mutations or induced by environmental agents—that lead to cancer initiation (11,154). Another approach would suggest that unlike the rigid hierarchy between normal SCs and differentiated cells, tumors could acquire plasticity, which allows progenitor or somatic cells to undergo dedifferentiation and gain SC characteristics to become CSCs. Once these cells acquire the adaptive ability to become CSCs, tumors can exploit this capacity in order to gain drug resistance and escape cancer therapy (46,149). The first theory is supported by the observation that SCs reside in the human body for a prolonged period of time—some dormant and others constantly dividing—and this raises the probability of undergoing malignant cell transformation. In addition, SCs possess intrinsic properties of self-renewal and ability to migrate inside or outside of their organ; such dynamic cellular processes may facilitate malignant transformation. The second theory is substantiated by the fact that the incidence of SCs is rather rare, with orders of magnitude less in comparison with the incidence of differentiated cells. Thus, the

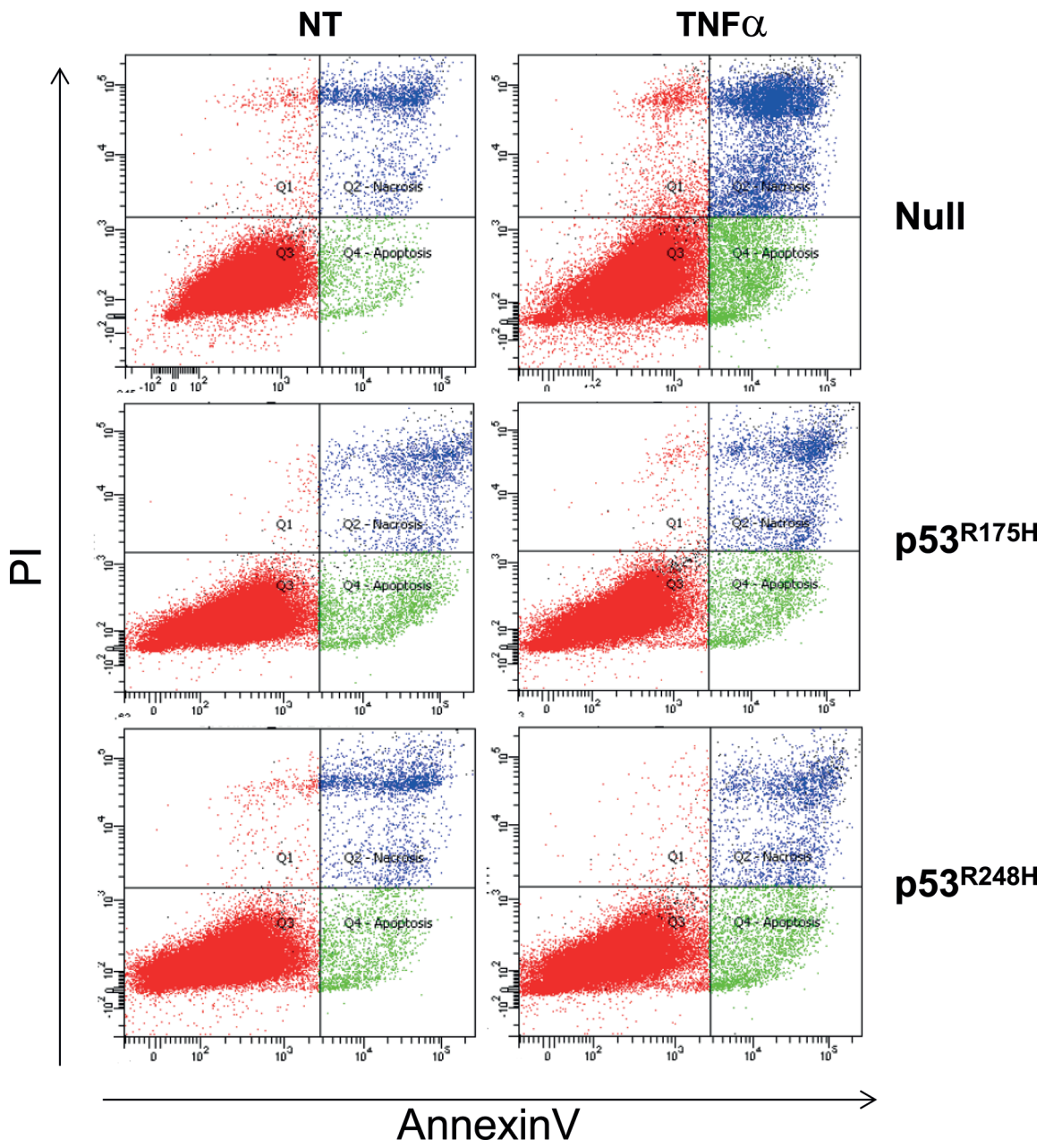

Fig. 1. Mutant p53 attenuates TNFα-induced cell death. H1299, non-small cell lung carcinoma, overexpressing mutant $p53^{R175H}$ and mutant $p53^{R248Q}$ were treated with 50 ng/ml TNFα for 72 h, followed by annexin V and propidium iodide staining. Analysis of cell death was performed by flow cytometry (fluorescence-activated cell sorting). Green dots represent the early apoptotic cells (Q4), and blue dots represent late apoptotic and necrotic cells (Q2). Cell death is presented as the sum of cells in Q2 and Q4.

occurrence of mutations in the resident SCs is at a very low frequency to affect the entire population. Furthermore, SCs were shown to have high genomic fidelity, lowering the chances of spontaneous transformation (155). Following the discovery of reprogramming, it became clear that dedifferentiation of transformed/normal somatic cells can explain the generation of CSCs. For example, ablation of respiratory SCs led luminal secretory cells to dedifferentiate into basal SCs (156), a normally occurring physiological process that, when uncontrolled, can contribute to a malignant phenotype. The state of the cell can be altered, similarly to cellular processes such as EMT and mesenchymal-to-epithelial transition, which under physiological conditions are involved in development and tissue repair (157). When these processes are out of control, they allow invasion and metastasis of cancer cells and generation of CSCs (158). In all, it seems that both SC transformation and dedifferentiation mostly depend on tumor type and context (46).

Functional perturbation in p53 leads to CSC generation. The emerging notion that mutations in p53 play a major role in CSC formation is greatly supported by the correlation between tumors with mutations in p53 and their undifferentiated phenotype. In thyroid gland carcinoma, for instance, p53 mutations were restricted to poorly differentiated tumors (159,160). Furthermore, one particular tumor showed different degrees of differentiation within regions, whereas overexpression of p53 was constrained to a less-differentiated area of the tumor (159). In addition, these studies suggested a link between mutations in p53, CSC formation and poor prognosis (159–162). Interestingly, it was shown that ESCs and undifferentiated tumors, such as breast, brain and bladder malignancies, express common specific gene signatures

and that similar transcription factors are shared among them (54). Moreover, mutations in p53 were shown to allow stem-like phenotype in breast and lung cancers (163). As mentioned above, several laboratories concomitantly suggested that WT p53 serves as a barrier in the reprogramming process by negatively regulating the rate of reprogramming (50,164). Such a regulatory activity agrees with the notion that self-renewal of SCs should be tightly controlled to attenuate the burst of accentuated excessive SC proliferation.

Accordingly, when mutant p53 knocked-in mouse embryonic fibroblasts were induced to reprogram, we found a GOF in the facilitated rate of reprogramming compared with that seen in the KO p53 mouse embryonic fibroblasts. Mutant p53 iPSCs exhibited the typical ESC markers, such as Nanog and Oct4, and underwent cell differentiation under *in vitro* conditions. Nevertheless, unlike WT p53 iPSCs that induced benign teratomas, the mutant p53 iPSCs induced the development of aggressive tumors *in vivo*. Global gene expression analysis indicated that mutant p53 iPSCs share expression patterns with pre-iPSCs (165). Interestingly, we found that p53-compromised iPSCs express gene members that are regarded as CSC markers and confer cells with drug resistance (unpublished data). This suggests that mutant p53 not only facilitates the reprogramming process but also affects the nature of the generated iPSCs. In all, p53 has control of the quality and quantity during the course of iPSC formation (50).

Interestingly, we have recently shown that heterozygous p53 cells have similar reprogramming efficiencies as WT p53, implying that WT p53 dominates over the mutant in the reprogramming process (75).

Another example that further connects the expression of mutant p53 and the malignant phenotype of CSCs is derived from glioma tumors. It was found that a p53 deletion is insufficient to make CSCs acquire their malignant phenotype. Rather, expression of mutant p53 (frequency of 26% in these tumor types) (76) is critical for the manifestation of the full malignant potential of these CSCs. In addition, this study provided an elegant proof that gliomas originate from neuronal stem cells in the subventricular zone (166). Recently, it was shown that not all neuronal stem cells are capable of initiating a neoplasm but that, specifically, the oligodendrocyte precursor cells alone could do so (167). In addition, MSCs were suggested to be the cell of origin in soft tissue sarcomas. Several mouse models have shown that MSCs lacking p53 formed malignant sarcomas (49,168–170). Furthermore, we have recently demonstrated that MSCs derived from heterozygous-mutant $p53^{R172H}$ adolescent mice that underwent p53 loss of heterozygosity do not form tumors *in vivo*, in comparison with cells derived from older mice, which induced malignant sarcoma when injected to immunodeficient mice (75). This implies that p53 loss of heterozygosity is an initiating event in the process of transforming MSCs, allowing other age-dependent perturbations to occur. Strikingly, we found that between 4 and 10% of the adherent bone marrow (BM) progenitors underwent p53 loss of heterozygosity *in vivo* in adult mice (75), suggesting that BM-derived MSCs are the origin of sarcomas. Nevertheless, Choi *et al.* (171) demonstrated that local MSCs, which reside within the tissue and not at the BM, are the cells that yield soft tissue sarcomas in an inactivated p53/Rb mouse model; yet, the lack of strictly defined markers of MSCs makes it difficult to rule out BM–tissue migration and the source of cells remains an open question (172).

Some pathways have been suggested to explain how p53, or its absence, exerts its effects on CSCs. It was shown that p53 represses the expression of CD44, which is commonly reported as a CSC marker and is involved in the metastasizing ability of CSCs (173,174). CD44 repression by p53 hampered the tumorigenic potential of CSCs in breast, lung and prostate tumors (175,176). Moreover, p53 was recently shown to repress the expression of c-KIT, another common CSC marker (146,177,178), through the p53 target miR-34a family. This downregulation resulted in reduction of sphere formation ability, chemoresistance and stemness phenotype in colorectal cancer (179). p53 was also shown to repress the expression of other SC genes Nanog and Oct4 (180). These two genes were shown to be crucial for the CSCs population in various tumors (181–183). Moreover, the repression of Nanog by p53 activation inhibits gliomagenesis *in vivo* (184,185). The EMT was recently suggested to be linked with the gain of SC properties by epithelial cells (158). p53 is known to negatively regulate the EMT process through transcriptional activation of miRs. For example, the miR-34 family targets the EMT activator Snail (186). Moreover, another p53 target, the miR-200 family, was shown to negatively regulate the expression of Zeb1 and Zeb2, EMT transcriptional activators (187,188). The attenuation of the EMT process by p53 may fulfill its role in restricting the SC pool. Finally, we have shown that WT p53 exerts a negative effect on reprogramming, which is mediated by the suppression of Klf4 that in turn suppresses mesenchymal-to-epithelial transition (189). Taken together, p53 is interwoven in the cellular circuits governing CSCs (Figure 3).

Several characteristics have been offered to describe CSCs. We have discussed the capability of CSCs to initiate new tumors; this is a crucial criterion for defining a cell as a CSC. Other characters are

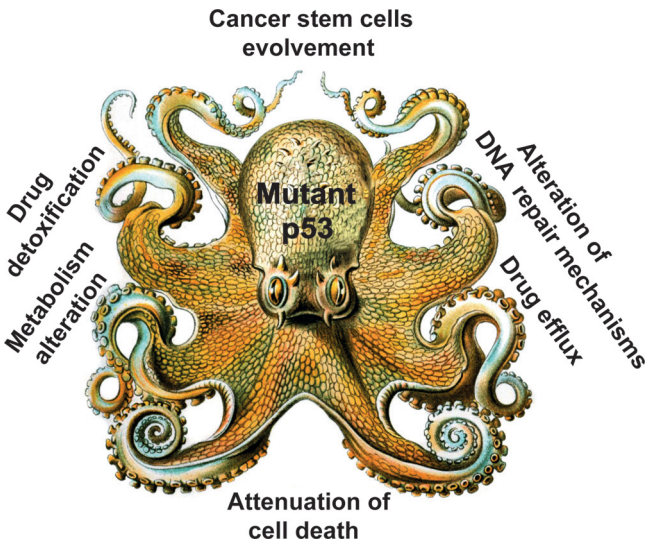

Fig. 2. Mutant p53 oncogenic activities pertaining to cell drug resistance. Mutant p53, as a 'multiarm' protein, confers cancer cells with drug resistance in several ways: enabling the evolvement of CSCs from differentiated cells and SCs and maintaining the CSC pool; elevating certain DNA repair mechanisms allowing the cells to survive; elevating expression of ABC transporters allowing efflux of drugs out of the cells; attenuating cell death by elevating the expression of antiapoptotic proteins and reducing expression of proapoptotic proteins; modulating expression of metabolic scavengers; and elevating expression of detoxifying enzymes.

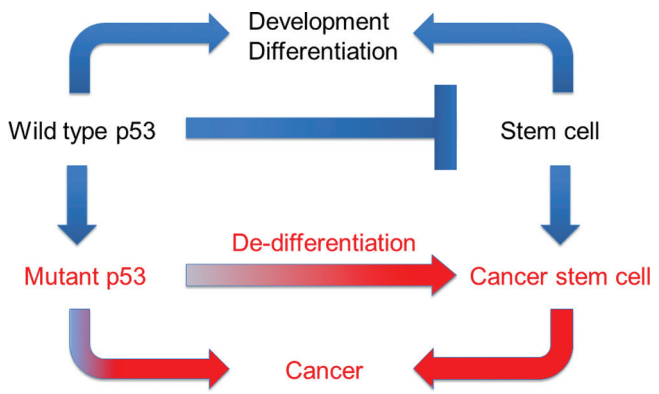

Fig. 3. The proposed p53–SCs circuit. WT p53 plays a regulatory role in the controlled development and differentiation of SCs. When p53 is mutated, it gains various oncogenic functions supporting tumorigenesis, including dedifferentiation of somatic cells into CSCs and transformation of SCs into CSCs.

heterogeneity, due to acquired changes in each cycle of the parental cell; asymmetric division, maintaining the CSC pool while contributing to the tumor bulk; quiescence, despite being transformed, it is believed that, to some extent, they remain in a slow cycling state and thus are resistant to agents that affect proliferating cells (190). However, the most potent hallmark of CSCs on cancer progression and relapse is drug resistance.

Drug resistance mechanisms of CSCs

It is well accepted that chemoresistance of cancer cells can be divided into two major categories: *de novo* and acquired chemoresistance (191). *De novo* chemoresistance is defined as the preexisting ability of cancer cells to resist chemotherapy, whereas acquired chemoresistance is defined as acquisition of drug resistance, which arises during chemotherapy treatment. The latter develops due to drug-induced selection pressure leading to clonal expansion based on survival advantage. There are several mechanisms that allow cancer cells to elude chemotherapy. This includes limiting drug influx, excessive drug efflux, alterations in apoptosis and survival signaling pathways, expression of detoxification enzymes and alterations in DNA repair mechanisms.

Drug efflux. The adenosine triphosphate (ATP)-binding cassette (ABC) transporter family is the most notable group executing the function of expelling anticancer drugs across the plasma membrane. There are three central members that have been extensively studied in relation to multidrug resistancein cancer: ABCB1 (multidrug resistance, MDR1), ABCC1 (MRP1) and ABCG2 (BCRP), which were shown to act on a broad range of conventional chemotherapy drugs (192,193) and to account for chemotherapy failure in various cancers (193,194). This phenotype of drug resistance is attributed to the CSC population that is contained within growing tumors (195). The ability of CSCs to expel drugs enabled their isolation in a method termed side population sorting method (151), which relies on the observation that somatic cancer cells, when stained, retain the dyes; however, CSCs expel the dyes, which is mediated by the ABC transporter proteins. In many primary tumors and cell lines, including breast cancer, lung cancer and brain tumors, side populations were detected (151). The expression of ABC transporters in CSCs is another trait shared with normal SCs. For example, hematopoietic stem cells express high levels of ABCG2 and/or ABCB1, in contrast to further differentiated cells of the hematopoietic system (195).

Drug detoxification. ALDH enzymes are also thought to be involved in chemoresistance of cancer cells. These proteins are members of the nicotinamide adenine dinucleotide (phosphate) -dependent enzymes that have a role in detoxifying a broad variety of endogenous aldehydes and xenobiotic aldehydes by oxidizing and converting them into carboxylic acids (196). Indeed, studies have shown that cells highly expressing ALDH genes, especially ALDH1A1 and ALDH3A1, exert drug resistance (197–199), whereas inhibition of activity of the ALDH enzymes leads to effective chemotherapy (200,201). However, the exact mode of action underlying this pathway is yet to be elucidated. High ALDH1 activity was found in several types of cancers, including head-and-neck, lung, liver, pancreas, cervix, ovaries, breast, prostate, colon and bladder cancers (202). Because ALDH is also expressed at variable levels in normal ASCs, it has been suggested to be a reliable marker for CSCs only in tissues that harbor ASCs expressing limited ALDH levels, such as breast, lung and colon tissues, and not in liver and pancreatic tissues wherein the residing ASCs express high ALDH levels (203).

Alterations in metabolism. In addition, cancer cells were shown to have the ability to inactivate different drugs, e.g. platinum drugs such as cisplatin and oxaliplatin, by covalently conjugating them with the thiol glutathione (GSH). The generated complex is a substrate for ABC transporter protein, resulting in inactivation of the drug (204). Accordingly, GSH was shown to be highly expressed in various cancers, providing them with chemoresistance ability (204–208). GSH was shown to be a critical cellular reducing agent and antioxidant that is responsible for reducing ROS levels. ROS are found at high levels in many cancer cells, contributing to the vicious cycle of aggravating damage to the DNA and other parts of the cell (209). In normal SCs, such as hematopoietic stem cells and mammary SCs, ROS are found at low levels, mainly due to elimination by scavengers such as GSH (210,211). Interestingly, it was shown that CSCs share a ROS-level phenotype similar to that in their normal SC counterparts. Several studies have shown that the CSC population contains low levels of ROS and higher capacity to synthesize ROS-scavenging molecules compared with somatic cancer cells. These low levels of ROS were shown to confer CSCs with resistance to radiotherapy (211).

DNA repair mechanisms. The ability of cancer cells to repair DNA damage significantly affects their response to chemotherapy. Several studies support the notion that alterations in DNA repair mechanisms confer chemoresistance to cancer cells. For example, excision repair cross-complementing 1, a crucial component of the nucleotide-excision repair pathway, was shown to be elevated in various tumors, thereby increasing chemoresistance of several cancer types, including non-small cell lung carcinoma and ovarian, colorectal and gastric cancers (212–216). However, mismatch repair deficiency has also been implicated in chemoresistance in a variety of cancers (217–219). This is due to the involvement of mismatch repair proteins in mediating cell cycle arrest and apoptosis in response to DNA damage (220,221). Interestingly, in CSCs contained within a given tumor, the DNA damage response is tilted toward enhanced DNA repair, as opposed to the situation in somatic cancer cells. For instance, glioma SCs expressing CD133 were shown to be resistant to γ-irradiation by elevation of the checkpoint activation in response to DNA damage. The phosphorylation of ataxia telangiectasia mutated, Rad17, Chk1 and Chk2 was higher in $CD133^+$ cells compared with the same in $CD133^-$ cells. Moreover, alkaline comet assay showed greater DNA repair efficiency in $CD133^+$ cells compared with that in $CD133^-$ cells (222). Elevated phosphorylation of Chk1 was also observed in colon and lung CSCs in response to chemotherapy (223,224). In addition, MCF-7-derived CSCs showed higher activation of the DNA single-strand break repair mechanism compared with the mechanism in the parental cells. This higher single-strand break repair activation was indicated by higher expression of single-strand break repair-associated protein APE1 (225).

Alterations in apoptosis and survival signaling pathways. Cancer therapy is aimed at the eradication of cancer cells, thereby challenging a central cancer hallmark, resistance to cell death (1). Indeed, cancer cells have developed multiple mechanisms to prevent cell death, e.g. regulation of the expression of Bcl-2 family members either by inducing antiapoptotic regulators including Bcl-2 and Bcl-x_L or by downregulating proapoptotic regulators such as Bax, Bim and Puma. Another important player in acquiring drug resistance is NF-κB, which promotes cell survival and exerts resistance to chemotherapy (194,226). In all, the alterations in cell death and survival signaling pathways mentioned above may hinder chemotherapy. Similarly, in glioma SCs, antiapoptotic Bcl-2 and Bcl-x_L, in addition to the inhibitor of apoptosis (IAP) family members X-linked inhibitor of apoptosis, cIAP1, cIAP2, neuronal apoptosis inhibitor protein, and survivin, were found at significantly higher levels in CD133-positive drug-resistant cells in contrast to their counterparts (227). In both colon (228) and hepatic CSCs (229), Bcl-2 contributes to chemoresistance, in addition to preferential activation of Akt/protein kinase B, in the CSCs of the liver (229), which is absent the activation of Akt/protein kinase B is absent in somatic cancer cells.

In all, CSCs adopt a variety of pathways to escape therapy. Indeed, much of the research made before the CSC hypothesis can now be explained in retrospect and can be shown to be attributable to the small CSC population.

Cross talk of mutant p53 and CSCs underlying drug resistance

Mutant p53 GOF. The fact that mutant p53 is frequently expressed in a variety of human tumors makes it an important target for cancer therapy (230). Mutant p53, the well-characterized genomic guardian, is known for its oncogenic GOF. Depending on the tumor-type specificity, p53 can be associated with various steps along the process

of malignant transformation. Mutant p53 was shown to modify the cell cycle checkpoints, accelerating proliferation, conferring genomic instability, affecting cell plasticity and inducing invasiveness and metastasis, in addition to being known for its non-cell autonomous affects, such as inflammation and angiogenesis (78,231). Mutant p53 acts as a multitask protein that simultaneously affects a number of pivotal pathways, all of which culminate in the acquisition of resistance to chemotherapeutic drugs (Figure 2). This feature of mutant p53 has long been known in the field of oncology, yet no approved therapy targeting mutant p53 in the Western world is available to date (232).

The p53 and SC connection. The initial observations that WT p53 plays a role in cell differentiation and development paved the way toward the understanding that WT p53, a cell cycle controller, plays an important role in restraining the normal repertoire of SCs. In recent experiments, taking advantage of cell reprogramming, it was shown that p53 acts as a barrier to the reprogramming of differentiated cells into the pluripotent state (50,59,61–63,233). This is in agreement with the notion that the reprogramming process shares some resemblance to malignant transformation. Furthermore, data derived from a variety of experimental models suggested that expression of mutant p53 in SCs might lead the way toward the evolvement of CSCs (166,169).

Mutant p53-CSCs share gene pathways. Of note, mutant p53 seems to affect specific pathways, which are central to the drug-resistant capacity of CSCs (Figure 2). For example, ABC transporters that are an important machinery in acquiring drug resistance by exporting drugs out of cells are often expressed in CSCs. Interestingly, MDR1, an important member of the ABC family, was shown to be upregulated by mutant p53 (128). Moreover, mutant p53 was shown not only to lose the capacity of WT p53 to induce apoptosis but also to gain function in augmenting the expression of antiapoptosis proteins (Bcl-2 and Bcl-x_L) and in reducing proapoptosis signals (Bax, Bad and Bid). Similarly to mutant p53, CSCs modify the Bcl-2 family to attenuate drug-induced death. WT p53 plays a major role in DNA repair mechanisms, such as nucleotide-excision repair, base-excision repair, mismatch repair, homologous recombination and non-homologous end joining (234). These repair mechanisms are impaired in somatic cancer cells; however, recently, we have found that murine p53-mutant-expressing MSCs that form malignant sarcomas exhibited elevated homologous recombination and non-homologous end-joining genes (75). As mentioned above, CSCs were shown in various models to express high levels of DNA-repair-related genes and to efficiently repair DNA damage, compared with somatic cancer cells.

Despite these similarities, not many studies of the role of p53 in drug resistance of CSCs were performed. It was shown that in colorectal cancer cells, attenuation of the SC marker c-Kit by the p53 target miR-34a sensitizes the cells to 5-fluorouracil (179). In addition, the anticancer phytochemical resveratrol reduces the tumor-initiating capacity of

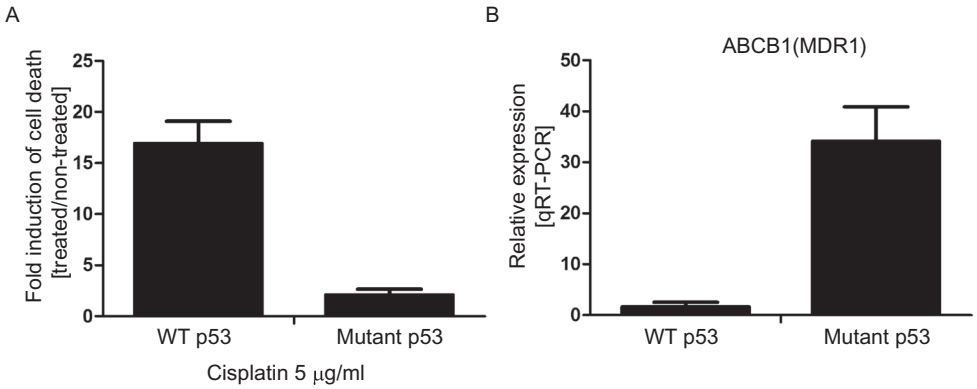

Fig. 4. Mutant p53-expressing MSCs exhibit resistance to cisplatin and express high levels of MDR1. MSCs were extracted from the BM of WT p53- and mutant p53-containing mice. (**A**) Cells were treated with cisplatin (5 μg/μl) for 24 h, followed by propidium iodide (PI) staining. Cell death was assessed according to PI exclusion by flow cytometry (fluorescence-activated cell sorting). (**B**) Relative mRNA expression of ABCB1A (MDR1) in WT p53- and mutant p53-containing MSCs, as measured by quantitative reverse transcriptase–polymerase chain reaction.

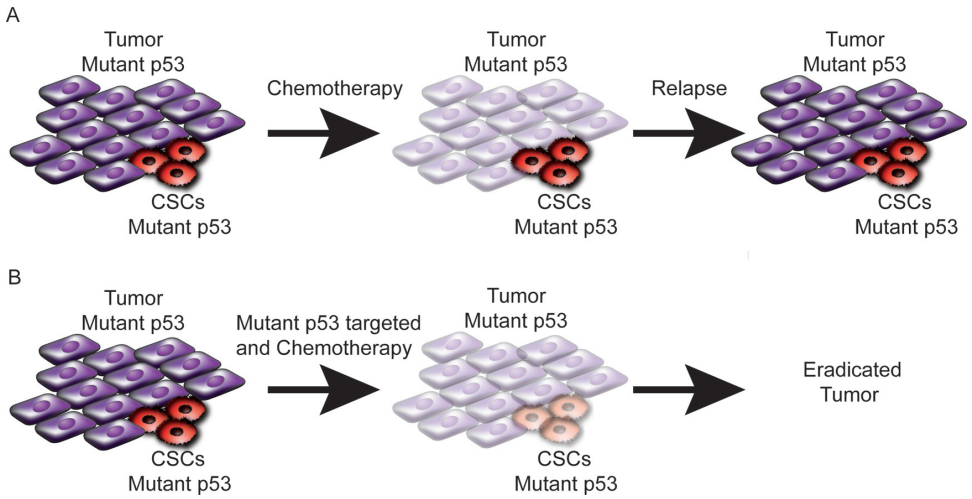

Fig. 5. Suggested model for combining mutant p53-targeted cancer therapy and conventional chemotherapy. (**A**) Tumor expressing mutant p53 when treated with chemotherapy will show regression due to elimination of the bulk tumor cells. However, the CSC compartment is resistant to chemotherapy-induced death, thus allowing tumor relapse. (**B**) Treatment with mutant p53-targeted therapy will convert the mutated p53 into intact p53 and sensitize CSCs to chemotherapy. Hence, both the bulk tumor cells and the CSCs will be eliminated and full eradication is expected.

glioma SCs by promoting the degradation of Nanog in a p53-dependent manner (184). A similar phenotype involving stemness-attenuating features was observed in the CSCs of nasopharyngeal carcinoma (235). Recently, it was shown that only in the absence of p53, colon CSCs are resistant to paclitaxel due to higher levels of autophagy and lower levels of apoptosis (236). All of these studies emphasize that much of the CSC resistance to chemotherapy is evident in conjunction with a cellular compromised-p53 status. In our recent studies, we found that iPSCs that express the mutant p53 and induce aggressive tumors in mice were found to highly express detoxifying enzymes associated with drug resistance. Furthermore, MSCs expressing mutant p53, which form aggressive tumors, exhibited drug resistance to cisplatin that correlated with the expression of MDR1, a central gene in acquiring drug resistance (Figure 4, unpublished data). This indicates that mutant p53 expression is important in inducing iPSCs and MSCs to acquire a transformed phenotype and drug resistance.

Therapeutic approach. In all, the notion that p53 plays a regulatory role in the life of SCs, coupled with the observations that p53 mutations may contribute to the evolvement of CSCs, makes it challenging to speculate that drug resistance and cancer recurrence are mediated by CSCs that express mutant p53. Accordingly, it may suggest that efficient cancer therapy in mutant p53-expressing tumors should be based on a combination of chemotherapy and a p53-based therapy. The chemotherapy will target the tumor bulk, whereas only the conversion of mutant p53 protein into WT p53 form will allow CSC eradication (Figure 5). We speculate that reverting mutant p53 in CSCs will render them sensitive to chemotherapy.

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Oncogenic Mutant p53 Gain of Function Nourishes the Vicious Cycle of Tumor Development and Cancer Stem-Cell Formation

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More than half of human tumors harbor an inactivated p53 tumor-suppressor gene. It is well accepted that mutant p53 shows an oncogenic gain-of-function (GOF) activity that facilitates the transformed phenotype of cancer cells. In addition, a growing body of evidence supports the notion that cancer stem cells comprise a seminal constituent in the initiation and progression of cancer development. Here, we elaborate on the mutant p53 oncogenic GOF leading toward the acquisition of a transformed phenotype, as well as placing mutant p53 as a major component in the establishment of cancer stem cell entity. Therefore, therapy targeted toward cancer stem cells harboring mutant p53 is expected to pave the way to eradicate tumor growth and recurrence.

During the past three and a half decades of p53 research, ample data have accumulated pertaining to the role of p53 in the regulation of various cellular processes and in preventing cancer development (Levine and Oren 2009; Bieging et al. 2014). Hence, the wild-type p53 (WT-p53) protein is considered the guardian of the genome (Lane 1992) and a key regulator of homeostasis (Vousden and Lane 2007) that exerts its activities both at the cell-autonomous and -nonautonomous levels (Lujambio et al. 2013). In fact, mutations in the p53 (mut-p53) gene are found in more than half of human cancer cases (Sigal and Rotter 2000; Brosh and Rotter 2009). It was widely shown that mutations in p53 result in loss of its tumor-suppressive function. Importantly, not only does mut-p53 interfere with the remaining WT-p53 protein via dominant negative mechanism, but also mutations empower p53 with oncogenic gain-of-function (GOF) effects that endow cells with tumorigenic potential (Brosh and Rotter 2009; Muller and Vousden 2014). This unique feature of mut-p53 in facilitation of malignant transformation indicates that p53 may be regarded as a proto-oncogene tumor suppressor.

The mutant p53 GOF characteristics and mechanisms of action have been broadly described (Brosh and Rotter 2009; Muller and Vousden 2014). Here, we will provide an update on the latest developments in this field and address the growing understanding of the complexity and heterogeneity of tumors, zooming in on the subpopulation of cancer stem cells (CSCs) within the tumors. Finally, we will discuss how mut-p53's unique features set it as a

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prime candidate for therapeutic targeting and how this relates to the latest developments in mut-p53 targeted therapy.

THE EVOLUTION OF THE mut-p53 GOF CONCEPT

Remarkably, one of the earliest observations in the field was that mut-p53 promotes cellular malignant transformation (Eliyahu et al. 1984; Jenkins et al. 1984; Parada et al. 1984; Wolf et al. 1984). This initial experimental evidence was obtained by using p53 cDNAs that originated from transformed cells that incorporated a mutant form of p53 (Wolf et al. 1984; Halevy et al. 1990; Shaulsky et al. 1991). Yet, these first reports did not consider the fact that cDNA may represent WT-p53 or mut-p53 sequences. The notion that a missense mutation in p53 confers oncogenic GOF that enhances tumorigenic potential was formally introduced several years later by Dittmer and colleagues: Overexpressed mut-p53 in cells lacking endogenous p53 expression resulted in malignant transformation of these cells both in vitro and in vivo (Dittmer et al. 1993). This was further confirmed by analysis of several experimental cellular models using either knockdown or overexpression of mut-p53. However, the final clincher in the establishment of the mut-p53 GOF concept was obtained through studies of transgenic mice expressing endogenous mut-p53. Although both mut-p53 and null-p53 mice showed facilitated tumor development, mice harboring mut-p53 displayed significantly higher incidence of metastasis (Lang et al. 2004; Olive et al. 2004). Further analysis of the aforementioned p53 models uncovered another important feature of the GOF mechanism of action—the requirement of mut-p53 to accumulate to exert its oncogenic function. Apparently, in normal mouse tissue, under physiological conditions, mut-p53, similarly to WT-p53, is maintained at low levels. However, on cellular insults, such as oncogene activation, DNA damage, or high reactive oxygen species (ROS) levels, mut-p53 undergoes constitutive stabilization and accumulates in the cell. In contrast to the WT-p53 protein, once mut-p53 is stabilized, it does not undergo degradation, mainly because of the inability of MDM2, a negative regulator of WT-p53, to polyubiquitinate mut-p53 (Frum and Grossman 2014). Indeed, augmented levels of p53 protein, manifested by mut-p53 accumulation in tumors and tumor lines, were suggested to serve as a clinical marker >30 years ago (Rotter 1983; Soussi and Beroud 2001).

Many tumors have shown an apparent dependency on specific gene expression to retain the malignant phenotype (Weinstein and Joe 2008). This phenomenon is referred as the Weinstein hypothesis of "oncogene addiction." Recently, by establishment of an elegant, novel system of mice harboring mut-p53, Alexandrova et al. (2015), showed that ablation of mut-p53 substantially diminished tumor burden, suggesting that mut-p53 stabilization confers tumor cells with an oncogenic addiction. This finding suggests that it might be sufficient to ablate the mutant p53 protein to obtain the required therapeutic outcome.

DIFFERENT p53 MUTATIONS SHOW VARIATIONS IN THEIR GOF

Approximately 2000 different mutations in the p53 gene were detected in sporadic tumors (Petitjean et al. 2007). Yet, only a small subset of missense mutations in the DNA-binding domain of p53 is modified frequently. These mutations, which lead to the formation of full-length protein, are referred as "hot-spot" mutations. They can be categorized into two groups: the conformational mutations such as R175H, G245S, R249S, and R282W and the DNA-contact mutations represented by R248Q and R273H (Fig. 1) (Petitjean et al. 2007). In addition to the occurrence of p53 mutations in somatic cells, germline mutations are associated with the rare familial cancer predisposition termed Li–Fraumeni syndrome (LFS). The LFS patients develop early onset of a wide spectrum of tumors (Malkin et al. 1990). These patients are heterozygous for mut-p53 (WT/mut-p53) in every cell of their body. Initial analysis of tumors derived from LFS patients showed that ~60% of tumors lost the remaining WT-p53 allele in a process termed loss of heterozygosity (LOH)

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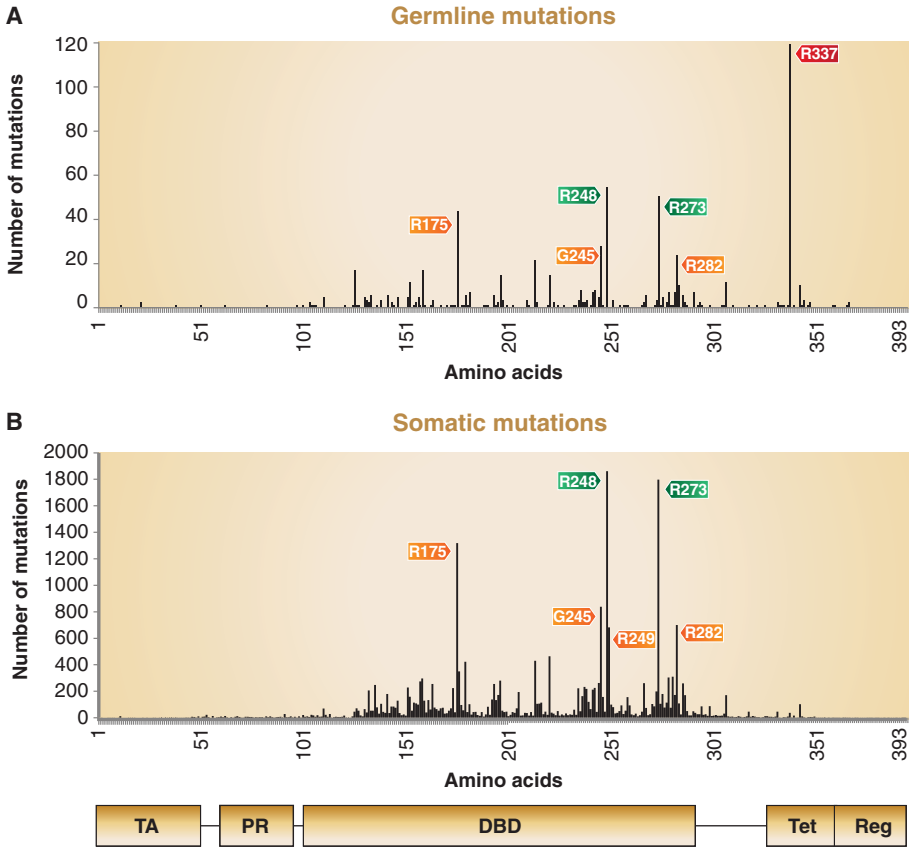

Figure 1. Distribution of somatic and germline p53 mutations. The distribution of reported missense mutations across 393 amino acids of the p53 protein. (*A*) The six most frequent "hot-spot" mutations detected in tumors obtained from Li–Fraumeni syndrome (LFS) patients ($N = 636$). (*B*) The six most frequent hot-spot mutations occurring in sporadic tumors ($N = 24{,}210$). The domain architecture of p53 is aligned below. Hot-spot mutations are highlighted in orange for conformational mutations, in green for DNA-contact mutations, and in red for tetramization mutation. Notably, five of the six hot-spot mutations are shared between somatic and germline mutations, indicating that they confer properties that contribute to their selection. R249 is unique to somatic mutations because of the mutagenic effect of aflatoxin B1 that is associated with food contamination. In contrast, R337 is unique to germline mutations because of a founder effect of a single source to its progeny. TA, Transactivation domain; PR, proline-rich domain; DBD, DNA-contact domain; Tet, tetramization domain; Reg, carboxy-terminal regulatory domain. (Data derived from the IARC TP53 mutation database, version R17, November 2013.)

(Varley et al. 1997). Interestingly, a recent study that performed p53-based genomic and transcriptomic meta-analyses using data from the Cancer Genome Atlas estimated that >93% of sporadic tumors with mut-p53 undergo p53 LOH (Parikh et al. 2014). These findings support the concept that p53 is a recessive tumor suppressor and loss of the remaining WT allele is required for tumor development.

Notably, five of the six hot-spot mutations, R175H, G245S, R248Q, R273H, and R282W, are shared between sporadic tumors and tumors obtained from LFS patients. This observation may suggest that the GOF effect of these p53 mutants predominantly contributes to their selection during tumorigenesis. In contrast, the conformational hot-spot p53 mutation R249S that is often induced by aflatoxin B1 was pref-

erentially found in somatic liver tumors (Aguilar et al. 1993). As this mutation is induced by food contamination, an environmental factor, it is rarely observed in germline-derived tumors. On the other hand, the R337H-specific germline mutation associated with multiple cancers of the LFS spectrum in the population of Southern Brazil is a result of a founder effect (Fig. 1) (see Garritano et al. 2010; Achatz and Zambetti 2016).

The effect and the magnitude of oncogenic GOF acquired by the different p53 mutants is a function of the mutation site, the type of nucleotide substitution, and the specific cell type, thus making the relationship between genotype and phenotype extremely complex (Freed-Pastor and Prives 2012; Bisio et al. 2014; Xu et al. 2014). Accordingly, we were able to show that, in human fibroblasts, various hot-spot p53 mutants cooperate in different ways with constitutively active H-RAS to promote cellular transformation. More specifically, the conformational mutants (R175H and H179R) induced a unique pattern of a cancer-related gene signature by elevating H-RAS activity through perturbation of BTG2, whereas DNA-contact mutants (R248Q and R273H) prompted cancer-related gene expression by cooperating with NF-κB. Notably, the L3 loop region conformational mutant G245S did not show an oncogenic GOF effect in this system (Solomon et al. 2012). In contrast to the latter, a recent study showed an oncogenic GOF effect of the G245S p53 mutation in osteosarcomas that were developed following reprogramming of fibroblasts obtained from LFS patients into induced pluripotent stem cells (iPSCs) that were further differentiated into osteoblasts. The mechanism of this GOF was manifested by suppressing the expression of the imprinted gene H19 during osteogenesis (Lee et al. 2015). Another interesting example of the variation in the p53 GOF effect resulting from different substitutions of a single amino acid at the same location of the p53 gene is represented in humanized p53 knockin (HUPKI) mouse models. Mice carrying the R248W mutation showed GOF in a broader spectrum of tumor types as well as more metastasis compared with p53-null mice, with no difference in their life span (Song et al. 2007), whereas mice harboring the R248Q mutation showed earlier tumor onset and shorter survival compared with their p53-null counterparts, thus showing significantly stronger GOF (Hanel et al. 2013).

MOLECULAR MECHANISMS UNDERLYING mut-p53 GOF

WT-p53 functions as a transcription factor (TF) that exerts the transactivation of its target genes via direct binding to its specific responsive elements entailed within the target gene loci (Raycroft et al. 1990; el-Deiry et al. 1993). In contrast, the majority of mut-p53 proteins lose the WT-p53 transactivation capacity because of alterations in their DNA-binding domain or conformation state that prevents their binding to the canonical responsive element of WT-p53. Therefore, mut-p53 function is often conveyed through protein–protein interactions (Brosh and Rotter 2009; Oren and Rotter 2010). One of the well-studied mechanisms underlying mut-p53 GOF effects can be attributed to mut-p53-dependent inactivation of its family members' p63 and p73 (Irwin 2004; Lunghi et al. 2009). In addition, it has been shown that the GOF effects of mut-p53 may be mediated by its interaction with numerous TFs, including SP1, NF-Y, VDR, SREBP1, Twist1, E2F family, and the ETS family, which serve also as WT-p53-interacting partners (Fig. 2) (Brosh and Rotter 2009; Menendez et al. 2009). Importantly, however, their cooperation with either WT- or mut-p53 usually leads to an opposite cell-fate outcome. For example, Di Agostino and colleagues have shown that mut-p53 interacts with the TF NF-Y to recruit histone acetyltransferase (p300) instead of the histone deacetylases (HDACs) commonly used by WT-p53 (Di Agostino et al. 2006; Oren and Rotter 2010). The mut-p53/NF-Y protein complex provokes aberrant transactivation of NF-Y target genes that eventually induces facilitated proliferation (Di Agostino et al. 2006). This is a perfect example emphasizing a mechanism frequently underlying the mut-p53 GOF effect, in which the WT-p53-interacting partner is "hijacked" by mut-p53 and used to support transformation by altering the epigenetic response.

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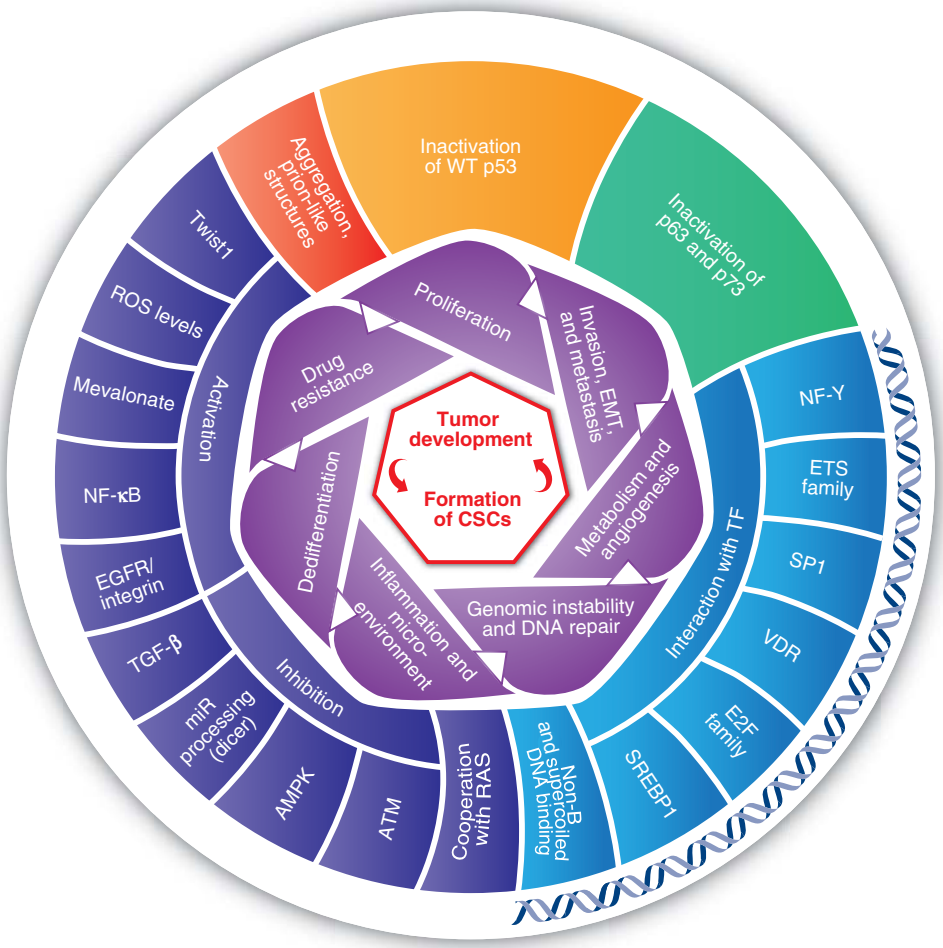

Figure 2. Selected phenotypes and mechanisms underlying oncogenic mut-p53 GOF (gain of function). The *inner* level (purple) represents the oncogenic GOF phenotypes associated with mut-p53. The *outer* level illustrates the pivotal mechanisms of mut-p53 GOF. Importantly, each phenotype can be associated with several underlying mechanisms. Mechanisms of mut-p53 interactions with DNA are depicted near a double-strand DNA. Because cancer stem cells (CSCs) are suggested to be the cell of origin of many human cancers, their formation constitutes the cornerstone of mut-p53 GOF in tumor initiation and progression capabilities. AMPK, 5′ AMP-activated protein kinase; ATM, ataxia telangiectasia mutated; EGFR, epidermal growth factor receptor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen species; SREBP-1, sterol regulatory element-binding protein; TGF-β, transforming growth factor β; VDR, vitamin D receptor.

Another facet associated with the understanding of mut-p53 GOF is related to microRNAs (miRs) (Donzelli et al. 2014), in which mut-p53 was found to affect Dicer, a pivotal regulator and processer of miRs (Muller et al. 2014) and noncoding RNAs, such as H19 (Lee et al. 2015). Furthermore, mut-p53 can bind to non-B DNA structure and supercoiled DNA with high affinity (Gohler et al. 2005; Brazdova et al. 2013) and thus might affect transcription by binding to DNA motifs. Finally, mut-p53 was shown to aggregate and to form prion-like structures (Ano Bom et al. 2012; Rangel et al. 2014). The importance of this phenomenon re-

quires further investigation to determine how and whether it confers cancer-related properties. Yet, it opens new horizons in the continuously evolving p53 field.

mut-p53 DEREGULATES CELL CYCLE AND ENHANCES PROLIFERATION

WT-p53 is a pivotal cell-cycle regulator. Indeed, it was shown that WT-p53 regulates the G_1/S checkpoint, by activating specific target genes, mainly *CDKN1A* (p21Waf1) and *GADD45α* (Kastan et al. 1992; el-Deiry et al. 1993). Accordingly, loss of WT-p53 activity abrogates the normal cell-cycle control that may lead to facilitated proliferation, a known cancer hallmark (Hanahan and Weinberg 2011). In an effort to unravel the basis for the mut-p53 oncogenic GOF, it became evident that mut-p53 disrupts the normal cell-cycle pattern. This appears to be the most prominent feature of mut-p53 GOF that constitutes a common denominator across the mutation spectrum (Brosh and Rotter 2010). Therefore, it is not surprising that mutations in p53 coincide with the Ki-67 proliferation marker (Olivier et al. 2005). Yet, although Ki-67 is only a single marker, it was shown that many of mut-p53 isoforms affect a cluster of cell-cycle-associated genes that has been termed "core proliferation signature." This set of genes is involved in DNA replication, spindle assembly and checkpoint, chromosome segregation, and mitotic processes (Whitfield et al. 2006). Several mechanisms underlying the molecular basis of mut-p53 GOF regarding enhanced proliferation were suggested. The transforming growth factor β (TGF-β), a key regulator of proliferation control, was proposed as an important candidate contributing to mut-p53 oncogenic GOF. In fact, TGF-β has a dual effect on cancer progression. While in early stages of cancer, TGF-β serves as an antiproliferation barrier in epithelial cells through interaction with the Smad pathway; at advanced stages, it promotes invasion and metastasis by enhancing proteolytic activity and the expression of cell-adhesion molecules (Blobe et al. 2000). In our previous studies, we found that mut-p53 reduces the expression of TGF-β receptor type II, thereby hampering the TGF-β/Smad pathway (Kalo et al. 2007). In contrast, Adorno and colleagues showed that mut-p53 increased TGF-β-mediated invasiveness and metastasis ability, by counteracting p63/Smad complex assembly (Adorno et al. 2009). This apparent discrepancy in the effects of mut-p53 on TGF-β action can be explained by the timing of p53 mutation appearance during the course of tumor development. In the event p53 mutation occurs early in tumorigenesis, when TGF-β serves as antiproliferation barrier, mut-p53 provides cells with clonal advantage via perturbing TGF-β function. However, if p53 mutation takes place in later stages of tumor progression, when TGF-β does not attenuate the cell cycle, cooperation between mut-p53 and TGF-β will induce invasiveness and metastasis. Recently, another molecular mechanism pertaining mut-p53 GOF was uncovered. Regγ is a proteasome 20S inducer that upon activation enhances proliferation via degradation of cell-cycle inhibitors such as p21Waf1, p16, and WT-p53. Regγ itself is a target of WT-p53 and TGF-β signaling. mut-p53 was shown to prevent TGF-β-mediated response by inhibiting Smad3 recruitment to the Regγ promoter in a GOF manner permitting the interaction with p300 to induce proliferation and drug resistance (Ali et al. 2013; Wang et al. 2015). Similarly, mut-p53 was shown to induce histone acetylation on the Axl promoter, a tyrosine kinase receptor that is involved in the stimulation of cell proliferation. Thus, these signals lead to a higher proliferation rate and increased motility (Vaughan et al. 2012b). In all, mut-p53 not only loses its normal role as cell-cycle regulator but also gains proliferative activities that facilitate tumorigenesis.

mut-p53 FACILITATES INVASION, EPITHELIAL–MESENCHYMAL TRANSITION, AND METASTASIS

The ability of the cancer cells to invade and metastasize is one of the typical hallmarks of malignant transformation. The plasticity of cancer cells permits them to undergo epithelial–mesenchymal transition (EMT) and thereby gain mesenchymal properties required to in-

vade the surrounding tissue and metastasize. A set of TFs, including Snail, Slug, Twist1, and Zeb1/2, orchestrates the EMT and related migratory processes during embryogenesis and tumorigenesis (Singh and Settleman 2010). Several reports indicated that mut-p53 shows an oncogenic GOF by promoting the EMT process in various cancer cells by enhancing the expression of EMT inducers. In agreement with this notion, we have shown that, in prostate cells, mut-p53 up-regulates a key activator of EMT, Twist1, via reduction of BMI-1-mediated methylation of the Twist1 promoter. This resulted in higher expression of mesenchymal markers, lower expression of epithelial markers, and enhanced invasive properties in vitro (Kogan-Sakin et al. 2011). Additionally, it was shown that mut-p53 induces EMT through miR-130b, a negative regulator of Zeb1 (Dong et al. 2013). An alternative mechanism used by mut-p53 GOF to promote EMT is attenuation of EMT suppressors. Indeed, Ali et al. (2013) showed that, in metastatic breast cancer cells, mut-p53 suppresses the expression of Klf17, a negative regulator of metastasis and EMT, leading to EMT-associated gene transcription and enhanced cancer progression.

Furthermore, mut-p53 enhances invasiveness and metastasis by exerting an oncogenic GOF involving the constitutive activation of EGFR/integrin signaling by inhibition of p63 (Adorno et al. 2009; Muller et al. 2009), as well as by up-regulation of Pla2g16 (Xiong et al. 2014). Additional mechanisms suggested for this phenomenon are mut-p53-mediated down-regulation of Dicer through p63-dependent and -independent means (Muller et al. 2014), inhibition of PDGFRβ by blocking the p73/NF-Y complex (Weissmueller et al. 2014), and interactions with NRG1 and Pin1 (Girardini et al. 2011; Coffill et al. 2012).

mut-p53 AFFECTS TUMOR STROMA AND PROMOTES CHRONIC INFLAMMATION

A tumor is a complex tissue composed of proliferating cancerous cells that reside in a rich microenvironment provided by resident fibroblasts and additional various nonmalignant cell types. This tumor microenvironment was shown to contribute substantially to the malignant process (Hanahan and Weinberg 2011). We have shown an interesting cross talk between the tumor and its microenvironment with respect to mut-p53. Lung carcinoma cells affect their co-cultured cancer-associated fibroblasts to secrete IFN-β, which normally prevents cancer cell migration. Notably, mut-p53-expressing cancer cells attenuate this response via SOCS1-mediated inhibition of STAT1 phosphorylation in a negative feedback loop. Remarkably, IFN-β reduces mut-p53 RNA levels by restricting WIG1, suggesting that patients with mut-p53 might benefit from IFN-β therapy (Madar et al. 2013). Mutations in p53 were also reported in tumor stroma of breast cancer patients (Patocs et al. 2007). Albeit there is some controversy as to their prevalence (Campbell et al. 2008; Roukos 2008; Zander and Soussi 2008), it was shown that mut-p53-expressing stromal cells promote tumorigenesis in prostate cancer cells better than their p53-null counterparts (Addadi et al. 2010).

Chronic inflammation has a role in tumorigenesis by creating a vicious cycle between the tumor and its microenvironment. Indeed, inflammation was recognized as one of the hallmarks of cancer (Hanahan and Weinberg 2011). One of the canonical pathways of inflammatory response is the tumor necrosis factor (TNF)-α/NF-κB pathway (Lawrence 2009). The role of NF-κB in cancer is complex; on the one hand, in some models NF-κB activation blocks tumor development, whereas in others it inhibits apoptosis and favors cell proliferation (Pikarsky and Ben-Neriah 2006). Activation of NF-κB by TNF-α can drive cancer progression in the context of chronic inflammation (Pikarsky et al. 2004). Several studies linked mut-p53 and chronic inflammation (Cooks et al. 2014). We have reported that, in cancer cells, mut-p53 induces NF-κB in response to TNF-α treatment. Accordingly, down-regulation of mut-p53 sensitized cancer cells to the apoptotic effects of TNF-α (Weisz et al. 2007). Recently, it was shown that mut-p53-mediated NF-κB activation by TNF-α is based on mut-p53-dependent

inhibition of the tumor suppressor DAB2IP in the cytoplasm (Di Minin et al. 2014). Another study, aiming to uncover the role of mut-p53 in inflammation-associated tumors, showed that mice heterozygous for mut-p53 (WT/mut-$p53^{R172H}$), but not hemizygous mice (WT/null-p53), are prone to develop invasive carcinoma upon induction of stress in their colons. This was a result of chronic inflammation and augmented NF-κB activation promoted by mut-p53 (Cooks et al. 2013). This correlates with the finding that in colitis-associated colorectal cancer, mutations in p53 are an early event (Cooks et al. 2013). Furthermore, we have shown that in non-small-cell lung carcinoma cells, mut-p53 cooperates with constitutively active H-RAS to up-regulate a proinflammatory gene signature, which leads to aggressive transformed phenotypes in vivo (Buganim et al. 2010; Solomon et al. 2012). Additional inflammatory mechanisms that mut-p53 isoforms use to promote carcinogenesis are suppression of the secreted IL1 receptor antagonist by binding to its promoter with the corepressor MAFF (Ubertini et al. 2015), induction of NF-κB2 activation via recruitment of CBP and STAT2 to acetylate NF-κB2 promoter (Vaughan et al. 2012a), and up-regulation of CXC chemokines (Yan and Chen 2009; Yeudall et al. 2012).

In conclusion, mut-p53 does not only facilitate invasion and metastasis in a cell-autonomous fashion but also affects the tumor microenvironment and contributes to the vicious cycle of chronic inflammation and tumorigenesis.

mut-p53 INDUCES CANCER-PROMOTING METABOLIC SHIFT AND ANGIOGENESIS

The initial observation that the majority of cancer cells display alterations in glucose processing was made in the beginning of the previous century. The term "Warburg effect" describes the phenomenon that cancer cells predominantly use glycolysis for energy production from glucose, instead of the oxidative phosphorylation used by normal cells. Despite being considerably less efficient, glycolysis enables the tumor cells to gain valuable building blocks to sustain their rapid proliferation rate (Vander Heiden et al. 2009). Accumulating data suggest that, in addition to alterations in glucose processing, tumor cells modify other metabolic pathways to maximize the malignant potential (Hsu and Sabatini 2008). As a part of its tumor-suppressive activities, WT-p53 was shown to directly influence various metabolic pathways, enabling cells to respond to metabolic stress (Vousden and Ryan 2009; Maddocks and Vousden 2011; Goldstein and Rotter 2012; Berkers et al. 2013; Maddocks et al. 2013). In contrast, mut-p53 facilitates cancer-promoting metabolic shift. For example, in head and neck, cancer cells upon nutrient deprivation, mut-p53, but not WT-p53, binds to one of the AMPK subunits, a major energy sensor, which in turn inhibits its function, leading to anabolic metabolism (Zhou et al. 2014). In addition, it was reported that in lung and breast carcinoma, mut-p53 stimulates glucose intake by up-regulating the RhoA-ROCK pathway that results in translocation of GLUT1 to the plasma membrane (Zhang et al. 2013). mut-p53 was also shown to modulate the mevalonate pathway by binding to the TF SREBP (Freed-Pastor et al. 2012). One explanation for the Warburg effect is that proliferating tumor cells generate hypoxic conditions, which confer an advantage for cells with decreased dependence on aerobic respiration (Hsu and Sabatini 2008). In addition, to overcome the shortage in oxygen supply, cancer cells induce angiogenesis (Hanahan and Weinberg 2011), which WT-p53 was shown to suppress (Dameron et al. 1994; Van Meir et al. 1994; Mukhopadhyay et al. 1995). In contrast, mut-p53 acts to enrich the nutrients and oxygen supply to the tumor through cobinding with E2F1 to ID4 promoter leading to neoangiogenesis (Fontemaggi et al. 2009). In agreement with these observations, we found that mut-p53 elevates ROS levels by attenuating the expression of phase 2 detoxifying enzymes, NQO1 and HO-1 (Kalo et al. 2012). High ROS levels lead to an increase of HIF1, which is responsible for up-regulation of VEGF-A, a pivotal angiogenesis signal (Khromova et al. 2009). In summary, mut-p53 mediates a tumorigenic metabolic shift and angiogenesis aiming to provide nutri-

ents, building blocks, and oxygen supply to support the developing tumor.

mut-p53 INTERFERES WITH DNA-REPAIR MECHANISMS AND MEDIATES GENOMIC INSTABILITY

Tumorigenesis is associated with compromised DNA-repair pathways. This perturbation results in reduced DNA-repair capacity and increased genetic instability in tumor cells (Helleday et al. 2008). In response to DNA damage and according to the type of perturbation and the cell-cycle state, WT-p53 mediates the proper DNA-repair response, including nucleotide-excision repair (NER), base-excision repair (BER), DNA-mismatch repair (MMR), nonhomologous end-joining (NHEJ), and homologous recombination (HR) (Offer et al. 1999; Zurer et al. 2004; Sengupta and Harris 2005). Thus, in the absence of WT-p53, genomic instability arises. HUPKI mice that harbor mut-p53 showed interchromosomal translocations rarely seen in p53-null mice (Song et al. 2007), indicating mut-p53 GOF. This observation of increased genomic instability was apparent in other mut-p53 mice models as well (Murphy et al. 2000; Hingorani et al. 2005; Caulin et al. 2007). Several mechanisms were suggested for mut-p53 GOF in modifying DNA-repair pathways. It was shown that mut-p53 inhibits the pathway downstream from ataxia telangiectasia mutated (ATM) by suppressing the establishment of Mre11-Rad50-NBS1 complex, which is essential for DNA double-stranded break repair. Alternatively, it was shown that mut-p53 cooperates with E2F4 in binding to BRCA1 and RAD17 promoters that result in their down-regulation (Valenti et al. 2015). Interestingly, a recent study showed that mut-p53 does not only affect transcription but also localization of the proteins. In breast cancer cells, DNA-repair genes, PARP1 and PCNA, were shown to be associated with the chromatin and absent in the cytosol only in the presence of mut-p53 (Polotskaia et al. 2015). Another mechanism that controls genomic stability in human cancer is the accumulation of ROS that can be mediated by mut-p53 (Kalo et al. 2012). It is well accepted that DNA perturbations that promote tumorigenic processes are acquired with time (Stratton et al. 2009). However, in certain cancer cases a short single catastrophic event, termed chromothripsis, was evident (Stephens et al. 2011). It was suggested that chromothripsis is formed by shattering of vast areas of chromosome(s), followed by an error-prone, NHEJ, reconstruction mechanism (Rausch et al. 2012). Despite being a rare event, it was found to be prevalent in specific tumor types, such as SHH (sonic-hedgehog-driven)-medulloblastoma of LFS patients and AML (acute myeloid leukemia), both expressing mut-p53 (Rausch et al. 2012). In all, these data suggest that mut-p53 actively contributes to a genome instability phenotype, mostly through modifications in DNA-repair pathways and elevated ROS levels.

mut-p53 PROTECTS FROM CELL DEATH AND MEDIATES DRUG RESISTANCE

WT-p53 serves as a central inducer of programmed cell death following anticancer therapy (Yonish-Rouach et al. 1991; Lowe et al. 1993). Perhaps the most devastating mut-p53 GOF is its ability to confer drug resistance (broadly described in Shetzer et al. 2014b), which is one of the reasons for the mut-p53 association with a poor prognosis (Olivier et al. 2005; Petitjean et al. 2007). mut-p53-mediated drug resistance was shown in many tumor types following treatment with different agents (Li et al. 1998; Blandino et al. 1999; Matas et al. 2001; Pugacheva et al. 2002; Capponcelli et al. 2005; Tsang et al. 2005; Bossi et al. 2006; Buganim et al. 2006; Kawamata et al. 2007; Wong et al. 2007; Do et al. 2012; Wang et al. 2014). Mechanistically, mut-p53 protects against apoptosis by affecting many proteins involved in the apoptotic pathway, both at the transcriptional level (Li et al. 1998) and by protein–protein interaction (Chee et al. 2013). Among these apoptotic proteins are Fas/Apo-1 (Gurova et al. 2003; Zalcenstein et al. 2003), caspase 9 (Chee et al. 2013), caspase 3 (Pohl et al. 1999; Wong et al. 2007), and Bcl-xL (Huang et al. 2013). Ample data indicate that tumors acquire various drug resistance mechanisms (Holohan et al. 2013). For

example, one of the canonical drug resistance mechanisms adopted by the cancer cells is the efflux of chemotherapeutics agents out of the cell (Gottesman 2002). In this respect, it was shown that mut-p53 enhances the expression of MDR1, ATP-binding cassette (ABC) transporters, notorious for coffering drug-resistance against xenobiotic compounds with broad substrate specificity (Chin et al. 1992; Dittmer et al. 1993). Other mechanisms of drug resistance used by cancer cells include activation of alternative signaling pathways and evasion of cell death (Holohan et al. 2013). Vitamin D_3 can also induce apoptosis and possess antiproliferative activities (Colston et al. 1992) and therefore is being extensively explored as a cancer-preventive and even a cancer-therapeutic agent. Strikingly, we showed that mut-p53 modulates the antiproliferative effects of vitamin D_3 by physical interaction with the vitamin D_3 receptor, thereby converting vitamin D_3 into an anti-apoptotic agent (Stambolsky et al. 2010). A number of interesting studies reported that mut-p53 mediates drug resistance via alteration of miRs expression. It was shown that in lung cancer cells, mut-p53 inhibits apoptosis and confers increased chemoresistance to multiple agents by induction of miR-128-2 expression that targets E2F5 (Donzelli et al. 2012). Similarly, Masciarelli and colleagues have shown that down-regulation of miR-223 expression by mut-p53 and the consequent up-regulation of stathmin-1 in breast and colon cancer cell lines sensitized these cells to treatment with DNA damaging agents (Masciarelli et al. 2014). Therefore, targeting the mechanisms underlying mut-p53-mediated drug resistance may be seen as a prime aim for overcoming cancer recurrence following chemotherapy.

mut-p53 GOF IN CSCs

Heterogeneity of cancer development at large can be explained by at least two main theories: the "stochastic" or "clonal evolution" model and the "hierarchical" model (Reya et al. 2001). The stochastic model postulates that every cancer cell within the tumor has the same potential to proliferate and to propagate into a tumor. However, the hierarchical model suggests that only a minor subset of cells within the tumor has the potential to generate new tumors that recapitulate the original one (Visvader and Lindeman 2008). This minor population is regarded as the tumor-initiating cell population that has the ability to self-renew and to differentiate into heterogeneous lineages (Vermeulen et al. 2008). In all, the latter theory served as the basis for coining the term "cancer stem cells" (or CSCs) (Lapidot et al. 1994; Bonnet and Dick 1997).

It is now well accepted that CSCs represent an important target population for anticancer therapeutics, as their survival following therapy is likely to result in disease relapse (Holohan et al. 2013). CSCs are characterized as quiescent cells within the tumors. Notably, cytotoxic agents are primarily effective against proliferative cells; therefore, these quiescent cells show a degree of drug insensitivity relative to cycling cells and persist following chemotherapy (Agarwal and Kaye 2003). Moreover, CSCs have the ability to efflux cytotoxic compounds, as well as to display high activity of aldehyde dehydrogenase (ALDH) detoxifying enzymes. Additional features of CSCs include the capacity to form spheres in soft agar and the expression of typical surface markers such as CD44, CD133, and many others (Magee et al. 2012).

Apparently, mut-p53 GOF and characteristics of CSCs seem to coincide (broadly described in Shetzer et al. 2014b). CSCs display tolerance to chemotherapy and play a crucial role in cancer recurrence (Visvader and Lindeman 2008); likewise p53 mutants show GOF in conferring drug resistance in numerous tumor types as elaborated above. mut-p53 shows GOF by up-regulating MDR1 (Dittmer et al. 1993), although these very same efflux pumps are considered to be pivotal means to detect and isolate CSCs. Additionally, mut-p53 confers apoptosis resistance by affecting Bcl-2 family members (Brosh and Rotter 2009; Huang et al. 2013). Similarly, CSCs show abundant expression of prosurvival proteins of the Bcl-2 family members compared with normal adult stem cells (ASCs) and somatic cells, allowing the former cells to sustain cellular stress (Merritt et al. 1995; Mandal et al. 2011). Another pathway that is

shared by CSCs and mut-p53 GOF is the ability to induce angiogenesis by its main regulator VEGF (Bao et al. 2006; Calabrese et al. 2007).

CSCs may originate from malignant transformation of normal ASCs or progenitor cells that underwent oncogenic genetic alterations or following dedifferentiation of somatic cells that already harbor precancer genetic defects (Sugihara and Saya 2013; Aloni-Grinstein et al. 2014). Ample data suggest that the emergence of CSCs occurs in part as a result of EMT. Transformed mammary epithelial cells that were induced to undergo EMT gave rise to cells with breast CSCs markers and features such as the increased capacity to form mammospheres, soft agar colonies, and tumors (Mani et al. 2008). Recently, a landmark report stated that there is a significant correlation between lifetime risk to develop a specific type of cancer and the number of lifetime stem cell (SC) divisions in the host tissue. Using meta-analysis, this study showed correlative evidence that SCs are the origin of two-thirds of the human cancer types examined (Tomasetti and Vogelstein 2015).

Importantly, WT-p53 blocks the formation of CSCs regardless of their origin. In fact, it was found that WT-p53 governs embryonic and ASCs properties. It ensures the genomic stability of SCs following genotoxic insults and also controls their differentiation and proliferation (Aloni-Grinstein et al. 1993; Lin et al. 2005; Molchadsky et al. 2008, 2010, 2013; Solozobova and Blattner 2011; Rivlin et al. 2014b). In contrast, p53 mutations in SCs seem to equip them with accentuated oncogenic activity. The initial evidence that linked mut-p53 GOF and dedifferentiation was the association of p53 mutations and poorly differentiated tumors such as thyroid carcinomas (Donghi et al. 1993; Fagin et al. 1993), gastric cancer (Han et al. 1993), chondrosarcomas (Yamaguchi et al. 1996), skin tumors (Kemp et al. 1993), adenoid cystic carcinomas (Nagao et al. 2003), and prostate cancer (Matsushima et al. 1998). These studies showed that accumulation of mut-p53 was restricted to high-grade/poorly differentiated tumors. Moreover, one tumor showed two distinct areas of differentiated and undifferentiated thyroid carcinoma, yet mut-p53 was detected only in the undifferentiated regions (Donghi et al. 1993). Nevertheless, mut-p53 GOF was not acknowledged as the driving force behind dedifferentiation, rather this data was interpreted as an association between mut-p53 and aggressiveness of the disease. As of today, CSCs with p53 perturbations were successfully isolated by different combination of CSC markers from various cancer types such as gliomas (Zheng et al. 2008; Wang et al. 2009; Friedmann-Morvinski et al. 2012), breast cancer (Vadakkan et al. 2014), and ovarian cancer (Motohara et al. 2011; Flesken-Nikitin et al. 2013).

In recent years, the development of reprogramming technology allowed the generation of iPSCs by dedifferentiation of somatic cells (Takahashi and Yamanaka 2006) and opened a new platform to study the potential contribution of various factors required for SC formation. Because reprogramming and tumorigenesis share overlapping mechanisms (Semi et al. 2013), the reprogramming technology may be used to mimic the process of CSC formation via dedifferentiation of somatic cells bearing oncogenic genetic aberrations. We and others have revealed that p53 functions as a reprogramming barrier (Krizhanovsky and Lowe 2009). This activity of WT-p53 is manifested by attenuation of cell proliferation (Hanna et al. 2009; Yi et al. 2012) and by inhibition of Klf4-induced mesenchymal–epithelial transition, essential in the early stages of iPSC generation (Brosh et al. 2013). In contrast to the suppressive actions of WT-p53, various mutations in p53 confer an opposite effect by promoting the reprogramming process and concomitantly displaying an oncogenic GOF (Sarig et al. 2010; Yi et al. 2012; Shetzer et al. 2014a). Indeed, we have shown that mouse embryonic fibroblasts carrying mut-p53^{R172H} undergo the reprogramming process with shorter latency and higher efficiency compared with their p53-deficient counterparts (Sarig et al. 2010). Importantly, although these mut-p53-expressing iPSCs were able to differentiate into the three germ layers in vitro, displaying the features of normal iPSCs, upon injection into immunocompromised mice, mut-p53 iPSCs formed malignant and invasive tumors instead of the benign teratomas generated by WT-p53

iPSCs. This illustrates the oncogenic GOF of mut-p53 that alters both the quantity and quality of the reprogramming process, permitting generation of CSCs. Interestingly, we showed that heterozygous mut-p53 iPSCs (WT/mut-p53^{R172H}) were comparable to WT-p53 iPSCs as manifested by similar reprogramming kinetics and formation of benign teratomas. Most of the clones retained heterozygosity for prolonged time in culture; however, the small percentage of clones that underwent p53 LOH formed malignant tumors in vivo. This intriguing observation suggests that, during the reprograming process of untransformed cells harboring endogenous mut-p53, WT-p53 dominates over the mut-p53 and only upon p53 LOH were these cells able to induce malignant tumors in mice (Shetzer et al. 2014a). Although iPSCs are known to show features that are comparable to authentic embryonic stem cells (ESCs) (Takahashi et al. 2007), we observed that murine ESCs heterozygous for mut-p53 do not undergo LOH in vitro and in vivo. Moreover, both mut-p53 heterozygous and homozygous ESCs generate benign teratomas following injection into immunosuppressed mice. This interesting phenomenon, reflecting the unique mechanism existing in ESCs that functions to protect against CSC formation, is mediated by the proteomic stabilization of mut-p53 and the conversion to WT conformation (Rivlin et al. 2014a).

Examination of humanized mouse models harboring mut-p53 that closely mimic LFS patients indicated an augmented self-renewal potential that is reflected by a higher number of mesenchymal and hematopoietic SCs compared with p53-deficient mice (Hanel et al. 2013). Mesenchymal stem cells (MSCs), which are known to be less genomically stable than ESCs and iPSCs (Krtolica 2005; Shetzer et al. 2014a), were proposed as the cell of origin of soft tissue and bone sarcoma in adult life (Li et al. 2009; Mohseny and Hogendoorn 2011; Rodriguez et al. 2012). Although sarcomas comprise $<$1.5% of human tumor burden (Zahm and Fraumeni 1997; Virtanen et al. 2006), in LFS patients the incidence of soft-tissue and bone sarcoma is the highest (Petitjean et al. 2007). We found that MSCs heterozygous for mut-p53 are prone to undergo p53 LOH, which may lead to sarcomagenesis (Shetzer et al. 2014a). Likewise, it was reported that, in the human osteosarcoma cell line, mut-p53 GOF resulted in facilitated CSC formation, promoted proliferation, invasiveness, and resistance to apoptosis (Di Fiore et al. 2014). Recently, another study showed the involvement of mut-p53 GOF in the development of osteosarcoma. LFS patients' fibroblasts (heterozygous for mut-p53^{G245D}) were reprogrammed into iPSCs and further induced to differentiate into MSCs and osteoblasts. Only osteoblasts were able to form tumors and recapitulate osteosarcoma of LFS patients characterized with defective osteoblastic differentiation and expression patterns (Lee et al. 2015). This discrepancy in the cell of origin of sarcoma between studies may result from either the different p53 mutation type (mut-p53^{R172H} vs. mut-p53^{G245D}) or from differences between human and mouse models.

Importantly, mutations in p53 are not a marker for CSCs. Nevertheless, p53 mutations augment the probability to generate CSCs by either malignant transformation of normal SCs or dedifferentiation of somatic cells. The observation that CSCs and mut-p53 share common features makes it tempting to speculate that the ability to form CSCs comprises the essence of mutant p53 GOF features (Fig. 2).

mut-p53-BASED THERAPEUTICS

The notion that p53 is the most frequently mutated gene in human cancer makes it an attractive target for cancer therapy. Numerous approaches and drugs directed to restore WT activity in mut-p53-bearing tumors are currently in different stages of preclinical and clinical trials (broadly discussed in Muller and Vousden 2014). However, today, there is no p53-based approved therapy. One of the approaches aiming to restore WT activity is based on reverting mut-p53 into WT conformation. Potentially, this strategy has two major advantages. On one hand, it will permit discarding of mut-p53 oncogenic GOF; on the other hand, it will allow reacquiring WT-p53 tumor-suppressor capabilities. This approach uses small mol-

ecules that change the structure of mut-p53 protein and enable it to partially retain transactivation activity. High-throughput screening led to identification of a group of small synthetic molecules such as APR-017 (PRIMA-1) and its methylated form APR-246 (PRIMA-1MET). These first-in-class drugs are able to interact with the DNA-binding domain of multiple p53 mutant proteins, promoting their folding into WT conformation and thereby inducing apoptosis and activating several p53 target genes in human tumor cells carrying mut-p53 (Bykov et al. 2002; Lehmann et al. 2012). Yet, the exact mechanism of action remains to be established (Lambert et al. 2009). Notably, PRIMA-1MET is the first drug of this class that has reached a clinical phase (Cheok et al. 2011). Other small molecules that interact specifically with mut-p53^{Y220C} and restore WT functionality are PhiKan083 and PK7088 (Boeckler et al. 2008; Liu et al. 2013). These molecules represent an example of structure-based drug design that can identify small molecules that stabilize oncogenic p53 mutants. We have attempted a different approach harnessing peptide-based therapy through a large phage display screening (P Tal, S Eizenberger, E Cohen et al., unpubl.). Additional strategies are based on induction of mut-p53 degradation. One option is proteasomal degradation by the ubiquitin ligase MDM2. It was shown that inhibiting HDAC by SAHA disrupts HSP90 and HSP70 protection, which leads to mut-p53 degradation (Li et al. 2011). However, one should bear in mind that HDAC inhibitors affect WT-p53 transcription as well (Murphy et al. 1999) with possible deleterious consequences. Another possibility is targeting mut-p53-interacting proteins, such as family members. One example for this strategy is RETRA, which induces the release of mut-p53-p73 interaction, therefore enabling p73 proper function and preventing mut-p53 oncogenic GOF (Kravchenko et al. 2008). Surprisingly, RETRA was shown to exert anticancer properties independent of p53 status (Sonnemann et al. 2015). Other approaches use degradation of mut-p53 through autophagy (Vakifahmetoglu-Norberg et al. 2013) and inhibition of mut-p53 downstream pathways such as receptor tyrosine kinase signaling (Muller et al. 2009, 2103) or cholesterol synthesis (Freed-Pastor et al. 2012).

CONCLUDING REMARKS

The concept that a single amino acid substitution in p53 leads to formation of an oncogenic protein that was first evident more than 30 years ago is now widely accepted. Oncogenic mut-p53 GOF is manifested in many fundamental aspects of the malignant transformation, the most pivotal of which are shown in Figure 2. The powers gained by p53 mutants facilitate enhanced proliferation and avoidance of cell death. They confer genome instability, as well as promote metabolic shift and angiogenesis that provide the tumor with its essential nutrients. Cells with mutations in p53 acquire plasticity that permits migration, invasion, and metastasis. Moreover, p53 mutants are able to modify the microenvironment and support chronic inflammation that further contributes to tumor development. We have addressed the various accepted mechanisms underlying mut-p53 GOF, including interactions with its family members and other TFs. The ample data accumulated in the field of mut-p53 GOF pertaining to the above-mentioned mechanisms mostly originate from the examination of tumor bulk population. Importantly, however, it is seems that mut-p53 enables the evolvement of CSCs, which serve as the cornerstone of initiation and progression of tumorigenesis. Furthermore, the notion that the CSCs are the drug resistance entity in tumors makes it tempting to suggest that, in addition to the conventional chemotherapeutic agents that eliminate the bulk of proliferating tumor cells, a second line of treatment that targets CSCs might prove beneficial. Our findings that ESCs expressing mut-p53 induce a shift toward a WT-p53 conformation and that ESCs heterozygous for mut-p53 do not undergo LOH, thus avoiding its oncogenic activity, suggest that ESCs have unique mechanisms that may suppress malignant transformation. The identification of these physiological mechanisms of preventing the onset of a mut-p53-dependent oncogenic process in ESCs may pave the way to novel cancer therapeutic approaches.

Finally, one could speculate that therapy aimed at mut-p53 conversion into its WT conformation could target a major driving force in the formation of CSCs. Hence, future efforts should be invested in this direction. Our growing understanding and experience gained in preclinical and clinical trials will permit future development of more sophisticated and efficient cancer therapies, at large, and p53-targeted cancer therapy, in particular.

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Oncogenic Mutant p53 Gain of Function Nourishes the Vicious Cycle of Tumor Development and Cancer Stem-Cell Formation

Yoav Shetzer, Alina Molchadsky and Varda Rotter

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Short Report

Immune deficiency augments the prevalence of p53 loss of heterozygosity in spontaneous tumors but not bi-directional loss of heterozygosity in bone marrow progenitors

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p53 loss of heterozygosity (LOH) is a frequent event in tumors of somatic and Li-Fraumeni syndrome patients harboring p53 mutation. Here, we focused on resolving a possible crosstalk between the immune-system and p53 LOH. Previously, we reported that p53 heterozygous bone-marrow mesenchymal progenitor cells undergo p53 LOH *in-vivo*. Surprisingly, the loss of either the wild-type p53 allele or mutant p53 allele was detected with a three-to-one ratio in favor of losing the mutant allele. In this study, we examined whether the immune-system can affect the LOH directionality in bone marrow progenitors. We found that mesenchymal progenitor cells derived from immune-deficient mice exhibited the same preference of losing the mutant p53 allele as immune-competent matched cells, nevertheless, these animals showed a significantly shorter tumor-free survival, indicating the possible involvement of immune surveillance in this model. Surprisingly, spontaneous tumors of p53 heterozygous immune-deficient mice exhibited a significantly higher incidence of p53 LOH compared to that observed in tumors derived of p53 heterozygous immune-competent mice. These findings indicate that the immune-system may affect the p53 LOH prevalence in spontaneous tumors. Thus suggesting that the immune-system may recognize and clear cells that underwent p53 LOH, whereas in immune-compromised mice, those cells will form tumors with shorter latency. In individuals with a competent immune-system, p53 LOH independent pathways may induce malignant transformation which requires a longer tumor latency. Moreover, this data may imply that the current immunotherapy treatment aimed at abrogating the inhibition of cellular immune checkpoints may be beneficial for LFS patients.

The function of the tumor suppressor p53 is abrogated in most human cancers,[1] typically by a missense mutation in the coding sequence of one of its alleles. In most cases, the remaining wild allele (WT) p53 allele is lost in a process named loss of heterozygosity (LOH).[2] Exposure to various types of cellular insults leads to stabilization and accumulation of the mutant p53 (Mutp53) protein, which gains novel oncogenic activity that facilitates cellular transformation. This unique phenomenon is termed oncogenic gain of function.[3,4] Germ-line mutations in p53 are associated with the rare familial cancer predisposition Li-Fraumeni syndrome (LFS).[5] The LFS patients develop early onset of neoplasms, predominantly sarcomas and breast carcinomas, as well as brain tumors and adrenocortical carcinomas.[6]

It is now well accepted that one of the hallmarks of cancer is the ability of malignant cells to escape immune surveillance.[7] Accordingly, recent breakthroughs in cancer therapy are aimed at abrogating the inhibition of cellular immune checkpoints. This clearly reiterate the importance of the immune system in elimination of cancerous cells.[8] Interestingly, it was shown that the presence of Mutp53 specific antibodies could be detected in sera of cancer patients,[9,10] suggesting a humoral response aimed against Mutp53. However, to date no direct link between immune response and mutant p53 mediated transformation was evident.

Previously, in order to study p53 LOH we used LFS murine model, harboring R172H Mutp53 that is homologous to human R175H hot-spot mutation.[11] This cohort of mice predominantly develop sarcomas and lymphomas within 15–18 months, whereas two third of spontaneous tumors undergoes p53 LOH.[11] Due to high prevalence of Sarcomas in our animal model and LFS patients, which are of mesenchymal

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What's new?
p53 loss of heterozygosity (LOH) is a frequent event in tumors of somatic and Li-Fraumeni syndrome (LFS) patients harboring p53 mutation. While the ability of malignant cells to escape immune surveillance is well accepted, a possible crosstalk between the immune system and p53 LOH remains to be clarified. This study suggests that the immune system may recognize and clear cells that underwent WTp53 LOH, whereas in immune compromised mice, those cells will form tumors with a reduced latency. Moreover, the data indicate that immunotherapy treatment aimed at abrogating the inhibition of cellular immune checkpoints may be beneficial for LFS patients.

origin we focused our studies on mesenchymal stem cells that were proposed as the cells of origin of mesenchymal neoplasms. We found that p53 heterozygous (HZp53) mesenchymal bone-marrow (BM) progenitor cells undergo p53 LOH *in-vivo*.[12] Surprisingly, the loss of either the WT or the mutant p53 alleles was detected with a ratio of three to one in favor of losing the mutant allele. Although this was the first report on the loss of the Mutp53 allele through LOH in an apparent healthy tissue, Mutp53 LOH was once noticed in two Li-Fraumeni tumors[13] and in other TSGs with a role in DNA repair.[14–18] We hypothesized that the outcome of dual LOH is a result of either cellular intrinsic mechanism or the byproduct of elimination of cells that underwent WTp53 LOH by the immune system.

In this study, we addressed the question of whether the immune system can recognize and eliminate BM progenitors that underwent WTp53 LOH. Herein, we established a novel Rag1 null Il2rγ null immune deficient (ID) mice carrying HZ $p53^{R172H}$ and matched immune competent (IC) siblings. We detected p53 LOH in all examined spontaneous tumors derived from HZp53 ID mice, whereas only two thirds of tumors obtained from HZp53 IC mice exhibited p53 LOH. Indeed, HZp53 ID mice showed significantly shorter tumor-free survival, indicating the important role of immune surveillance in this model. As far as we know, this is the first report linking LOH and immune surveillance. These findings imply that the immune system may affect the prevalence of p53 LOH during the development of spontaneous tumors.

Material and Methods

Mice strains

The following mice strains were used in this study: C57BL/6 HZp53 for $p53^{R172H}$ (kindly provided by Professor G. Lozano) and NOD.Cg-$Rag1^{tm1Mom}Il2^{rgtm1Wjl}$/SzJ ID mice (The Jackson Laboratory, Sacramento, CA). ID mice lacking Rag1/Il2rγ have diminished number of Th, Tc, B, macrophages and NK cells in their spleen.[19] These strains were crossed to create mice which are ID and IC HZp53. Animal protocols were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Preparation of BM cells suspension: colony-forming units - fibroblasts (CFU-fs)

CFU-fs were established as previously described[12] from BM nucleated cells from femurs and tibias of 6–13 months IC ($n = 6$) and ID ($n = 6$) HZp53 mice. Cells were incubated at 37°C in a humidified atmosphere of 10% CO_2 and re-fed once a week without further treatment. At 14–21 days, un-fixed colonies were subjected to quantitative genomic DNA genotyping.

Genomic DNA extraction

DNA was extracted using QUICK gDNA Miniprep (Zymo Research, Irvin, CA), according to manufacturer's protocol. The genomic DNA was eluted in double distilled water.

p53 SNP genomic real time melt curve genotyping

The p53 R172H SNP was detected using the SimpleProbe TaqMan assay (Roche, Switzerland) according to the manufacturer's protocol. Known percentage of WTp53 and Mutp53 DNA samples were mixed to create a six points' standard curve for the unknown samples. The fluorescence was measured using the LightCycler® 480 instrument (Roche). An algorithm was applied to analyze the heterozygous percentage of cells in the population, based on the slope between the WTp53 and the Mutp53 picks.

Melt-curve genotyping analysis

Analysis was performed using an algorithm that was developed with the help of Alex Kagan from the physics department. This algorithm detects the melting temperatures local maxima for the WTp53 and Mutp53 alleles and calculates their ratio. This ratio is then compared to the standard curve, resulting in high resolution genotyping of the unknown samples.

Tumor samples

A cohort of mice was monitored for signs of illness or obvious tumor burden. Moribund mice were sacrificed, and tumors were fixed with 4% paraformaldehyde in phosphate-buffered saline. Tissues were paraffin embedded and sectioned at 10 μm. All sections were stained with hematoxylin and eosin prior to pathological analysis.

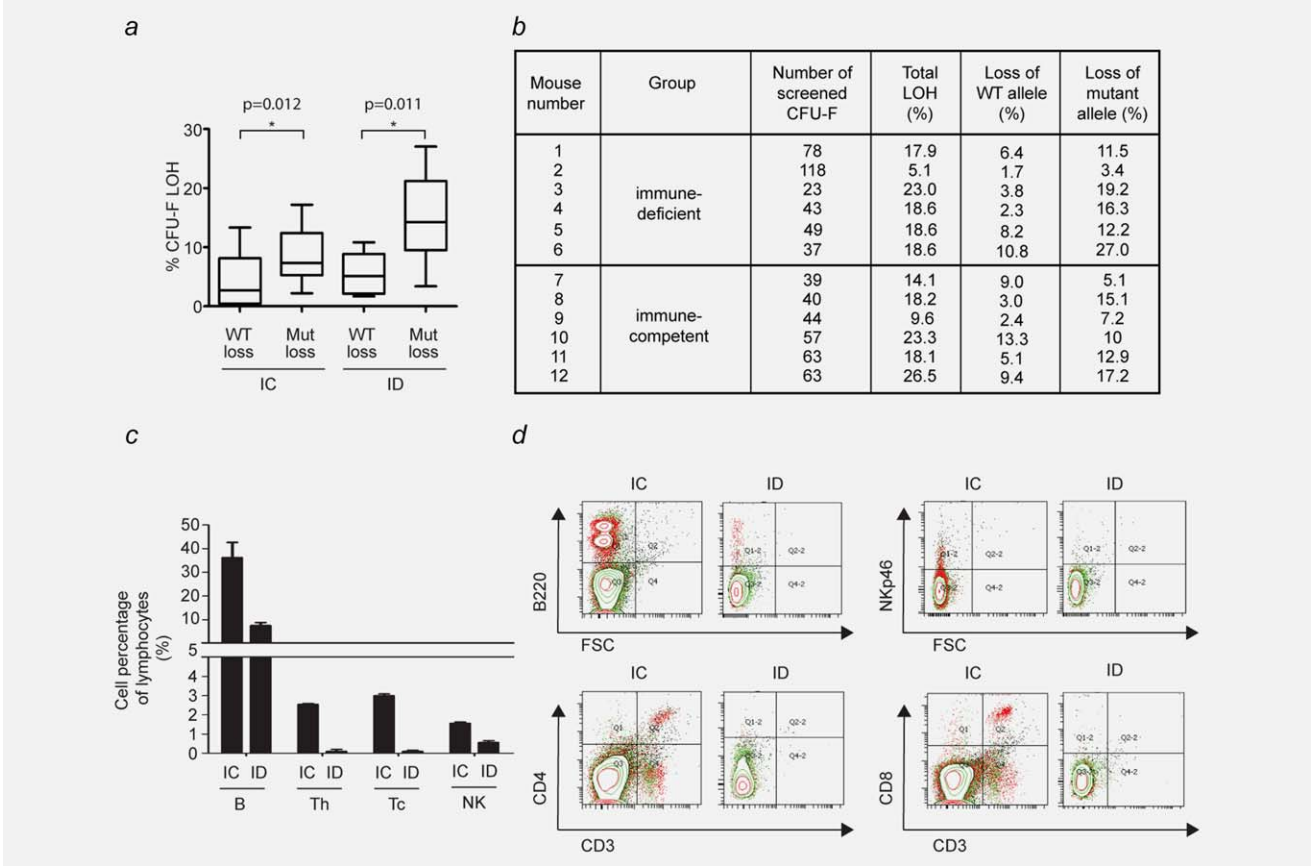
Statistical analysis

The Prism 5 program (GraphPad Software, San Diego, CA) was used for statistical analysis. Differences were considered statistically significant with a p values <0.05.

Results

Directionality of dual p53 LOH is not a result of immune system clearance

p53 LOH is a common phenomenon that facilities malignant transformation. Yet, little is known about the molecular



Mouse number	Group	Number of screened CFU-F	Total LOH (%)	Loss of WT allele (%)	Loss of mutant allele (%)
1	immune-deficient	78	17.9	6.4	11.5
2		118	5.1	1.7	3.4
3		23	23.0	3.8	19.2
4		43	18.6	2.3	16.3
5		49	18.6	8.2	12.2
6		37	18.6	10.8	27.0
7	immune-competent	39	14.1	9.0	5.1
8		40	18.2	3.0	15.1
9		44	9.6	2.4	7.2
10		57	23.3	13.3	10
11		63	18.1	5.1	12.9
12		63	26.5	9.4	17.2

Figure 1. CFU-Fs derived from both HZp53 IC and HZp53 ID mice display increased frequency of Mutp53 LOH. BM was isolated from HZp53 IC and HZp53, *Rag1* null and *Il2rγ* null, ID, mice. CFU-F formation was assessed. Two weeks later, colonies were quantitatively genotyped by Q-PCR. (*a*) Percentage of BM-derived CFU-F colonies that underwent p53 LOH, derived from IC and ID mice that lost either WTp53 or Mutp53 allele. Box plots represent median, 25th percentile, 75th percentile and extreme values. *p* values represents ANOVA post hoc Tukey's multiple comparison test. (*b*) Table summarizing the results obtained from genotyped CFU-Fs presented in panel A. (*c*) BM cells from 8 to 11 month-old ID and IC male mice ($n = 3$) were analyzed by flow cytometry for expression of mouse lineage cell surface characteristics as described in Material and methods. B220 represents B cells, NKp46 represents NK cells, CD3/CD4 represent Th cells, CD3/CD8 represent Tc cells. (*d*) Representative images of flow cytometry of the different populations (B cells, NK cells, Th cells and Tc cells) of IC and ID mice.

events occurring in pre-malignant cells that lead to transformation.[12] It is of interest to examine whether the immune system may recognize the WTp53 LOH associated cellular alterations and thus, as a consequence, to execute clearance of these cells. To address this issue we studied ID mice lacking *Rag1,* which is involved in V(D)J recombination, but not in DNA repair homologues recombination repair pathway. These mice are also deficient in *Il2rγ*. The mice were crossed with Mutp53^{R172H} harboring mice in order to generate HZp53 ID mice. Fluorescence-activated cell sorting (FACS) analysis indicated the deficiency of T, B and NK cells in the BM of HZp53 ID mice is comparable with the original ID mice colony (Figs. 1*c* and 1*d*). To assess the incidence of p53 LOH and examine whether the immune system has an effect on its directionality we compared CFU-Fs derived from HZp53 IC and HZp53 ID mice using a novel developed quantitative genotyping approach. This technique utilized chemically modified Taqman probe that specifically anneals to either WTp53 or Mutp53 alleles in different temperatures. Consequently, a designated algorithm calculates the percentage of cells within the population that underwent p53 LOH, by comparison of the obtained data to known ratios of WTp53 and Mutp53 alleles. It was found that CFU-Fs derived from HZp53 ID mice underwent p53 LOH in a similar frequency as their counterparts from HZp53 IC mice (Fig. 1). Furthermore, the trait of a higher frequency of Mutp53 allele loss was detected in CFU-Fs from both HZp53 IC (*p* values= 0.012) and HZp53 ID mice (*p* values = 0.011), suggesting that p53 LOH directionality is not affected by the immune system (Figs. 1*a* and 1*b*).

Immune deficiency accentuates p53 LOH prevalence in spontaneous tumors

The notion that the immune system plays a role in tumor surveillance,[20] was mostly based on studies of carcinogenic-induced mice models, where ID animals display shorter

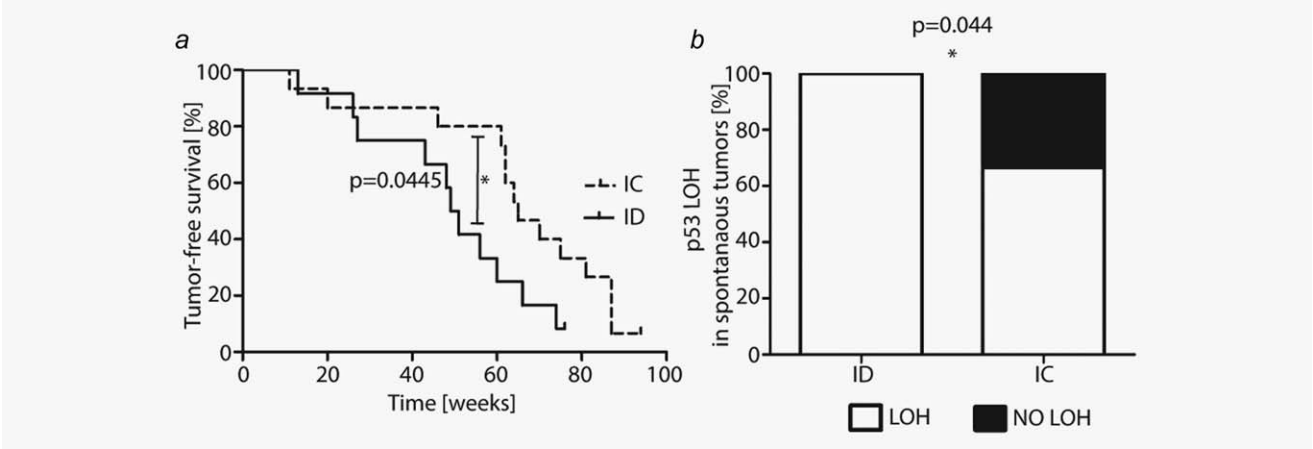


Figure 2. HZp53 ID mice exhibit shorter tumor free survival that correlates with increased p53 LOH in spontaneous tumors. Tumor appearance was monitored in HZp53 immuno-competent (IC) and immune-deficient, *Rag1* null and *Il2rγ* null mice (ID) and quantitatively genotyped by Q-PCR. (*a*) Tumor-free survival curve of IC ($n = 16$) and ID ($n = 12$). *p* values represents Survival log-rank (Mantel-Cox) test. (*b*) Percentage of tumors derived from IC mice ($n = 9$) and ID ($n = 9$) that show p53 LOH or p53 LOH – free in spontaneous tumors. >30% of tumor cells underwent p53 LOH as measured by quantitative genotyping. *p* values represents Two-tailed two proportions Z Test.

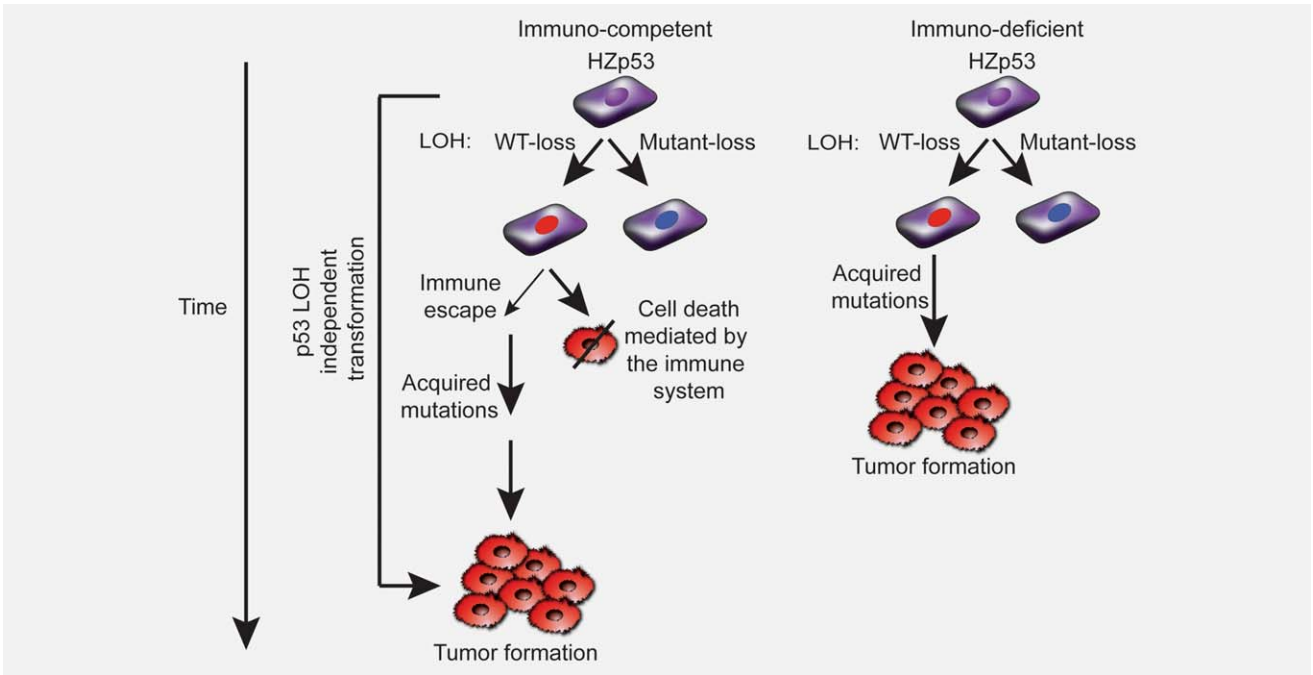


Figure 3. Proposed model: immune escape following WT p53 LOH require longer latency of tumor formation due to p53 LOH independent transformation. The immune system can recognize and clear cells that underwent p53 LOH, whereas in its absence, those cells will form tumors in shorter latency. In an individual with a competent immune system p53 LOH independent pathways are utilized in the transformation process, which require longer period of time to form tumors.

tumor-free survival compared to matched IC animals. Conversely, Rogers *et al.* reported that adaptive immune system is not efficiently suppressing tumor formation in a Sleeping Beauty (SB) transposon mutagenesis mouse model of spontaneous tumors.[21] Thus, it was interesting to examine whether the immune system affects spontaneous tumor latency and p53 LOH status in spontaneous tumors of p53HZ IC and p53HZ ID mice cohort. For this aim, mice were monitored for tumor appearance. Once tumor appeared, LOH status of the tumor was assessed. p53HZ ID mice showed a significantly shorter tumor-free survival compared to p53HZ IC mice, mean of 50 weeks versus mean of 65 weeks, respectively (Fig. 2*a*). This is in agreement with the tumor-free survival that was previously described of double *Rag1* p53 deficient mice.[22] Since the latter mice colony lack only B and T cells, this suggests that these are the cells that are crucial for immune-surveillance in the absence of p53.

The tumor spectra of the novel crossed bread colony of ID/IC mice with C57BL/6 – NOD background was similar to the original C57BL/6, including soft-tissue and osteosarcomas, lymphomas, adeno and squamous-cell carcinoma. However, the incidence of carcinomas was much more prevalent in p53HZ ID mice compared with p53HZ IC mice, ~50% ($n = 14$) versus 17% ($n = 23$), respectively (Supporting Information Table 1).

Surprisingly, in contrast to HZp53 original mice colony and in LFS patients about two third of tumors undergo p53 LOH[11,23] all tumors derived from p53HZ ID mice underwent p53 LOH ($n = 9$) compared with only 66.7% of tumors derived from p53HZ IC mice that exhibited p53 LOH (Fig. 2*b*, p values = 0.044). This may indicate that the immune system can recognize and clear cells that underwent p53 LOH, whereas in its absence, those cells will form tumors in shorter latency. In an individual with a competent immune system, either mice or theoretically LFS patient, other pathways are utilized in the transformation process which takes longer period of time to form tumors (Fig. 3).

Discussion

Most LFS patients harbor a mutated p53 allele in their germline.[5] Although these individuals develop normally, they have a high risk to develop tumors, with an incidence of 40–60% of tumors undergo p53 LOH.[13] Soft-tissue and bone sarcomas, which are of mesenchymal origin are predominant in LFS families.[6] The intriguing findings that BM progenitors derived from HZp53 IC mice undergo bi-directional p53 LOH *in-vivo* with higher incidence of losing the Mutp53 allele[12] led us to examine whether the immune system plays a role in eliminating pre-malignant cells following p53 LOH. Early studies in the field of p53 detected antibodies against p53 in patients' serum,[9] indicating a humoral response against p53. We hypothesized that the loss of either WTp53 or Mutp53 allele is stochastic. While upon WTp53 loss the cell undergoes cellular alterations that are recognized by the immune system that eliminates them, thus leaving higher percentage of cells that lost the Mutp53 allele. However, when we examined this hypothesis by using CFU-Fs derived HZp53 immune deficient mice, the same preference of losing the Mutp53 allele were found. This finding may indicate that the adaptive immune system does not play a role in the elimination of cells that lost WTp53. Still, it could also be that the NK cells, which are part of the innate immune system, despite being in reduced numbers, cleared those cells. Another possibility may be that cell autonomous death is triggered following the loss of WT p53, despite the presence of mutant p53. Further research is needed to resolve this question.

Another intriguing observation in LFS research is the strikingly high prevalence of soft-tissue and osteosarcomas in these patients, ~30% versus ~1% of all adult solid malignant cancers.[6,24] It was demonstrated that alternative genetic background of distinct mouse colonies knocked-in with Mutp53^{R172H} affects the spectrum of developing tumors types. For example, HZ p53 C57BL/6 mice predominantly develop sarcomas and lymphomas,[11] whereas HZp53 129S/Sv develop a variety of carcinomas, soft tissue and bone sarcomas, leukemia, and even a glioblastoma multiforme.[23] Furthermore, backcrossing HZp53 C57BL/6 mice to BALB/C background gave rise to mostly osteosarcomas and mammary carcinomas.[25] Herein, our specific established mice colony of ID/IC HZp53 generated by crossing C57BL/6 and NOD background exhibited similar tumor trait as their progeny, the C57BL/6 HZp53 genotype. Indeed, they share a similar tumor spectra and tumor-free survival. However the percentage of adeno- and squamous cell carcinomas was elevated, suggesting that C57BL/6 background has an intrinsic resistance to carcinomas, as suggested before.[26] The two colonies, the progeny C57BL/6 HZp53 and the novel C57BL/6/NOD IC HZp53, showed similar incidence of p53 LOH and its directionality in BM progenitors, suggesting that this phenomenon is background independent.

Interestingly, HZp53 ID mice of our cohort exhibited shorter tumor free survival and augmented prevalence of p53 LOH in spontaneous tumors compared to their IC counterparts. Pathological analysis revealed that these traits are not mediated by increased cell growth or decreased cell death in ID mice compared to IC mice (Supporting Information Table 1). Thus, it is tempting to speculate that in HZp53 ID mice, cells that have lost WTp53 generate tumors in a shorter latency compared to IC mice as a result of immune-surveillance, elimination of cells that lost the WTp53. It is plausible that cells, which evade the immune system, develop additional genetic aberrations, which require time to evolve, thus mice have a longer tumor free survival and lower percentage of tumors that underwent p53 LOH. This suggests that immune checkpoints inhibitors, such as anti-PD1 or anti-CTLA-4, which showed remarkable results in clinical trials[27] may augment the immune response against pre-malignant cells that underwent p53 LOH in LFS patients. Therefore, immune checkpoints blockade could be utilize both for prevention and treatment of LFS patient. However, current FDA-approved tumor types, that is, melanoma, squamous cell carcinoma, renal cell carcinoma and classical Hodgkin lymphoma[27] do not overlap with LFS tumor spectra. Second, as a preventative medicine, the costly treatment and adverse effects argue against this notion. Yet, the diverse capability and lasting effects of this therapy may be beneficial as a first-line treatment of first tumor, which may prevent frequently occurring second or third tumors in those patients.

Giving together, this appears to be the first report linking LOH prevalence and immune surveillance. Furthermore, the shorter tumor-free survival in HZp53 ID mice may implicate that augmentation of the immune response against tumors of LFS patients may benefit from the current immune therapy aimed against the inhibition of immune checkpoints.

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