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To cite this version:

Yannick Saintigny, Bernard Lopez. Homologous recombination induced by replication inhibition, is stimulated by expression of mutant p53. Oncogene, Nature Publishing Group, 2002, 21 (3), pp.488 - 492. 10.1038/sj.onc.1205040 . cea-01938162

HAL Id: cea-01938162

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Oncogene (2002) 21, 488–492
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www.nature.com/onc

Homologous recombination induced by replication inhibition, is stimulated by expression of mutant p53

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Cell cycle control, faithful DNA replication, repair and recombination are associated in a network of pathways controlling genome maintenance. In mammalian cells, inhibition of replication produces DNA breaks and induces *RAD51*-dependent recombination, in a late step. Here we examine whether the status of p53 affects this process in mouse L-cells containing a recombination substrate. We show that expression of the mutant ^{His175}p53 strongly stimulates recombination induced by aphidicolin, in a late step (kinetically related to the *RAD51* step). Mutant p53 stimulates recombination induced by the replication elongation inhibitors (aphidicolin, hydroxyurea and Ara-C) but is without effect on recombination induced by the initiation inhibitors (mimosine and ciclopirox olamine). We compared the impact of several p53 mutations showing different effects on the G1 checkpoint and on recombination. We show that the mutant ^{Pro273}p53 protein, which does not alter the G1 checkpoint, strongly stimulates recombination induced by elongation inhibitors. These results show that p53 can act on recombination induced by replication arrest independently of its role in the G1 checkpoint. An action of p53 via the *RAD51* pathway is discussed.
Oncogene (2002) **21,** 488–492 DOI: 10.1038/sj/onc/1205040

Keywords: homologous recombination; replication inhibition; p53; RAD51

Faithful genome transmission during cell division requires the association of cell cycle checkpoints, DNA replication and repair in a network of pathways preventing genome instability. Replication forks are routinely inactivated by a broad variety of stresses (for review see Hyrien, 2000; Rothstein *et al*., 2000). Cell cycle control participates in genome maintenance by optimizing DNA repair prior to DNA replication or mitosis. Arrest in G1 phase allows cells to repair their genomes to ensure an intact DNA matrix during S phase. Defects in the G1 checkpoints would permit replication to take place on non-repaired DNA matrix. Replication forks could then be blocked by the remaining damage, leading to DNA breaks, efficient substrates for homologous and illegitimate recombination. Thus, defects in the G1 checkpoint should lead to genome instability and to tumor predisposition (Hartwell, 1992; Hartwell and Kastan, 1994). Consistent with this are the facts that (i) the tumor suppressor p53 protein plays a pivotal role in the G1 checkpoint and is the most frequently mutated gene in tumors (Hollstein *et al*., 1991; Levine *et al*., 1991); (ii) alteration of p53 function leads to genetic instability (Bouffler *et al*., 1995) and to an increase in homologous recombination (Wiesmuller *et al*., 1996; Bertrand *et al*., 1997; Mekeel *et al*., 1997; Saintigny *et al*., 1999). However, this view should be carefully re-evaluated since mutant p53 can stimulate spontaneous and radiation-induced homologous recombination independently of G1 checkpoint alterations and of transactivation activity (Saintigny *et al*., 1999; Dudenhoffer *et al*., 1999; Willers *et al*., 2000). Moreover, treatment with replication inhibitors such as hydroxyurea (HU) leads to accumulation of p53 (similarly to γ-rays) but is inactive on its transactivation activity, in contrast with γ-rays (Gottifredi *et al*., 2001). Replication is a very particular and elaborate process and the DNA interruption caused by replication arrest may differ from the DNA damage induced by radiation. It is important to know which role is related to this accumulation of p53 induced by hydroxyurea (HU). Homologous recombination (HR) may be a good candidate since the effect of p53 on HR is independent of its role in the G1 checkpoint and of its transactivation activity (Saintigny *et al*., 1999; Dudenhoffer *et al*., 1999; Willers *et al*., 2000). In addition, we recently showed that prolonged inhibition of replication elongation by HU or aphidicolin produces DNA breaks and actually stimulates *RAD51*-dependent recombination in a late step. In contrast, inhibitors of replication initiation did not stimulate HR (Saintigny *et al*., 2001). Here we tested whether the status of p53 may affect recombination induced by replication inhibition (RIRI) and whether p53 and *RAD51* could act on the same step (late step of the RIRI). We used a collection of cell lines containing one single copy of a

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Received 3 September 2001; revised 1 October 2001; accepted 9 October 2001

recombination substrate (Figure 1), all of them derived from the same ancestor cell line (mouse L-cells). The cell lines express an endogenous wild-type p53 protein and different ectopic mutant p53 proteins affecting G1 arrest after irradiation and/or recombination (Saintigny *et al.*, 1999). Cell lines and the effect of the various mutant p53 proteins are summarized in Table 1.

To investigate the effect of p53 status on recombination induced by replication inhibitors (RIRI), we treated parental cell lines or mutant p53-expressing derivatives (i) with different concentrations and times of contact with aphidicolin; (ii) we compared the effect of inhibitors of replication initiation (mimosine, ciclopirox olamine) to inhibitors of replication elongation (aphidicolin, hydroxyurea, Ara-c); (iii) we compared the impact of different p53 mutations on the RIRI provoked by replication elongation inhibitors. After treatment, it is noteworthy that the expression of the different p53 mutant proteins affected neither the repartition of cells in the cell cycle phases nor the cloning efficiency, compared to the control lines.

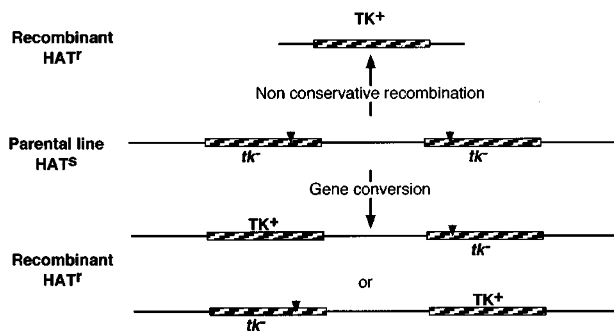

Figure 1 Recombination substrates. The recombination substrates and cell lines have already been described (Liskay *et al.*, 1984). All the cell lines derive from mouse L-tk^- cells containing a direct repeat of two Herpes Simplex Virus type I thymidine kinase (TK) genes (hatched boxes) inactivated by linker insertions (black triangles). The cells are tk^- and thus sensitive to HAT medium. Recombination will re-create a functional TK gene; thus, the recombinant cells are resistant to HAT. Mouse L cells (pJS3-10, pJS4-7-1 and their derivatives) were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. TK^+ clones were selected in HAT medium (100 μM hypoxanthine, 2 μM aminopterin, 15 μM thymidine) as described (Liskay *et al.*, 1984). The p53 derivative lines have been described (Saintigny *et al.*, 1999) and are listed in Table 1

Mutant p53 proteins stimulate the RIRI as a function of the duration of replication block

Aphidicolin is a specific inhibitor of replication elongation (Levenson and Hamlin, 1993) which is frequently used for synchronization experiments. Inhibition of replication by this drug leads to stimulation of recombination in two phases, as a function of the time of contact with the drug, i.e. the duration of replication block. Moreover, this effect is specific to cells in the S phase (Saintigny *et al.*, 2001). In CHO cells as well as in mouse L-cells (data not shown), the G2 peak disappears as soon as 6 h of contact with aphidicolin, leaving 40–50% of cells arrested in the S phase; the same proportion were observed after 24 h of treatment (see Saintigny *et al.*, 2001).

Two independent clones (HDR102 and HDR211) were used both of which express the mutant ^{His175}p53 protein leading to a defect in G1 arrest and to stimulation of recombination after irradiation. Up to 12 h of contact with aphidicolin, RIRI was very moderate and of the same extent in the two independent mutant p53 clones compared to the control cell line (Figure 2). RIRI increased substantially with replication block of 18 h (Figure 2 and Saintigny *et al.*, 2001). Expression of the mutant p53 protein strongly stimulated the RIRI when aphidicolin was added for more than 18 h. Indeed, after 24 h of treatment with aphidicolin, recombination was 3–5-fold more efficient in cell lines expressing the mutant ^{His175}p53 protein compared to the control wild-type p53 line (Figure 2). In the parental pJS3-10 line (wild-type p53) as well as in the mutant p53 lines, the effect on the RIRI was not dependent on the aphidicolin concentration since treatment with 6 or 30 μM aphidicolin gave the same results (compare Figure 2A,B).

Thus, the mutant p53 amplified the phenomenon observed with the parental (wild-type p53) line. The effect was independent of the drug concentration and was a function of the time of contact with the drug. The mutant p53 did not modify the kinetics of RIRI but strongly stimulated recombination when the RIRI was more pronounced, i.e. after 18 h of replication block. This phase corresponds to the *RAD51*-dependent phase (Saintigny *et al.*, 2001).

One hypothesis suggests that p53 acts on recombination via G1 arrest control. A defect in G1 arrest would allow replication to take place on damaged DNA

Table 1 The different cell lines used (effect of the p53 status on recombination)

Cell lines	*Mutation of p53*	*G1 arrest after γ rays*	*Homologous recombination*[a] *Spontaneous*	*Radiation-induced*
pJS 3.10	None	+	/	/
H175 DR 102	175 (Arg→His)	–	+ +	+ + +
H175 DR 211	175 (Arg→His)	–	+ +	+ + +
G175 DR 8	175 (Arg→Gly)	+	/	+
Q248 DR 4	248 (Arg→Gln)	+	+ +	+/
H273 DR 11	273 (Arg→His)	+	/	/
P273 DR 8	273 (Arg→Pro)	+	+ +	+ + +

[a]Data from Saintigny *et al.* (1999); +: stimulation of recombination; /: no stimulation of recombination.

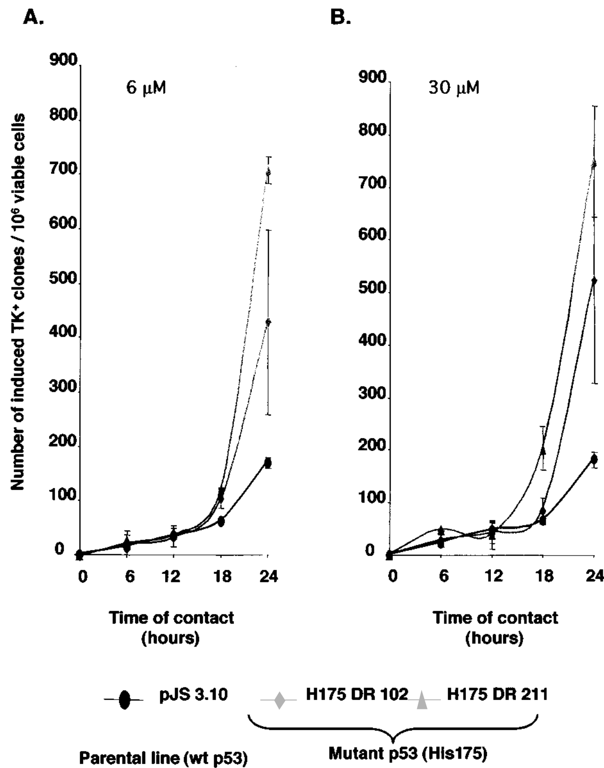

Figure 2 Stimulation of recombination as a function of time of contact with aphidicolin. (**A**) 6 μM aphidicolin, (**B**) 30 μM aphidicolin. The names of the cell lines and the p53 status are indicated on the figure. Cells were incubated with inhibitors for the times indicated. They were then trypsinized and divided into two fractions. The first fraction was used to calculate the viability by measuring the plating efficiency. The second fraction was plated under HAT selection to measure the frequency of TK^+ clones. The recombination frequency was estimated by the ratio: number of TK^+ clones over the total number of viable clones. The number of induced clones corresponds to the number of TK^+ clones in treated cells corrected for the number of TK^+ clones in untreated cells for 10^6 viable cells

matrix leading to replication arrest that could stimulate recombination. If this hypothesis is true, a mutant p53 that diminishes the G1 block would stimulate spontaneous recombination but not RIRI. In the present experiments, the mutant ^{His175}p53 affected the G1 and stimulated spontaneous HR (Saintigny *et al.*, 1999), but also stimulated the RIRI. This result indicates that the effect of p53 on recombination is additive to recombination stimulation by a G1 block defect and that the two processes should be unlinked.

p53 acts on the RIRI independently of the G1 checkpoint alteration

To support this interpretation we then analysed the impact of expression of different p53 mutants on RIRI. Some of the mutants stimulated spontaneous as well as radiation-induced recombination. One mutant (His175) affected the G1 checkpoint after irradiation whereas other mutants had no effect on the G1 arrest after radiation (Table 1 and Saintigny *et al.*, 1999). RIRI was measured in the cell lines expressing the different mutant p53 proteins, after treatment with either aphidicolin or hydroxyurea, two elongation inhibitors.

p53 mutants (His175 and Pro273) that stimulated spontaneous and radiation-induced recombination also significantly stimulated RIRI, and p53 mutants that did not stimulate spontaneous or radiation-induced recombination (His273) did not stimulate RIRI (compare Figure 3 and Table 1). The mutant Gln248 stimulated spontaneous recombination but only slightly radiation-induced recombination (Saintigny *et al.*, 1999). This mutation had very little effect on the RIRI (Figure 3), similarly to radiation-induced recombination. Since His175 affects the G1 arrest whereas Pro273 had no effect on the G1 arrest (Table 1 and Saintigny *et al.*, 1999), this confirms that the effect of p53 on RIRI is independent of its role in the G1 checkpoint.

p53 mutants act on RIRI by replication elongation inhibitors but not on initiation inhibitors

In order to compare the effect on the RIRI of replication initiation inhibition with replication elonga-

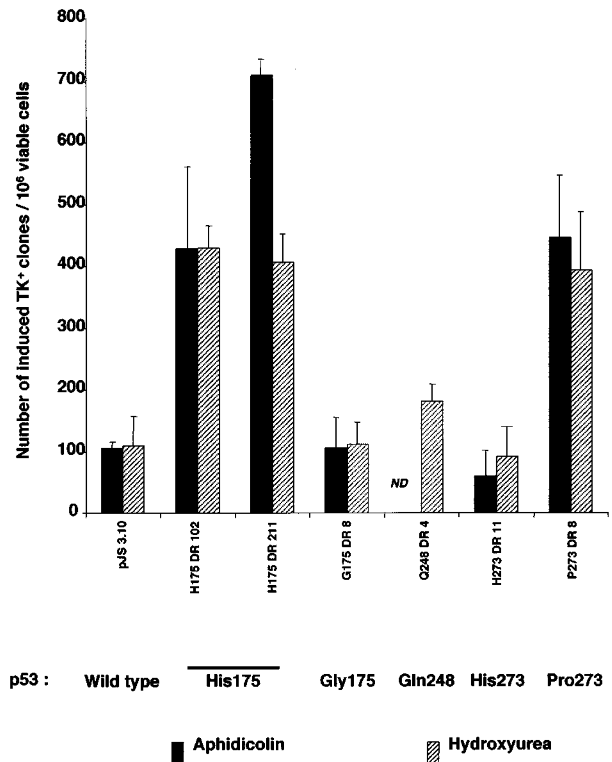

Figure 3 Effect of the different p53 mutations on the induction of recombination by aphidicolin or hydroxyurea (two elongation inhibitors). Cells were treated for 24 h with 6 μM aphidicolin (black bars) or 1 mM hydroxyurea (hatched bars). The names of the cell lines and the p53 status are indicated on the figure. The number of induced clones corresponds to the number of TK^+ clones in treated cells corrected for the number of TK^+ clones in untreated cells for 10^6 viable cells

tion inhibition, we used different drugs known to act specifically on these steps. Mimosine and ciclopirox olamine were shown to inhibit replicon initiation, whereas aphidicolin, hydroxyurea (HU) and Ara-C inhibit replicon elongation (Levenson and Hamlin, 1993). We thus treated the parental pJS3-10 (wild-type 53) and two derivative lines, (HDR102 and HDR211) both expressing the mutant ^{His175}p53 protein, with the different replication inhibitors. In the parental line (pJS3-10) without mutant p53 proteins, the three replication elongation inhibitors (aphidicolin, hydroxyurea, Ara-C) stimulated recombination twofold more efficiently than the replication initiation inhibitors (mimosine, ciclopirox olamine). Expression of the mutant ^{His175}p53 protein had no effect on recombination induced by initiation inhibitors (Figure 4). In contrast, ^{His175}p53 strongly stimulated recombination induced by the different replication elongation inhibitors.

Thus, p53 acts on recombination when replication elongation is stalled, whereas it has no effect on recombination induced by a block of replication initiation.

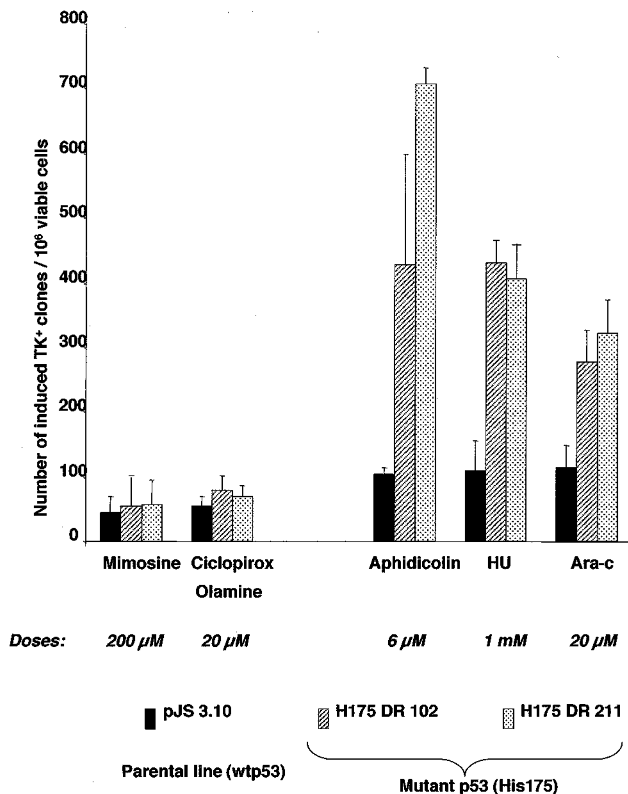

Figure 4 Stimulation of recombination by different replication inhibitors. Cells were treated for 24 h at the concentrations indicated. The names of the cell lines and the p53 status are indicated on the figure. Mimosine and ciclopirox olamine inhibit replicon initiation; aphidicolin, hydroxyurea and Ara-C inhibit replicon elongation (Levenson and Hamlin, 1993). The number of induced clones corresponds to the number of TK^+ clones in treated cells corrected for the number of TK^+ clones in untreated cells for 10^6 viable cells

Taken together these results suggest that the p53 alteration and replication arrest are two independent pathways for the recombination stimulation. This interpretation is consistent with the fact that (i) p53 mutants do not act at the initiation step but at the elongation step of replication, a step more far from G1/S transition; (ii) mutants that do not affect the G1 block after radiation, such as the Pro273, as well as mutants that alter the G1 arrest, such as His175 (Saintigny *et al.*, 1999), also stimulate the RIRI. These results show a separation between the role of p53 in the G1 checkpoint and its role in the RIRI.

Interestingly, the same p53 mutants stimulate spontaneous as well as radiation-induced recombination and RIRI; the same p53 mutants show no effect on spontaneous, radiation-induced recombination and RIRI. RIRI, spontaneous and radiation-induced recombination are all controlled by the *RAD51* pathway (Lambert and Lopez, 2000; Saintigny *et al.*, 1999). In particular, p53 and *RAD51* act similarly on the RIRI: they act weakly on inhibitors of replication initiation and both affect the same step of the RIRI, i.e. the late step. These data may suggest a role of p53 in regulation of homologous recombination via the *RAD51* pathway. In line with this, interactions between *RAD51* and p53 proteins have been reported (Sturzbecher *et al.*, 1996; Buchhop *et al.*, 1997; Susse *et al.*, 2000). Moreover, wild-type p53 protein has recently been found to play an anti-recombination role by degradation of the *RAD51*-recombination heteroduplexes containing mismatches, i.e. a direct role of p53 in the recombination mechanism (Dudenhoffer *et al.*, 1999; Susse *et al.*, 2000). The present results do not disagree with a direct role of p53 in recombination. Finally, the fact that p53 acts at the elongation step but not at the initiation step of replication is also consistent with this hypothesis. Indeed, the mechanistic role of *RAD51* protein in recombination is to promote the invasion of a double-strand DNA by a homologous single-stranded DNA (Baumann *et al.*, 1996; Baumann and West, 1998). One can thus suggest that strand exchange requires an elongated nascent DNA. In line with this, we have previously shown that the *RAD51*-dependent recombination is induced more efficiently by elongation inhibitors than by initiation inhibitors (Saintigny *et al.*, 2001). Consequently, an action of p53 on RIRI via the *RAD51* pathway should act more efficiently on elongation inhibition than on initiation inhibition of replication. Remarkably, the results presented here are fully consistent with this hypothesis. Taken together, the present results are consistent with a role of p53 in recombination, via the *RAD51* pathway.

p53 plays a fundamental role in cancer prevention. The p53 gene is the most frequently mutated gene in human tumors (Hollstein *et al.*, 1991; Levine *et al.*, 1991) and inactivation of this gene leads to cancer predisposition (Donehower *et al.*, 1992). The transactivation function of p53 is very important in tumor prevention (Jimenez *et al.*, 2000). However, these results are not conflicting with our data showing a

separation between the G1 block and the recombination regulation functions. First, some null p21 model mice are defective in G1 block without developing spontaneous malignancies (Deng *et al*., 1995). Second, the transactivation-deficient mouse model Trp53^{QS} may develop malignacies less efficiently than null p53 mutant and possibly with a different tumor spectrum (Jimenez *et al*., 2000). Third, Trp53^{QS} MEFs can be transformed *in vitro* by cooperating oncogenes suggesting that additional genetic changes were required for transformation, as discussed (Jimenez *et al*., 2000). Thus the role of p53 in genes expression and in cell cycle checkpoint should be very important in cancer protection, although, an additional role in recombination control may also be essential for tumor prevention. Indeed, excess recombination can result in various diseases (for review see Purandare and Patel, 1997), and loss of heterozygosity or oncogene translocation via homologous recombination have been proposed (Cavenee *et al*., 1983; Onno *et al*., 1992). In addition, the present results suggest that p53 acts via the *RAD51* pathway. *RAD51* has also been shown to interact with the breast cancer-preventing proteins BRCA1 and BRCA2 (Mizuta *et al*., 1997; Scully *et al*., 1997; Marmorstein *et al*., 1998). While up-regulation of *RAD51* protein has been described in human tumors (Maacke *et al*., 2000), over-expression of *RAD51* stimulates spontaneous as well as induced recombination (Lambert and Lopez, 2000). These results reveal an additional potential role for the tumor suppressor p53 protein in the regulation of the connections between replication arrest and homologous recombination. This role may be of crucial importance in the control of genome maintenance and transmission.

Acknowledgments
We thank D Rouillard (Institut Curie, Paris) for FACS expertise. We are grateful to Drs P Bertrand, D Marsh, R Monnat for critical reading of the manuscript. Thanks are due to M Liskay for providing the pJS3-10 cell lines. Y Saintigny was supported by a fellowship from 'la Fondation pour la Recherche Médicale'. This work was supported by Electricité de France, ARC (9822 and 9238).

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