Discriminatory suppression of homologous recombination by p53

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ABSTRACT
Homologous recombination (HR) is used in vertebrate somatic cells for essential, RAD51-dependent, repair of DNA double-strand-breaks (DSBs), but inappropriate HR can cause genome instability. A transcriptional transactivation-independent role for p53 in suppressing HR has been established, but is not detected in all HR assays. To address the basis of such exceptions, and the possibility that suppression by p53 may be discriminatory, we have conducted a controlled comparison of the effects of p53 depletion on three different kinds of HR. We show that, within the same cells, p53 depletion promotes both intra-chromosomal HR (ICHR) and extra-chromosomal HR (ECHR), but not homologous DNA integration (gene targeting; GT). This conclusion holds true for both spontaneous and DSB-induced ICHR and GT. We show further that non-conservative ICHR is more susceptible than conservative ICHR to inhibition by p53. These results provide strong evidence that p53 can discriminate between different forms of HR and, despite the fact that GT is used experimentally for gene disruption, is consistent with the possibility that p53 preferentially suppresses genome destabilizing forms of HR. While the mechanism of suppression by p53 remains unclear, our data suggest that it is independent of mismatch repair and of changes in RAD51 protein levels.

INTRODUCTION
Homology-directed repair (HDR) of DNA uses homologous recombination (HR) for the accurate repair of DNA double-strand breaks (DSBs) and requires an intact DNA template homologous to the damaged locus (1). During the S and G2 phases of the cell cycle, templates with perfect homology are available throughout the cell cycle, on chromosome homologues or, for repeat sequences, elsewhere in the genome, but their use is genome-destabilizing, leading to loss of heterozygosity or genome rearrangements (4,5). Mechanisms for restricting the amount, or perhaps the type, of HR have therefore evolved. Thus many proteins have been implicated in suppressing HR, including products of the oncogenes bcl-2 (6), bcr-abl (7) or bcl-x(L) (8) and the tumour suppressors MSH2 (9–11) BRCA2 (12–14), BLM and WRN (15,16) and p53 (17).

Stimulation of HR in response to p53 inactivation was first described for an extra-chromosomal HR (ECHR) assay involving replicating SV40 genomes (18), but has also been demonstrated for ECHR assays with non-replicating plasmids (19,20). Intra-chromosomal HR (ICHR) assays are consistently stimulated by p53 inactivation (21–26), and it has been established that this effect depends on parts of the p53 protein distinct from those required for transcriptional transactivation, G1 arrest and apoptosis in response to DNA damage (23–27). The mechanism by which p53 suppresses HR remains uncertain, but many biochemical properties of p53 that may be relevant have been described. These include exonuclease, DNA renaturation and DNA strand-transfer activities, an ability to bind DNA recombination intermediates, and associations with proteins implicated in HR (RAD51, RAD52, RAD54, RPA) or its control (BLM, WRN, BRCA1, BRCA2, MSH2) [see (20) and (28) and references therein]. In the case of RAD51, there is evidence that a direct interaction with p53 is required for suppression of HR (29,30), and that RAD51 over-expression promotes various kinds of HR [reviewed in (31)], p53 could conceivably suppress HR by reducing RAD51 expression or stability.

An important question concerning the suppression of HR by p53 is whether it is discriminatory. It is conceivable that p53 globally suppresses HR, leaving sufficient activity to maintain cell viability. Alternatively, p53 may discriminate between genome-stabilizing and genome destabilizing HR events, suppressing only the latter. Such discrimination offers obvious advantages, and could even be essential, given the lethality of RAD51 disruption. There is evidence (28,32,33) that p53 may, like the mismatch repair (MMR) system (10,11), preferentially suppress HR between imperfectly homologous sequences, and this would certainly represent a discriminatory, genome-stabilizing suppression of HR. The possibility that p53 may
discriminate on the basis of variables other than the extent of homology, such as the relative positions of HR substrates, remains to be tested, however. A small number of studies (34–36) describe HR assays that were p53-inhibitive, but it remains unclear whether these can be taken as evidence of discriminatory suppression or whether some aspect of the host cells or assay conditions used precluded detection of p53-sensitivity. In one study (35), for example, it was pointed out that the p53-inseptivity of gene targeting (GT; HR between chromosomal and extra-chromosomal DNA) might be attributable to the unusual cytoplasmic location of p53 in the mouse ES cells used. Similarly, further HR assays must be carried out to determine whether all HR in HCT116 (colun carcinoma) cells is p53-inseptive, or whether the reported p53-inseptivity of GT and sister chromatid exchange (SCE) in these cells (34) is indicative of discriminatory suppression. Also, the p53-inseptivity of ECHR in mouse embryo fibroblasts (36) contrasts with its p53-sensitivity in others cell lines (18–20), suggesting that some unusual aspect of the host cells or ECHR assay conditions used, may have precluded detection of p53-sensitivity.

Evidence for discriminatory suppression of HR by p53 therefore requires further experiments in which different kinds of HR assays are compared in the same cell lines. Accordingly, we describe here a comparison of ICHR, ECHR and GT in both human fibrosarcoma (HT1080) and embryonic kidney (HEK293) cell lines and show that GT is inseptive to p53 depletion by RNA interference, whereas ECHR and ICHR are both stimulated. Furthermore, we find that ECHR is p53-inseptive in colon carcinoma (HCT116) cells where GT and SCE were previously shown to be p53-inseptive (34), and that non-conservative ICHR is suppressed more than conservative ICHR. Our data therefore provide strong evidence that suppression of HR by p53 is indeed discriminatory, and is characterized by a remarkable inactivity towards GT. Despite the experimental use of GT for genome disruption, we argue that such discrimination is compatible with the notion that p53 preferentially suppresses genome-destablizing HR. Finally, because we find no evidence of RAD51 accumulation following p53-depletion, and because ECHR suppression is detectable in MMR-defective HCT116 cells, we conclude that suppression by p53 is MMR-independent and cannot be explained in terms of RAD51 depletion.

MATERIALS AND METHODS

Cells

HT1080 and HEK293 cell lines were from the American Tissue Culture Collection, except for GT experiments in HEK293 where a derivative (HEK293E, Invitrogen) expressing the Epstein Barr virus protein EBNA-1 was used. Conditions of culture were as described previously (37).

Plasmids

- pCMV3xnsI-SceI (38), pCMVβ (BD Biosciences), pCX-EGFP, p451-2 and p429-1 (39) and pPHRThyg (37) have been described. A version of pDrNneo (40) was used in which the original XhoI site was changed to a HindIII site. To make pSUPER-p53/neo, the p53 shRNA cassette was removed from pSUPER-p53 (41) as a 247 bp HincII/PstI fragment and cloned into pEGFP-C1 (Invitrogen), which had been cut with PstI, and AseI to remove the EGFP cassette. A similar procedure was used to make pSUPER.neo using the equivalent HincII/PstI fragment from pSUPER (41). The pPHRThyg derivative pPHRThygBcl was generated by linearization at the unique BclI site (see Figure 5), followed by end-filling and religation.

- pPU-I-RO/zeo was made in three steps. First, pBL-Puro/R was made by cloning a 1.3 kb PvuII/BamHI fragment, carrying the Puro expression cassette from pPUR (Clontech), into the EcoRV site of pBSKS(+) (Stratagene), with the PvuII end oriented adjacent to the EcoRV site of pBSKS(+). Second, sites for I-SceI and SacI (annealed oligonucleotides 5'-AATTACCCCTGTATCCCTAGAAGGTCTCT-3' and 5'-AGACTCTTACGTTAAAAGGATTTATTACGTTGTGTAATC-3') were cloned into the MscI site of pBL-PUR/R to make pPU-I-RO. Third, a 1.4 kb NotI/XhoI fragment, carrying the Zeo cassette from pfoxZeo (see below), was cloned into the NotI (e/f) site of pPU-I-RO, to make pPU-I-RO/zeo. Three steps were required to make pfoxZeo. First, pBSloXP was made by cloning a loxP site (annealed oligonucleotides 5'-CTAGATAACTTCCGTATATGATGTATGACTACGAAATTAC-3' and 5'-ACTAGATAACTTCCGTATATGATGTATGACTACGAAATTAC-3') into the SpeI site of pBSKS(+). Second, a 1.3 kb SspI/BamHI fragment, carrying the Zeo cassette from pZeoSV (Invitrogen), was cloned into the EcoRV/BamHI site of pBSloXP to form pBSZeoLoXP. Third, MscI and loxP sites (annealed oligonucleotides 5'-TGGATGCTGCAATATACTTCCGTATATGATGTATGACTACGAAATTAC-3' and 5'-AGCTTACGTTAAAAGGATTTATTACGTTGTGTAATC-3') were cloned into the HindIII/XhoI site of pBSZeoLoXP to form pfoxZeo, whose loxP site are in the same orientation.

Stable transfection

Electroporation was used as described in (37) for Gene targeting because lipofection is less efficient in this context (42), and for other stable transfections because it favours single integrations. For HT1080 cells, 10 μg of each plasmid was used per electroporation. For HEK293 cells, 2.5 μg of each plasmid was used per electroporation. Electroporation typically caused some cell death (e.g. 10–20%), but no systematic differences in killing were evident for the various treatments used prior to electroporation. The following concentrations were used for selecting drug-resistant colonies: G418 (200 μg/ml active concentration), Hygromycin (100 μg/ml), zeocin (200 μg/ml) and puromycin (0.8 μg/ml) 6TG (45 μg/ml). pSUPER-p53/neo and pSUPER/neo were delivered by lipofection and G418R clones were screened by western blot: all (6/6) pSUPER/neo transfectants had normal p53 levels whereas 19/36 pSUPER-p53/neo transfectants showed substantial p53 knockdown. DRneo was delivered by electroporation after linearization with Bsal. HygroR colonies were cloned or pooled (≥1000 per pool) for further analysis. Clones with a single integration were identified by Southern analysis. pPU-I-RO/zeo was
delivered by electroporation after linearization with SpeI and
cloned p293PU-I-6.2 was one of several a zeocin-resistant clones
shown by Southern analysis to have a single integration. Un-
less stated otherwise, p5′APURO was linearized with BsiWI,
and pHPRTThyg with Sall, prior to electroporation.

**siRNA transfection**

RNA oligonucleotide pairs specific for p53 were p5′-CGUACGGAGAUACUUGAdTdT-3′ and p5′-UCGAA-
CUAUCCGGCUAGdTdT-3′, and for luciferase were p5′-UUGCAUGGAUUAUUGACdTdT-3′ and p5′-GCA-
ACUAUCAUAUGCAAdTdT-3′, were obtained pre-
 annealed from Dharmacon and delivered by oligofectamine
(Invitrogen) according to manufacturer’s instructions. Cells
were plated at 50% confluence in 24-well plates or in large
(15 cm diameter) plates. The following day, a mixture contain-
ing siRNA (1200 nM, unless stated otherwise) and oligofec-
tamine (3%, v/v) in OptiMEM was prepared according to
manufacturer’s instructions, and added to cells (100 µl/well,
or 2 ml/15 cm plate) with 5 volumes of medium, to give a final
siRNA concentration of 200 nM. After 48 h, the siRNA-
containing medium was replaced with normal medium; if
necessary at this stage, cells were trypsinized and replated
or 2 ml/15 cm plate) with 5 volumes of medium, to give a final

**Western blots**

Immunoblots were as described previously (39). For p53
detection, a primary monoclonal antibody to human p53 I
(DAKO, M7001; 1:1000 dilution) and a secondary, horser-
adish peroxidase-conjugated goat anti-mouse immunoglobulin
antibody (DAKO, P0447; 1:1000 dilution) were used.

**γ-irradiation**

Cells were irradiated with a 137Cs source (CIS BIO IBL 367
irradiator) at a dose rate of 1.85 Gy/min while still attached to
the wells of a 6-well plate.

**Flow cytometry**

Flow cytometric analysis of cells for DNA content or EGFP
expression was as described previously (39).

**Southern blots**

Standard methods were used as described elsewhere. Single
integrations of DRneo were identified by BamH1 digests and
probed with a neo probe; colonies with only one fragment
were chosen. Single pPU-I-RO/zeo integrations were identified by
SacI digests probed with a puro probe: colonies with only one
fragment in addition to the 3.5 kb fragment were chosen. The
neo probe was a 400 bp PCR product from the S2neo cassette,
adjacent to the HindIII site. The Puro probe was a 1 kb HindIII
fragment from pBL-Puro/R. The hygro probe was a 945 bp
AatII/Sacl fragment of the hygromycin open reading frame.

**ICHR assays**

DRneo-transfected cells were transfected with siRNA (see
above) in the absence of hygromycin selection. Cells were
then trypsinized, counted, electroporated (3–7 million per
electroporation) with pCMV3xlns-I-SceI or pCMVβ
and replated at ~3 million cells per 15 cm plate. After a further
24–48 h, selection in G418 was started, and continued for 10–
14 days when drug-resistant colonies were counted. To score
for conservative ICHR only, selection was continued for an-
other 5–7 days in the presence of both G418 and hygromycin,
and the number of surviving colonies counted. Frequencies are
expressed as colonies per million cells electroporated.

**ECHR assays**

The previously described ECHR assay was used (39) except
that HR DNA substrates were delivered by lipofectamine 2000
and this was preceded by siRNA transfection. Briefly, siRNA-
transfected cells (as above) were processed immediately, if in
24-well plates or, if in 15 cm plates, were trypsinized and
distributed into wells of 24-well plate at ~50% confluence
and allowed to attach for 4–6 h before proceeding. Cells were
transfected with a positive control plasmid (pCX-EGFP), a
negative control plasmid (p451-2) or equal weights of
ECHR substrates (p451-2 and Sall-linearized p429-1). For
each experiment, a single amount of control plasmid was
transfected (0.8 or 1 µg per well), whereas varying amounts of
ECHR substrates (0.125–2 µg of each) were used.

Cells were analysed for EGFP expression by flow cytometry
24 h after the addition of DNA. The % ECHR was calculated
as [{(% EGFP + cells after p451-2/p429-1 cotransfection) −
(EGFP + cells after p429-1 transfection)]} × 100%
EGFP + cells after pCX-EGFP transfection). Values for (%
EGFP + cells after p429-1 transfection) were usually zero.

**GT and RI assays**

The HPRT-GT assay was used as previously described (37)
and, in some instances (see Supplementary Material, Table 2),
was preceded by siRNA transfection in 15 cm plates (see
above). For each electroporation, ~10–25 million cells were
used, 95% of which were selected in hygromycin and
6TG, the remaining 5% selected in hygromycin only. For
PURO-GT, which was always preceded by siRNA transfection
in 15 cm plates, cells were trypsinized and counted. For each
electroporation, 1–5 million cells were co-electroporated with
p5′DPURO and pCMV3xlns-I-SceI or pCMVβ β. All or most
(95%) of the electroporated cells were replated (~3 million per
15 cm plate) for selection in puromycin. Remaining cells were
replated (~0.5 million per 9 cm plate) for selection in hygro-
mycin. Selection was started 24–48 h after electroporation and
continued for 7–14 days and colonies counted. To score for
insertion events, puromycin-resistant colonies were selected
for a further 10 days in the presence of both hygromycin and
puromycin, and colonies recounted. GT and RI frequencies are
expressed as colonies per million cells electroporated.

**RESULTS**

**Transient and stable knockdown of p53 expression in HT1080 and HEK 293 cells**

Knockdown of p53 expression by RNA interference (RNAi)
was achieved in both HT1080 and HEK293 cells. In HT1080
cells, both transient and stable knockdown were demonstrated.
itors of transcriptional activation by p53 (45–48), we detected
the adenovirus E1A and E1B genes (44), known inhib-
Consistent with the fact that HEK293 cells constitutively
resistant (G418 R) clones were screened by western analyses
against p53 mRNA and the neomycin phosphotransferase gene
PER-p53 (41), which expresses a short hairpin RNA (shRNA)
resistance with pSUPER-p53/neo, a modified version of pSU-
For stable knockdown, cells were transfected to G418-
Transient knockdown was used as a control, the average fold
in the absence of hygromycin before siRNA treatment, the base-
and HT/DRneoP3) that had been extensively passaged in
ICHR, the plasmid DRneo (40) was introduced into both
HT1080 and HEK293 cells and stably transfected cells
experiments described below showing HR to be stimulated.

**Stimulation of spontaneous and I-SceI-induced ICHR
by p53 knockdown**

Several measurements of the effect of p53 depletion on ICHR
were made (Supplementary Material, Table 1) and represent-
ative data are shown in Figure 3. To measure the frequency of
ICHR, the plasmid DRneo (40) was introduced into both
HT1080 and HEK293 cells stably transfected cells
were selected in hygromycin. Pools of hygromycin-resistant
whether or not they are transfected with siRNA against luci-
control (luciferase) or experimental (p53)
and HT/DRneoC22) were ana-
were selected in hygromycin. Pools of hygromycin-resistant

   **Figure 1. Stable and transient p53 knockdown in HT1080 and HEK293 cells.** Immunoblots for p53, RAD51 and actin expression were carried out on HT1080 clones stably transfected with pSUPER derivatives (lanes 1–12), or on HT1080 and HEK293 cells transiently transfected with siRNA specific for p53 or luciferase, as indicated (lanes 13–28). LF, Lipofectamine only, NT, no treatment. In lane 28, luciferase siRNA was used at 200 nM.

To confirm that the reduction in p53 was sufficient to have
physiological consequences, DNA damage-induced cell cycle
in G1, a well-known p53-mediated response (43), was
tested. Parental HT1080 cells show a typical reduction in
G1 arrest in G1, a well-known p53-mediated response (43), was
accumulation predominates (Figure 2c and d). Consistent with the fact that HEK293 cells constitutively
express the adenovirus E1A and E1B genes (44), known inhib-
itors of transcriptional activation by p53 (45–48), we detected
no DNA damage-induced G1 arrest in HEK293 cells
(Figure 2e–g). G2 arrest was barely if at all detectable, sug-
gesting that p53-independent G2 arrest is somehow weakened
in HEK293 cells. That p53 knockdown was physiologically
effective in HEK293 cells was therefore established only in the
experiments described below showing HR to be stimulated.
respectively, and not significantly different ($p = 0.35$). Southern analysis of G418R colonies derived from HT/DRneoP2 indicated that 12/12 had undergone non-conservative ICHR, a result consistent with the predominance of non-conservative events, although in larger sample, a proportion (~25%) of conservative events would have been expected. As expected, all (8/8) G418R HygR colonies analysed had undergone conservative events. Representative Southern analyses are shown in Figure 3e.

Similar results were obtained in HEK293 pools 293/DRneoP1 and 293/DRneoP2 (Figure 3d; Supplementary Material, Table 1, Expts 6 and 7) providing clear evidence that p53 depletion stimulates both spontaneous and I-SceI-induced ICHR. As in HT1080 cells, p53-sensitivity was evident whether selection was for total ICHR or for conservative ICHR events only (Figure 3c), and the fold increases were greater for the former than for the latter (8.6 versus 2.9 and 15.1 versus 4.2). Together these results indicate that, in this ICHR system, non-conservative ICHR is more frequent than conservative ICHR and that, while p53 can suppress both, it preferentially suppresses the former.

**ECHR is stimulated by p53 knockdown**

To measure ECHR, we co-transfected two plasmids (p451-2 and p429-1) with different defective EGFP cassettes that can undergo intermolecular HR to form a functional EGFP cassette (39). Cells were transfected with siRNA 72 h before introducing the recombination substrates, and analysed by flow cytometry for EGFP expression 24 h after. The ability of p53 knockdown to stimulate ECHR was evident in both HT1080 and HEK293 cells, though more pronounced in the latter cells (Figure 4). Interestingly, the degree of sensitivity to p53-depletion was dependent on the concentration of DNA used, sensitivity being lower at high concentrations, especially in HT1080 cells. The maximum levels of ECHR were ~4-fold higher in HEK293 cells than in HT1080 cells (Figure 4). The ability to detect an effect of p53 depletion on ECHR may therefore depend on the HR capacity of the cells used and, where this is low, require the use of low concentrations of DNA substrates. Using the same assay, we also found ECHR to be stimulated by p53 depletion in HCT116 cells (Figure 4).

**GT and random integration are not affected by p53 knockdown**

To assess the effects of p53 knockdown on GT in HT1080 cells, we used a previously described targeting construct (pHPRThyg) for disruption of the HPRT gene (37). Targeted integration of pHPRThyg at the HPRT locus results in cells that are resistant to both 6-thioguanine (6TG) and hygromycin whereas random integration (RI) generates cells that are only hygroR (Figure 5a). In a series of electroporations (Table 1), the effects of p53 knockdown, stably, transiently or both, on random and targeted integration frequencies were measured. Transient p53 knockdown was achieved by lipofection of siRNA 72 h prior to the introduction, by electroporation, of the pHPRThyg (methods). Western analyses on samples taken at the time of electroporation confirmed that p53 depletions similar to those shown in Figure 1 were achieved, and Southern analyses confirmed that colonies resistant to both drugs had undergone GT at the HPRT locus (data not shown). The results, whether taken individually or averaged for the three experiments, show that, in contrast to ICHR and ECHR, both GT and RI are insensitive to p53 status (Table 2 and Figure 5b, Supplementary Material).

A different system was required to assess GT in HEK293 cells. An HEK293 transfectant (293PU-I-6.2), carrying a
single copy of the target construct pPU-I-RO/zeo (Figure 6a, i), was transfected with the targeting construct p50DPURO (Figure 6a, ii). HR between the defective puromycin-resistance cassettes (puro) in the target and targeting constructs regenerates a functional cassette. In principle, this can occur via replacement- or insertion-type GT mechanisms (Figure 6a) and Southern analyses of PuroR clones confirmed that both types of events occur (Figure 6b). The puro cassette in pPU-I-RO/zeo is defective because an I-SceI site has been inserted into the coding sequence. GT can therefore be greatly stimulated by I-SceI-catalysed DSB at the target locus, as has been shown in other GT systems (49,50). Accordingly, the targeting construct was co-electroporated with pCMV-I-SceI or with a control construct (pCMVb). Transient p53 knockdown was again achieved by siRNA lipofection 72 h prior to electroporation, and western analysis of samples taken at the time of electroporation were used to confirm p53 knockdown (data not shown). The frequency of hygroR colonies (which is overwhelmingly made up of random integrants) was unaffected by p53 depletion, with or without I-SceI expression.

Figure 3. ICHR assays. (a) The DRneo substrate is depicted before and after conservative or non-conservative ICHR. Thick arrows represent neo (white) and hygro (stippled) expression cassettes; circles represent their associated promoter regions. Relevant sites for HindIII (H) and fragments it generates are shown. The 3' neo cassette lacks 5' sequences including the promoter region and the S2neo cassette is disrupted by insertion of an I-SceI site at the NcoI (N) site. (b–d) The effect of control (black) or p53 (white or grey) siRNA on frequencies of G418^- (grey background) or G418^-/Hygro^- (white background) colonies derived from the indicated DRneo-carrying cells, with or without I-SceI expression, as indicated. (d) Southern analysis of three G418^- and three G418^-/Hygro^- colonies derived from HT/DRneoP2. Control (C) DNA was 10 pg of DRneo. All DNA was digested with HindIII and probed with the neo cassette probe indicated in (a).
(Table 2 and Figure 6c). Without I-SceI stimulation, the total (insertion plus replacement) GT frequency, as indicated by the appearance of puroR colonies, was barely measurable, but I-SceI-stimulated total GT was readily measurable and found to be unaffected by p53 knockdown (Table 2, Expt B). The frequency of insertional GT, as indicated by the appearance of puroR/hygroR colonies, was approximately one-third of the total GT frequency, and also unaffected by p53 knockdown (Table 2, Expt B). Southern analyses similar to those in Figure 6b confirmed that puroR/hygroR colonies had the structure expected for insertional GT while Puro R colonies represented a mixture of insertion and replacement GT events (data not shown).

Different sensitivities to p53 do not correspond to differences in the degree of DNA mismatch

It is known that HR is inhibited by the mismatch repair (MMR) system if sequence mismatches are generated during HR (10). Thus, for example, GT is made more efficient by inactivation of the MMR system, but only if mismatches exist between the target locus and targeting construct (11,51,52). It has been proposed that p53 inhibits HR in a similar way, perhaps in cooperation with the MMR system (28,32). None of the particular HR assays we have used here is expected to involve single base-pair mismatches, including HPRT-GT (the targeting construct was made with HT1080 genomic DNA). Insertion/deletion loops (IDLs), which can also be recognized by the MMR system, of the following nucleotide lengths could be generated, however: 25 nt (ECHR), 14 nt (ICHR), 27 nt (puro-GT) and 2119 nt (HPRT-GT). By considering the first three of these, which are all of a similar size, it is clear that p53 insensitivity of GT cannot simply be explained in terms of differences in the extent of DNA mismatches. In the case of HPRT-GT, it is possible that the large IDL is recognized less efficiently by the MMR system and that this might explain p53 insensitivity. To test this directly, we introduced a small (4 bp) insertion into the left-hand arm of homology of pHPRThyg to make pHPRTBclI- (see Methods) and repeated the GT, but again found no evidence for a stimulation of GT following p53 knockdown (Table 3).

DISCUSSION

The results described here not only support the proposed transactivation-independent role for p53 in suppressing HR, but also provide first clear evidence that p53 can discriminate between different forms of HR. In particular, GT is shown to be unaffected by p53 depletion while, in the same cells, ICHR and ECHR are stimulated. Such differential p53-sensitivity holds for a variety of assays in each of the three cell lines where it has been analysed: HT1080, HEK293 and HCT116. Thus GT was found to be p53-insensitive whether it was
spontaneous or DSB-induced, or whether it occurred by an insertion or replacement type mechanism. Conversely, ICHR was found to be p53-sensitive regardless of whether it was spontaneous or DSB-induced or whether it occurred by a conservative or non-conservative mechanism (though the latter was more sensitive).

An ability to discriminate between different forms of HR may be useful to cells for suppressing genome-destabilizing forms of HR whilst allowing genome-stabilizing forms to proceed. It is interesting to consider how the observed direction of discriminatory suppression may be compatible with such a scheme. The suppression of non-conservative ICHR, which causes deletion, duplications or inversions, is clearly consistent with this scheme. Conservative ICHR, while not leading to such gross rearrangements, is capable of generating mutations [e.g. gene conversion of a normal allele by a pseudogene (17)] and, notwithstanding its value and widespread use as an assay for HDR, must therefore be regarded as genome-stabilizing, and appropriately suppressed by p53.

There is no obvious natural correlate for ECHR, but as discussed below, it involves the same mechanism as non-conservative ICHR, and this may explain its sensitivity to p53. GT is widely used in the laboratory for the purpose of gene disruption, and might therefore be regarded as genome-destabilizing, making its lack of suppression by p53 surprising. Under normal circumstances, however, opportunities for GT to occur are extremely rare, compared, for example, to HR between chromosomal repeat sequences. GT therefore probably occurs via an HR pathway that has evolved for another purpose, and we speculate, based on its insensitivity to suppression by p53, that it employs a genome-stabilizing pathway. For example, GT constructs may be able to associate, albeit at low efficiency, with recombination complexes involved in the normal HDR between single-copy sequences on sister chromatids. Unfortunately, there is no direct assay for such genome-stabilizing HDR between non-repeat sequences. The nearest assay is arguably SCE and this also appears to be p53-insensitive (34). Thus a rational physiological basis for the observed discriminatory suppression by p53 can be envisaged, but further work is required to test its validity.

Ultimately, some structural difference at the DNA/chromatin level between GT and ICHR or ECHR must be recognized by p53, directly or indirectly, and the question remains as to what this difference may be. It has been suggested that p53 may suppress HR in concert with the MMR system (28,32). To explain the differential suppression, we have observed in terms of MMR, would require that the ICHR and ECHR assays used here generate heteroduplexes with mismatches while GT assays do not. As already pointed out, however (see results), this is not the case. Furthermore, the HCT116 cell line, in which we observed p53-dependent suppression of its own HR activity (33,34), is MLH1-deficient and therefore defective for MMR (53). These considerations argue against a role for MMR, not only in differential HR suppression by p53, but also in p53-dependent HR suppression generally.

A more likely possibility is that p53 can differentiate between strand invasion (SI) and single-strand annealing (SSA) mechanisms of HR, preferentially suppressing the latter. SI is thought to be RAD51-dependent and SSA to be RAD51/54-independent (54). There is evidence that BRCA2 differentially influences these two forms of HR (55), and it is conceivable that p53 works in a related way. This would certainly be consistent with p53-insensitivity of GT that is generally believed to occur by SI, being RAD54-dependent (56)

### Table 1. Targeted and random DNA integration are unaffected by p53 knockdown in HT1080

<table>
<thead>
<tr>
<th>Expt</th>
<th>Cells</th>
<th>p53</th>
<th>n</th>
<th>RI (×10^3)</th>
<th>TI (×10^4)</th>
<th>TI/RI (%)</th>
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<tr>
<td>A</td>
<td>Clone 26</td>
<td>hi</td>
<td>3</td>
<td>6.4 ± 0.02</td>
<td>3.33 ± 0.57</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td>B</td>
<td>Clones 6, 8 or 14</td>
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<td>4</td>
<td>3.5 ± 0.46</td>
<td>2.41 ± 0.02</td>
<td>0.67 ± 0.09</td>
</tr>
<tr>
<td>A–C</td>
<td>Clones 2, 24 or parental HT1080</td>
<td>lo</td>
<td>6</td>
<td>2.2 ± 0.46</td>
<td>2.1 ± 0.43</td>
<td>1.00 ± 0.25</td>
</tr>
<tr>
<td>C</td>
<td>Clones 6, 8, 14, or 26</td>
<td>hi</td>
<td>4</td>
<td>2.1 ± 0.14</td>
<td>2.4 ± 0.55</td>
<td>1.1 ± 0.28</td>
</tr>
</tbody>
</table>

### Table 2. Targeted and random DNA integration are unaffected by p53 knockdown in HEK293

<table>
<thead>
<tr>
<th>Expt</th>
<th>EP</th>
<th>siRNA</th>
<th>I-SceI</th>
<th>N (×10^6)</th>
<th>RI (hygromycin) (×10^3)</th>
<th>TI (puro) (×10^4)</th>
<th>TI (puro/hygromycin) (×10^6)</th>
<th>TI/RI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>luc</td>
<td>–</td>
<td>5.09</td>
<td>5.5 ± 0.61</td>
<td>0.13 ± 0.11</td>
<td>ND</td>
<td>0.025 ± 0.022</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>luc</td>
<td>+</td>
<td>4.31</td>
<td>ND</td>
<td>23.3 ± 4.00</td>
<td>9.5 ± 4.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

### Footnotes

aThe indicated clones were grown individually or as combined cultures. In some cases, luciferase or p53 siRNA were used. For full details see Supplementary Material, Table 2.

bNumber of electroporations; 0.9–2.4 × 10^7 cells per electroporation.

*Targeted integration (TI) and random integration (RI) frequencies (average ± SD).
transfection of BsiWI-linearized p50
(MscI (M) and BsiW1(B), and DNA fragments generated by them, are shown. Associated promoter regions (circles) are shown. Relevant sites for SacI (S), hygromycin (grey) resistance cassettes (thick arrows) including their pPU-I-RO/zeo, respectively. Puromycine (black), zeocin (white) and grey lines represent vector (pBSIIKS(+) replacement (iii) or insertional (iv) GT events, are shown. Thick black and indicated), are shown. The data are from experiment A in Table 2.

Figure 6. GT and RI in HEK293 cells are unaffected by p53 knockdown. (a) Maps of the target plasmid pPU-I-RO/zeo as integrated in clone 293PUI-6.2 (i), the targeting construct p5'ΔPURO (ii) and the product of replacement (iii) or insertion (iv) GT events, are shown. Thick black and grey lines represent vector (pBSIIKS(+) vector), and chromosomal DNA flanking pPU-I-RO/zeo, respectively. Puromycin (black), zeocin (white) and hygromycin (grey) resistance cassettes (thick arrows) including their associated promoter regions (circles) are shown. Relevant sites for SacI (S), MscI (M) and BsiW1(B), and DNA fragments generated by them, are shown. (b) Southern analysis of DNA from clone 293PUI-6.2 (P) and three PuroR clones after co-transfection of uncut p5'ΔPURO and pCMV3x3xS-I-Sce1. DNA was digested as indicated and probed with puro (left) or hygro (right) probes. (c) The frequencies of random and targeted integration after transfection of BsiW1-linearized p5'ΔPURO into clone 293PUI-6.2, with (white) or without (black) p53 knockdown, or I-Sce1 expression (as indicated), are shown. The data are from experiment A in Table 2.

Table 3. p53 knockdown does not stimulate HPR-GT involving mismatches

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>N (×10^-5)</th>
<th>n</th>
<th>p53</th>
<th>RI (×10^0)</th>
<th>TI (×10^6)</th>
<th>TI/RI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHPRThygBclIF</td>
<td>2.5</td>
<td>2</td>
<td>hi</td>
<td>2.2 ± 0.5</td>
<td>4.8 ± 0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>pHPRThygBclIF</td>
<td>2.5</td>
<td>2</td>
<td>lo</td>
<td>2.0 ± 0.7</td>
<td>2.2 ± 0.84</td>
<td>1.1</td>
</tr>
</tbody>
</table>

stimulated by RAD51 over-expression (35,37). It would also be consistent with the p53-sensitivity of most ECHR, which occurs predominately by SSA (57,58). Indeed, in one study of ECHR in H1T1080 cells (19), HR between direct repeats was found to be p53-sensitive, whereas HR between inverted repeats (which cannot occur by SSA) was not. Furthermore, the fact that non-conservative ICHR, which most likely occurs by SSA, was more p53-sensitive than conservative ICHR, which cannot occur by a simple SSA mechanism, is again consistent with preferential suppression of SSA. Conservative ICHR was nevertheless clearly p53-sensitive, suggesting that discrimination between SI and SSA cannot fully account for the observed pattern of p53-sensitivities.

It was notable that suppression of HR by p53 was still detectable in HEK293 cells (e.g. Figures 3d and 4) even though these cells express the adenoviral E1A and E1B genes (44) whose products prevent transcriptional transactivation (45,46,48) by binding, at least in the case of E1B, to the N-terminus of p53 (47). We therefore conclude that the transforming activities of E1A/E1B do not include genome destabilization through loss of p53-mediated HR suppression. This observation is also fully consistent with the proposed mechanism for suppression of HR that involves p53 residues quite distinct from those involved in transcriptional transactivation, and probably required for direct interactions with RAD51 (17). Nevertheless, our results do appear to rule out p53-induced RAD51 depletion as the mechanism, because we saw no RAD51 accumulation in response to p53 depletion. Although this contrasts with reports of RAD51 up-regulation in p53-depleted cells (29,30), it is consistent with the fact that RAD51 over-expression stimulates GT (35,37), while p53-depletion does not.

In previous studies where GT was found to be insensitive to p53 status, in HCT116 (34) and ES cells (35), ICHR and ECHR were not analysed, and the possibility that p53-sensitivity may depend on the type of HR assays was not considered. Our observation that ECHR is stimulated by p53 depletion in HCT116 cells (Figure 4) argues against the idea of global p53-insensitivity of HR in these cells and in favour of discriminatory suppression. In the case of ES cells, the unusual cytoplasmic localization of p53 appeared to provide a good explanation for p53-insensitivity. Our results suggest, however, that even if p53 had been nuclear, GT may have remained unaffected by p53 depletion. An analysis of the effects of p53 knockouts on ICHR or ECHR in ES cells would therefore be of interest.

In a previous report of differential HR suppression by p53 (36), ECHR appeared to be unaffected to p53 inactivation while, in the same mouse embryo fibroblast (MEF) cells, ICHR was stimulated. On this basis, it was suggested that p53 might suppress only HR involving chromatinized HR substrates. In contrast to that study, our results and those of others (18–20) clearly show ECHR to be p53-sensitive. It is possible that this discrepancy reflects an important difference between the MEF cells used by Willers et al. and the various cell lines used in other studies. Alternatively, given the tendency for p53 sensitivity to be less pronounced at high cell lines used in other studies. It would also be consistent with the p53-sensitivity of most ECHR, which occurs predominately by SSA (57,58). Indeed, in one study of ECHR in H1T1080 cells (19), HR between direct repeats was found to be p53-sensitive, whereas HR between inverted repeats (which cannot occur by SSA) was not. Furthermore, the fact that non-conservative ICHR, which most likely occurs by SSA, was more p53-sensitive than conservative ICHR, which cannot occur by a simple SSA mechanism, is again consistent with preferential suppression of SSA. Conservative ICHR was nevertheless clearly p53-sensitive, suggesting that discrimination between SI and SSA cannot fully account for the observed pattern of p53-sensitivities.

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<td>1.1</td>
</tr>
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</table>
DNA, random or targeted, may occur during certain viral infections (61,62) or as a result of apoptosis (63), and can be considered a form of natural mutagenesis. It is therefore interesting to note that, despite its ability to suppress other genome destabilizing events, p53 suppresses neither random nor targeted DNA integration.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS
We are grateful to Maria Jasim (pDRNeo and pCMV3xns-I-SceI) and to Reuven Agami (pSUPER and pSUPER-p53) for generously supplying plasmids.

REFERENCES