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p53 Null Fluorescent Yellow Direct Repeat (FYDR) Mice have Normal Levels of Homologous Recombination

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Abstract

The tumor suppressor p53 is a transcription factor whose function is critical for maintaining genomic stability in mammalian cells. In response to DNA damage, p53 initiates a signaling cascade that results in cell cycle arrest, DNA repair or, if the damage is severe, programmed cell death. In addition, p53 interacts with repair proteins involved in homologous recombination. Mitotic homologous recombination (HR) plays an essential role in the repair of double-strand breaks (DSBs) and broken replication forks. Loss of function of either p53 or HR leads to an increased risk of cancer. Given the importance of both p53 and HR in maintaining genomic integrity, we analyzed the effect of p53 on HR in vivo using Fluorescent Yellow Direct Repeat (FYDR) mice as well as with the sister chromatid exchange (SCE) assay. FYDR mice carry a direct repeat substrate in which an HR event can yield a fluorescent phenotype. Here, we show that p53 status does not significantly affect spontaneous HR in adult pancreatic cells in vivo or in primary fibroblasts in vitro when assessed using the FYDR substrate and SCEs. In addition, primary fibroblasts from p53 null mice do not show increased susceptibility to DNA damage-induced HR when challenged with mitomycin C. Taken together, the FYDR direct repeat assay and SCE analysis indicate that, for some tissues and cell types, p53 status does not greatly impact HR.

Keywords

homologous recombination; p53; genomic stability; fluorescence; mitomycin C

1. Introduction

The p53 tumor suppressor gene is a transcription factor that plays an essential role in maintaining genomic stability. Indeed, greater than half of all tumors have lost p53 function [1–5]. Inherited mutations in p53 cause Li-Fraumeni syndrome, a genetic disorder characterized by an early incidence of cancer [6, 7], and p53 null mice develop tumors at an accelerated rate [8]. Together, these data indicate that p53 is a key inhibitor of tumor formation, and loss of p53 function provides cells with critical selective advantages for tumor formation.
p53 mediates cell cycle arrest and/or apoptosis in response to DNA damage. Cells are constantly exposed to endogenous and exogenous agents that can damage DNA [9]. Of the many lesions that form, DNA double-strand breaks (DSBs) are among the most cytotoxic and mutagenic, and mitotic homologous recombination (HR) provides a critical pathway for their repair [9]. In addition, HR provides the only pathway for accurate repair of broken replication forks [10]. Thus, HR is critical for preventing tumor-promoting sequence rearrangements.

As with inherited mutations in p53, germline mutations in genes that modulate HR are also associated with an increased risk of cancer [11–15]. Exchanges between misaligned sequences can lead to tumorigenic rearrangements and loss of heterozygosity. While too much HR can be problematic [15–17], too little HR can also lead to genomic instability. In the absence of HR, misrepair can lead to large-scale sequence rearrangements. Indeed, germline mutations that suppress HR (i.e., BRCA1 [18], BRCA2 [19], FANCC [20]) increase the risk of cancer [15, 21, 22]. Thus, maintaining the accuracy and the rate of HR is critical for preventing tumor formation.

Given the importance of both p53 and HR in maintaining genomic stability, a number of studies have focused on the effect of p53 status on HR. While some in vitro studies suggest that p53 suppresses HR [23–29], many other studies do not show any effect of p53 on HR [30–35]. Although the number of studies performed in vivo is limited, several investigators have nevertheless studied HR in p53 null mice in vivo. These studies show either no effect or a slight suppressive effect of p53 on HR [11, 36, 37]. Taken together, there is limited information about the in vivo effect of p53 on HR in adult tissues.

Here, we investigate the impact of p53 on HR. We used the Fluorescent Yellow Direct Repeat (FYDR) mice, in which an HR event at an integrated transgene yields a fluorescent signal [38]. Analysis of pancreata from Fydr<sup>y/y:p53<sup>+/+</sup></sup> and Fydr<sup>y/y:p53<sup>−/−</sup></sup> mice shows that the frequency of recombinant cells is not affected by p53 in vivo. Since HR plays a critical role in the repair of broken replication forks, we further analyzed the effect of p53 on HR in rapidly dividing primary fibroblasts. However, p53 had no effect on the rate of spontaneous or damage-induced HR in vitro. Taken together, loss of p53 does not significantly impact SCEs or HR at the FYDR substrate in vitro or in vivo. These data call attention to the possibility that the impact of p53 on HR is cell type-dependent.

2. Materials and Methods

2.1 Animals

FYDR [38] and p53 mice [39] were described previously. 9 week old Fydr<sup>y/y:p53<sup>+/+</sup></sup> and Fydr<sup>y/y:p53<sup>−/−</sup></sup> littermates were compared in a sex-matched fashion.

2.2 Flow Cytometry

Pancreatic cells were disaggregated as described previously [40]. Disaggregated pancreatic cells were pelleted and resuspended in 350 µl OptiMEM (Invitrogen), filtered (35 µm), and analyzed with a Becton Dickinson FACScan flow cytometer (excitation 488 nm, argon laser). On average, ~1 million cells were analyzed per sample.

2.3 Imaging

Pancreata were imaged as described previously [40]. Briefly, whole pancreata were compressed to 0.5 mm and imaged using a fixed aperture time (1x objective). Filters included: visible light; UV-2E/C (Ex:330–380 nm, Em:420 nm); Red (Ex:540/25 nm, Em: 605/55 nm); and EYFP (Ex:460–500 nm, Em:510–560 nm). Foci were counted in a blinded...
2.4 Isolation of Ear Fibroblasts

Ears were isolated, minced, and incubated at 37°C in 4 mg/ml collagenase/dispase (Roche Applied Sciences). After 1 hour, two volumes of fibroblast medium were added [Dulbecco’s Modified Eagle’s Medium, 15% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.3 mg/ml L-glutamine, 0.1mM Non-Essential Amino Acids, 5 µg/ml amphotericin B (Sigma)]. After 24 hours at 37°C and 5% CO₂, cells were triturated, filtered (70-µm mesh; Falcon), and seeded into dishes.

2.5 SCE analysis

Primary ear fibroblasts from age and sex-matched \textit{Fydr}^{y/y};\textit{p53}^{+/+} and \textit{Fydr}^{y/y};\textit{p53}^{−/−} littermates were seeded at 2 × 10^5 cells per well. After 24 hours, cells were cultured in10 µM BrdU in McCoys media. After ~22 hr, cells were incubated for ~2 hr in 0.1 µg/ml Colcemid, and SCEs were analyzed as previously described [41]. SCEs from 25–30 metaphase spreads per genotype were counted in a blinded fashion. Analysis was performed on passage-matched samples (<5 passages).

2.6 Calculation of Rate in Primary Fibroblasts

Primary ear fibroblasts were isolated from sex-matched \textit{Fydr}^{y/y};\textit{p53}^{+/+} and \textit{Fydr}^{y/y};\textit{p53}^{−/−} littermates and rate experiments were performed in parallel. For rate experiments, ~10^4 cells were seeded into each of 24 independent cultures. Cultures were expanded to ~10^6 cells prior to flow cytometry. Freshly isolated fibroblasts were used for each experiment (passage 2). The MSS Maximum Likelihood Method was used to calculate the rate of recombination, as described [42]. Rate experiments were repeated with fibroblasts from seven different pairs of mice.

2.7 Quantification of spontaneous and DNA damage-induced recombinant cell frequency

Primary ear fibroblasts from sex-matched \textit{Fydr}^{y/y};\textit{p53}^{+/+} and \textit{Fydr}^{y/y};\textit{p53}^{−/−} littermates were seeded at 5 × 10^5 cells per 100 mm dish. After 24 hours, quadruplicate samples were cultured in Dulbecco’s Modified Eagle’s Medium or exposed to medium supplemented with 0.5 µg/ml mitomycin-C (CAS No 50-07-7) for 1 hour. After 72 hours, samples were analyzed by flow cytometry. Population growth was determined using a Coulter counter. Experiments were repeated with fibroblasts from three different cohorts.

3. Results

3.1 Effect of p53 status on spontaneous homologous recombination in pancreatic cells

To study HR \textit{in vivo}, we previously developed FYDR mice that carry a direct repeat recombination substrate containing two differently mutated copies of the coding sequence for enhanced yellow fluorescent protein (EYFP). An HR event can restore full length \textit{EYFP} coding sequence (Fig. 1) [38]. One method for measuring the \textit{in vivo} frequency of fluorescent recombinant cells is to analyze disaggregated tissue by flow cytometry [40]. To determine the effect of p53 status on HR \textit{in vivo}, we analyzed recombinant cells in pancreata of 9 week-old \textit{Fydr}^{y/y};\textit{p53}^{+/+} and \textit{Fydr}^{y/y};\textit{p53}^{−/−} mice. The median frequency of recombinant pancreatic cells is not significantly different between the two cohorts (Fig. 2A).

Analysis of FYDR pancreata by flow cytometry requires tissue disaggregation; thus, the contribution of clonal expansion versus independent HR events on recombinant cell frequency cannot be determined [40, 43, 44]. We previously developed \textit{in situ} imaging
techniques that enable the direct detection of fluorescent recombinant foci (recombination events; see Fig. 1 [40]). For the Fydr<sup>y/y</sup>;p53<sup>+/+</sup> and Fydr<sup>y/y</sup>;p53<sup>−/−</sup> mice, the median number of recombinant foci is not significantly different between the two cohorts (Fig. 2B). Additionally, because the sizes of pancreata vary among mice within each cohort (data not shown), we analyzed the number of foci per unit surface area, and again found that the frequencies of recombinant foci per cm<sup>2</sup> are not significantly different (Fig. 2C). Although there is significant variation among individual animals, previous studies show that relatively subtle differences in the median value can nevertheless be detected. For example, a ~3 fold difference in recombination frequency was observed using fewer mice than in this study [40]. Thus, these data indicate that p53 status does not significantly impact the susceptibility of adult pancreatic cells to HR in vivo.

### 3.2 Effect of p53 Status on HR in Primary Fibroblasts in vitro

HR activity is most active during S phase and late S/G2 [45], however, in the pancreas, the vast majority of cells are not in S phase [46]. To explore the effect of p53 status on HR in rapidly dividing cells, we created primary ear fibroblast cultures from Fydr<sup>y/y</sup>;p53<sup>+/+</sup> and Fydr<sup>y/y</sup>;p53<sup>−/−</sup> mice, and analyzed genome-wide HR events using the sister chromatid exchange (SCE) assay [47]. There is not a significant difference in the frequency of spontaneous SCEs in Fydr<sup>y/y</sup>;p53<sup>+/+</sup> and Fydr<sup>y/y</sup>;p53<sup>−/−</sup> primary ear fibroblasts (Fig 3A). Primary fibroblasts were also analyzed via flow cytometry. No difference was observed in the frequency of fluorescent recombinant cells among p53<sup>+/+</sup> and p53<sup>−/−</sup> cultures (Fig. 3B).

As another independent approach to explore p53’s potential effects on HR, we measured the rate of HR per cell division. For each experiment, 24 independent cultures were allowed to expand prior to analysis and the recombination rate was calculated using the MSS Maximum Likelihood Method [42]. We found that the rate of homologous recombination is not significantly different between Fydr<sup>y/y</sup>;p53<sup>−/−</sup> and Fydr<sup>y/y</sup>;p53<sup>+/+</sup> fibroblasts (Fig. 3C). Thus, taken together, these studies of HR using the FYDR system and the traditional SCE assay show that p53 status does not affect the spontaneous frequency or rate of HR in primary fibroblasts in vitro.

To determine if p53 impacts HR induced by an exogenous agent, we treated primary fibroblasts with a potent recombinogen, mitomycin-C (MMC), and assayed both cell proliferation and HR frequency. Consistent with being resistant to cell cycle arrest and to apoptosis, untreated p53<sup>−/−</sup> cells had a proliferative advantage (compare black bars in Fig. 3D) [48]. When challenged with MMC, Fydr<sup>y/y</sup>;p53<sup>+/+</sup> and Fydr<sup>y/y</sup>;p53<sup>−/−</sup> fibroblasts both show significant growth inhibition (see black versus gray bars in Fig. 3D). We next queried MMC-induced HR. For both Fydr<sup>y/y</sup>;p53<sup>+/+</sup> and Fydr<sup>y/y</sup>;p53<sup>−/−</sup> fibroblasts, there is a statistically significant increase in the frequency of recombinant cells for MMC-treated cultures (compare black and gray bars in Fig. 3E). However, p53<sup>−/−</sup> cells did not show an increase in susceptibility to MMC-induced HR events.

### 4. Discussion

As guardian of the genome, p53 function is important for maintaining genomic integrity and loss of p53 results in an early onset and increased frequency of many types of cancers [6, 7, 39]. In response to DNA damage, p53 promotes the repair of DNA damage by inducing cell cycle arrest and activating DNA repair proteins. However, in the presence of extensive DNA damage, p53 signals for the elimination of damaged cells through apoptosis [49]. Due to p53’s critical role in suppressing cancer, we set out to understand the mechanisms through which loss of p53 promotes genomic instability.
A number of studies have shown the direct involvement of p53 in HR. Specifically, p53 may act at stalled replication forks to prevent breakdown [50, 51]. Additionally, p53 can directly interact with and inhibit the function of HR proteins, including RPA [52], Rad51 [53, 54], BLM and WRN [55–57]. Finally, p53 has been shown to bind to Holliday Junctions [57–59]. Thus, a clear interaction between p53 and HR exists and some of its activities suggest that p53 would suppress HR. However, using FYDR mice we found that p53 status has no effect on HR in vivo in pancreatic cells or in vitro in primary fibroblasts, suggesting that the role of p53 in modulating spontaneous HR in vivo is minimal in at least some cell types. Thus, p53’s tumor suppressor function may be more reliant on suppression of other forms of genomic instability and/or on other attributes of p53, rather than on its potential to suppress HR.

Using two different mouse models, previous studies examining the effect of p53 status on HR in vivo show different results. In pink-eyed unstable (per) mice, HR at a direct repeat during embryonic development can give rise to a dark spot on the fur or on the retinal epithelium [60, 61]. Using the fur spot assay, p53 had no effect on HR in vivo [36]. However, another study using the more sensitive per eye-spot assay showed that loss of p53 increased HR events, specifically during early embryonic development [11]. Because clonal expansion of recombined cells is required to detect spots on fur and retinal epithelium, per mice can only be used to detect recombination events that occur during embryogenesis. In contrast, FYDR mice can be used to detect recombination events that occur in adult tissues in vivo. Therefore, p53 may be less important in suppressing HR in adult versus embryonic tissues.

In addition to possible differences among stages of development, the effect of p53 on HR may be cell type specific. Indeed, studies using the adenine phosphoribosyltransferase (Aprt) mouse model to detect recombination events in vivo showed that while fibroblasts derived from p53−/− mice had ~2 fold increase in spontaneous mutation events due to mitotic recombination; there was no difference in p53−/− compared to p53+/+ T lymphocytes [37, 62]. Together, these data show that p53 may have different effects on HR in different cell types.

When considering the in vivo data, it is important to consider the limitations of the assay used here. It is noteworthy that there is a significant amount of variability from mouse to mouse. Some of this variation is due to stochastic factors, such as the timing of the recombination event, which can influence the total number of recombinant cells as a consequence of clonal expansion. Despite this variance, in older animals and in animals exposed to DNA damaging agents, a change in the median value has been observed, indicating that this approach can be used effectively to detect endogenous and exogenous factors that impact homologous recombination [40, 43]. Given that we did not detect an impact of p53 status on HR, even when analyzed with multiple approaches, we conclude that p53 has at most a subtle impact on HR in adult animals, at least in some tissues.

The effect of p53 function on HR has also been examined in vitro in multiple cell types. In studies using systems that detect HR events at specific loci (e.g., Tk, EGFP, SV40), the majority suggest that p53 suppresses HR in vitro [23–26, 28, 29, 63–65]. In contrast, virtually all studies analyzing the effect of p53 on HR by examining SCEs show no effect of p53 function on HR [32, 34, 35, 66–70]. Additional SCE studies reveal that p53 does not significantly impact damage-induced HR when cells are exposed to a variety of agents (mitomycin-C, nitric oxide, low dose radiation and low dose UV) [66–70]. Thus, the observation here that p53 does not impact SCEs is consistent with previous studies.
It is clear that HR is dependent on multiple factors, including stage of development, cell type, and detection method. Interestingly, Slebos and Taylor found that p53 status influenced one type of HR reporter system, but not another [31]. Furthermore, p53 appears to act differently in different types of recombination events, depending on the amount of homology between the sequences [23, 28, 71, 72]. Finally, different types of mutations in the p53 gene (e.g., null versus a point mutation) have different effects on HR [23, 29, 64, 73]. Here, we have shown that in cells and tissues of mice lacking p53, there is not a significant effect on recombination in vivo or in vitro when assessed at a direct repeat or via SCEs. Taken together, previous studies and the results presented here suggest a rather complex relationship between p53 and HR, which is consistent with p53’s highly pleiotropic roles in cell behavior. Finally, this study, in combination with the results from several others [34–37, 72], emphasizes the extent to which HR is independent of p53 in many normal cells and tissues.

**Highlights**

► p53 does not impact homologous recombination at a direct repeat in mouse pancreata
► p53 null fibroblasts show normal levels of homologous recombination and sister chromatid exchanges. ► p53 does not appear to be a major factor in modulating HR in some cell types in vivo.

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**References**


Fig 1. FYDR system and analysis of the effect of p53 on HR in vivo. (A) Arrangement of the FYDR recombination substrate: large arrows indicate expression cassettes; yellow boxes show coding sequences; black boxes show positions of deleted sequences (deletion sizes not to scale). (B) Compiled image for representative FYDR pancreata. Cell nuclei were stained with Hoechst and imaged using a UV-2E/C filter (435–485 nm). Image was collected at 1x using an EYFP filter (510–560 nm). Insert is an individual fluorescent cell.
Fig 2. Effects of p53 status on Homologous recombination in vivo. (A) Spontaneous frequency of recombinant pancreatic cells per million as determined by flow cytometry for Fydy^{+/+}p53^{+/+} (n=49) and Fydy^{+/−}p53^{-/-} (n=47) mice. Points on the x-axis indicate individual mice with zero recombinant cells. (B) Spontaneous recombinant foci per pancreas detected by in situ image analysis for Fydy^{+/+}p53^{+/+} (n=48) and Fydy^{+/−}p53^{-/-} (n=47) mice. (C) Spontaneous recombinant foci per cm² detected by in situ image analysis for Fydy^{+/+}p53^{+/+} (n=47) and Fydy^{+/−}p53^{-/-} (n=47) mice. Medians are indicated by black bars.
Fig 3.
Effects of p53 status on HR in vitro (A) Effect of p53 status on SCEs. Frequency of SCE events in primary mouse ear fibroblasts (p=0.35, 2-tailed Student’s t-test). (B) Frequency of recombinant fluorescent primary fibroblasts. Three independent experiments were performed in quadruplicate, and the average of the three experiments is shown (p=0.32, 2-tailed Student’s t-test). (C) Rate of HR in primary fibroblasts as determined by the MSS Maximum Likelihood Method [42] (p=0.16, 2-tailed Student’s t-test). Each bar indicates the average of 7 independent experiments. (D) Cell density (black bars) and MMC-treated (gray bars) primary fibroblasts. Each bar indicates the average of three independent experiments, each performed in quadruplicate. * p<0.0001, 2-tailed Student’s t-test. (E) Frequency of

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recombinant cells for mock- (black bars) and MMC-treated (gray bars) fibroblasts. Each bar indicates the average of three independent experiments, each performed in quadruplicate. Note that data for the mocktreated samples is also shown in part B. * p<0.0005, 2-tailed Student’s t-test. Error bars indicate 1 SD.