Improved methods for the generation of human gene knockout and knockin cell lines

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ABSTRACT

Recent studies have demonstrated the utility of recombinant adeno-associated viral (rAAV) vectors in the generation of human knockout cell lines. The efficiency with which such cell lines can be generated using rAAV, in comparison with more extensively described plasmid-based approaches, has not been directly tested. In this report, we demonstrate that targeting constructs delivered by rAAV vectors were nearly 25-fold more efficient than transfected plasmids that target the same exon. In addition, we describe a novel vector configuration which we term the synthetic exon promoter trap (SEPT). This targeting element further improved the efficiency of knockout generation and uniquely facilitated the generation of knockin alterations. An rAAV-based SEPT targeting construct was used to transfer a mutant CTNNB1 allele, encoding an oncogenic form of β-catenin, from one cell line to another. This versatile method was thus shown to facilitate the efficient integration of small, defined sequence alterations into the chromosomes of cultured human cells.

INTRODUCTION

Gene targeting via homologous recombination is the most definitive means of assessing gene function. While routinely applied to diverse organisms such as bacteria, yeast and rodents, established methods of gene targeting have been significantly less efficient when applied to human cell lines (1). The comparatively low rate at which homologous recombinants can be recovered from human cell populations seems to be due to a lower rate at which exogenous DNA can be introduced into cells and integrated into the genome, and possibly to a lower rate of homologous recombination as well. There have been three successful approaches to circumventing these obstacles. One approach has been to optimize the delivery of intact targeting DNAs so that they are introduced into a large proportion of the target cell population while avoiding intracellular degradation (2). A second approach has been to configure targeting vectors so that they preferentially allow growth, under selection, of clones that result from homologous integration rather than random, non-homologous integration (3). A third approach, has been to increase the rate of homologous recombination at the locus of interest. Recently, it has been shown that this can be accomplished by the generation of site-specific DNA breaks (4).

The most common means of DNA delivery is by electroporation or lipofection of linearized plasmid DNA. Electroporation of targeting DNAs has been shown to result in an improved rate of homologous recombination, possibly due to a reduction in the extent to which exogenous DNA is degraded under these conditions (2). Because such methods are relatively inefficient in many human cell types, lipofection has been the method of choice for the generation of transgenic and knockout human cell lines. The efficient generation of human cell knockouts was recently achieved by the use of recombinant adeno-associated viruses (rAAV) (5). The enormous potential of this method was underscored by the demonstration that targeted human cells could be detected without selection for integration, suggesting that both integration and homologous recombination were greatly favored.

A major advance in the targeting of human genes was the development of the promoter-trap targeting construct (6). Most targeting methods use the expression of a marker gene as a means of selecting clones in which the targeting construct has stably integrated into the genome. The type of construct used most commonly to target mouse genes includes a constitutively active promoter that drives expression of the selectable marker gene (3). Such a construct does not have to integrate near an endogenous promoter in order to give
rise to a drug resistant transgenic clone. The promoter trap strategy, in contrast, requires integration of a promoterless construct in the proximity of an active endogenous promoter, which then drives the expression of the marker gene (6). Accordingly, promoter trap vectors result in the generation of a smaller number of drug resistant clones, but in many cases a high proportion of these clones are found to result from homologous integration. First described by Hanson and Sedivy (6), promoter-trap targeting vectors have been successfully employed in the targeting of many human loci. Such techniques are generally considered the method of choice for plasmid-based gene targeting.

This high targeting efficiency of promoter-trap constructs comes at the price of limited versatility. The use of a promoter trap requires the disruption of an exon and the insertion of a selectable marker, expressed either as an in-frame fusion with the endogenous gene or in a separate cistron by means of an internal ribosomal entry site (IRES). Unless the targeted exon is painstakingly reproduced elsewhere in the targeting construct, the disruption of an exogenous exon will result in a loss of gene function. Subtle alterations that preserve endogenous exons are not generally feasible by current methods.

Attempts to use rAAV to introduce human gene mutations have yielded promising results. Using rAAV to target the human HPRT gene, Russell and coworkers (7) succeeded in introducing single base mutations that conferred resistance to the selectable agent 6-thioguanine. While these results are encouraging, most desired genetic alterations would not cause a selectable growth advantage. The targeting of a nonselectable gene, and subsequent recovery of a knockin cell line, would clearly require a method that facilitates high-efficiency gene targeting.

One of the major goals of this study was to improve the versatility of the promoter-trap approach and to devise a general method for altering human genes. In this report, we describe a targeting strategy, the synthetic exon promoter trap (SEPT), that can be used in conjunction with rAAV-based gene targeting. We demonstrate the broad utility of this approach by transferring a functional, pathogenic mutation upstream of these loci and the inserted marker cassette. Approximately 5 × 10⁸ AAV-293 cells in a 75 cm² flask were transfected with a mixture of 2.5 µg of each of the above three plasmids, using 54 µl of Lipofectamine (Invitrogen) as per manufacturer’s protocol. The transfection was performed in serum-free conditions using OptiMEM medium (Invitrogen). Two days after transfection, cells were scraped into 1 ml of phosphate-buffered saline and frozen and thawed three times. The crude lysate was clarified by centrifugation and the supernatant was divided into aliquots and stored at −80°C. The titer of these rAAV stocks was ~10⁹ viral particles/ml.

**Packaging of rAAV targeting constructs**

Infectious rAAV stocks were produced with the AAV Helper-Free System (Stratagene) according to the manufacturer’s protocols. Briefly, ITR-containing targeting constructs were co-transfected with the plasmids pAAV-RC and pHELPER. Approximately 5 × 10⁶ AAV-293 cells in a 75 cm² flask were transfected with a mixture of 25% of each of the above three plasmids, using 54 µl of Lipofectamine (Invitrogen) as per manufacturer’s protocol. The titer of these rAAV stocks was ~10⁹ viral particles/ml.

**Gene targeting and isolation of recombinant cell lines**

Cells were grown in 25 cm² flasks and infected with rAAV when ~75% confluent. At the time of infection, medium was aspirated and 4 ml of medium containing 50–250 µl of rAAV lysate (0.5–2.5 × 10⁵ viral particles) was added to each flask. Cells were washed with Hanks buffered saline solution and detached with trypsin (Invitrogen), 24 h after infection. Cells were replated in eight 96-well plates in medium containing genetin (Invitrogen) at a final concentration of 0.4 mg/ml. Drug resistant colonies were grown for 3–4 weeks. At the end of the selection period, genomic DNA was extracted from single clones growing in 96-well plates using the Wizard SV 96 Genomic DNA Purification System (Promega) and eluted in 100 µl of the supplied elution buffer. Locus-specific integration was assessed by PCR using a primer that annealed outside the homology region and another that annealed within neo. Positive clones were confirmed by PCR across both homology arms.
Cre-mediated excision of selectable marker elements and Southern blot analysis

To remove the SEPT cassette from correctly targeted clones, cells were infected with an adeno virus that expresses the Cre recombinase, as described (10). Cells were plated at limiting dilution in nonselective medium, 24 h after infection. After 2 weeks, single cell clones were plated in duplicate and 0.4 mg/ml geneticin was added to one set of wells. After 1 week of growth, clones that were geneticin-sensitive were expanded for further analysis. Southern blot analysis was performed as in (10). Genomic DNA was digested with XbaI. Blots were probed with a 565 bp probe derived from CTNNB1 intron 6.

RT–PCR and DNA sequencing

Total RNA was isolated using the Qiagen RNeasy kit (Qiagen). RT–PCR was performed with the SuperScript One-Step with Platinum Taq System (Invitrogen), using 0.5 μg of RNA as template. Primer-specific cDNA was amplified for 40 cycles at an annealing temperature of 58°C, as per manufacturer’s recommendations. The primers specific for the β-catenin gene transcript were 5’-AAATCCAGCGTGGACAATGG-3’, which anneals within exon 2, and 5’-CTAGAGCAGACAGATAGGCACC-3’, which anneals within exon 7.

RESULTS AND DISCUSSION

Delivery of targeting constructs: rAAV versus plasmid DNA

Gene targeting in human cells has been accomplished with various types of targeting constructs and vectors (1). Most reports of human cell knockouts employ only one targeting strategy, which makes it difficult to compare the efficiencies of different methods. It is clear that targeting efficiencies vary broadly between loci, a fact that further complicates a comparison of different approaches (1).

In a recent study of rAAV-mediated gene targeting, Kohli et al. (11) reported achieving efficiencies that ranged between 0.4 and 13% using promoter-containing constructs. In comparison, efficiencies of gene targeting with plasmid constructs have been reported to range from 0.01 to 20% (12,13). However, most of these studies employed promoter trap constructs. Thus, while rAAV-mediated gene targeting is clearly highly efficient, there is in fact a significant amount of overlap between published rates at which homologous recombinant clones have been recovered using the rAAV- and plasmid-based methods. A recent study that assessed the ability of exogenous DNA to quantitatively alter an integrated reporter gene showed that rAAV was significantly more efficient at gene targeting when compared with plasmid DNA (4). Taken together, these disparate studies suggest that rAAV-based methods should yield a higher proportion of desired recombinant cell lines than plasmid-based methods, yet the published rates at which targeted clones have been recovered do not demonstrate a clear difference. We speculated that the overlap in the published targeting efficiencies might therefore be due to the differential utilization of promoter-traps in different laboratories. Differences between the recombination rates at different loci may further cloud this issue. The factors that contribute to targeting efficiency at a given locus are unknown, but it has been speculated that the presence of repeat elements within regions of homology may reduce efficiency (11).

To directly compare rAAV and plasmid transfection methods at the same locus, we measured the rate of recovery of homologous recombinant clones resulting from the insertion of a neo cassette into exon 3 of the CTNNB1 gene, which encodes β-catenin. Both the targeting plasmid and the rAAV had an identical configuration at the point of insertion, and were designed to disrupt exon 3 and thereby knockout gene function. In this scheme, the neo gene, flanked by regions of homology to CTNNB1, was expressed as an in-frame fusion with the first 32 amino acids encoded by β-catenin and driven by the endogenous promoter that was outside the region of homology.

To maximize the potential efficiency of the CTNNB1 targeting plasmid, we incorporated a lengthy region of chromosomal homology, spanning a total of 8.5 kb, into the plasmid construct pBCATN (Figure 1). The packaging capacity of rAAV is ~4.7 kb, which limited the maximum amount of chromosomal homology that could be incorporated into the viral construct, pAAV-BCATN. Homology arms were accordingly shorter and a total of 2229 bp of homology was used.

We chose to focus our initial efforts on gene targeting in the cell line HCT116. This cell line is a widely used diploid colorectal cancer cell line and has been employed in the majority of human gene targeting studies to date. Both rAAV- and plasmid-based approaches have been used successfully in this line. Which method is more efficient? To address this important question, HCT116 cells were either infected with rAAV derived from pAAV-BCATN or transfected with the targeting plasmid pBCATN. Two days later, cells were detached and reseeded into multiwell plates containing selective medium and then grown under selection for an additional

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**Figure 1.** Comparison of gene targeting at CTNNB1 using a targeting plasmid, pBCATN (upper construct) and an rAAV derived from pAAV-BCATN (lower construct). Both constructs have the identical configuration around the point of insertion into exon 3. The lengths of the homology arms are as indicated. PCR with the primer pairs P1 and P2 (for rAAV) and P1 and P3 (for plasmid pBCATN) were used to screen for homologous integration. Exons are blue boxes; LoxP sites are indicated by red triangles. ITR, inverted terminal repeat; neo, neomycin transferase ORF; pA, polyadenylation signal sequence.
splice acceptor followed by an IRES, a exon into the chromosomal locus of interest by homologous endogenous exon is duplicated and then removed (16). While powerful, this approach requires either the disruption of less probable to result in expression of the selectable marker. contains no promoter and therefore random integration is within an actively transcribed gene. The construct itself marker gene can only be expressed if the construct integrates can be obtained with this approach because the selectable significantly higher proportion of homologous recombinants tated the targeted disruption of numerous human genes is a As described above, an important development that has facili- 

| Cells treated | Colony formation rate | Colonies PCR positive screened colonies | Targeting efficiency |
|--------------|-----------------------|----------------------------------------|----------------------|
| Plasmid\(^{a}\) | 6 x 10^7 | 2.5 x 10^{-5} | 576 | 5 | 0.009 |
| rAAV\(^{b}\) | 5 x 10^6 | 1 x 10^{-3} | 60 | 14 | 0.23 |

\(^{a}\)Targeting efficiency is defined as the proportion of colonies screened that were homologous recombinants.

\(^{b}\)Targeting plasmid pBCATN, as described in Materials and Methods.

\(^{c}\)Generated by packaging pAAV-BCATN plasmid, as described in Materials and Methods.

2 weeks. More colonies were obtained after infection with rAAV than transfection of the plasmid (Table 1), indicating a higher rate of stable neo integration. It is important to note that while the plasmid transfection was performed under previously optimized conditions, the rAAV infection was performed with an arbitrary amount of a low-titer viral stock. It is therefore probable that much higher colony formation rates may be achieved by adjusting the multiplicity of infection (MOI). Importantly, increasing the MOI not only increases the amount of viral gene delivery but has also been reported to increase the frequency of targeting (14).

Screening the neo resistant clones by PCR to assess homologous integration revealed correct targeting in 1% of the clones generated by integration of the plasmid construct, while 23% of the rAAV derived clones were targeted (Table 1). A previous study has reported a targeting efficiency at CTNNB1 of 12% using a promoterless plasmid-based construct (15). The previously described construct was significantly different from the one used here; it was designed to delete a large region of CTNNB1 and therefore the homology arms encompass a different region of the gene. Indeed, the difficulties inherent in comparing different constructs provided the rationale for this experiment. Comparison of these studies suggest that gene targeting efficiency varies not only between genes, but can also vary at different positions within a gene.

**Gene targeting using a synthetic exon promoter trap**

As described above, an important development that has facilitated the targeted disruption of numerous human genes is a feature of targeting constructs known as a promoter trap (3). A significantly higher proportion of homologous recombinants can be obtained with this approach because the selectable marker gene can only be expressed if the construct integrates within an actively transcribed gene. The construct itself contains no promoter and therefore random integration is less probable to result in expression of the selectable marker. While powerful, this approach requires either the disruption of an endogenous exon or a more complex strategy wherein an endogenous exon is duplicated and then removed (16).

We designed a targeting element that we call the SEPT. As its name implies, the SEPT strategy is to integrate a synthetic exon into the chromosomal locus of interest by homologous recombination. This exogenously introduced exon contains a splice acceptor followed by an IRES, a neo ORF and a polyadenylation signal sequence (Figure 2A and B). The exon cassette is preceded by a short synthetic intron and flanked by LoxP sites, so that all introduced sequences can be easily removed from the genomic locus by expression of the Cre recombinase. SEPT is a promoterless configuration and therefore expression of the neo marker requires integration into the proximity of an active promoter. A similar vector configuration has been used in mouse cells to target and trap developmentally-regulated genes (17).

We compared the efficiency of the SEPT approach in human cancer cells with similar constructs in which neo expression was driven by the herpesvirus thymidine kinase (tk) gene promoter. We examined targeting at two different loci: P53, which encodes the tumor suppressor protein p53, and CTNNB1, which encodes β-catenin. For each locus, the two targeting constructs tested had identical homology arms.

To test the capabilities of the SEPT approach, the CTNNB1 targeting constructs were designed to knockin a previously described oncogenic mutation (Figure 2A). The P53 targeting constructs were designed to delete endogenous exon 2, thereby resulting in a knockout allele (Figure 2B). Homologous recombination of these constructs at the endogenous locus results in the integration of the selectable marker into intron 2.
of CTNNB1. HCT116 harbors an oncogenic mutation in exon 3 of CTNNB1, a 3 bp deletion in exon 3 that deletes the codon for serine 45 (18). The right homology arm of the two CTNNB1 constructs was derived from the mutated allele of HCT116 and thus contained the oncogenic mutation.

As shown in Table 2, infection with the tk promoter-containing constructs resulted in greater numbers of drug resistant colonies than did the SEPT constructs. The high rate of colony formation is consistent with previous observations that integration of a promoterless plasmid construct is less probable to give rise to a drug resistant clone (3). This is presumably because integration into a random location is much less probable to result in expression of the selectable marker. The efficiency of targeting, as measured by the proportion of the drug resistant clones that resulted from homologous recombination, was higher for the SEPT constructs (Table 2). Compared with the tk promoter-containing constructs, the SEPT constructs were ~5- and 7-fold more efficient at targeting the CTNNB1 and P53 loci, respectively.

Table 2. Gene targeting with the SEPT element

| Locus   | Cells infected | Colony formation rate | Colonies screened | PCR positive colonies | Targeting efficiency/a |
|---------|----------------|-----------------------|-------------------|-----------------------|------------------------|
| CTNNB1  |                |                       |                   |                       |                        |
| tk promoter | 5 \times 10^6 | 0.033                 | 741               | 3                     | 0.004                  |
| SEPT    |                | 5 \times 10^6         | 0.004             | 170                   | 3                      | 0.018                  |
| P53     |                |                       |                   |                       |                        |
| tk promoter | 5 \times 10^6 | 0.015                 | 95                | 4                     | 0.042                  |
| SEPT    |                | 5 \times 10^6         | 0.006             | 94                    | 26                     | 0.28                   |

*aTargeting efficiency is defined as the proportion of colonies screened that were homologous recombinants.

Derivation of a knockin human cell line using rAAV and SEPT

While we were able to quantify targeting of CTNNB1 in HCT116, the mutated allele that is already present in this cell line made it less suitable for demonstrating a functional knockin alteration. To show that we could alter CTNNB1 and achieve expression of the altered allele, we used the SEPT-containing rAAV in DLD1, a colorectal cancer cell line that has two wild-type CTNNB1 alleles (18). We used exactly the same methods to generate and screen transgenic clones that had been used in HCT116. Screening 10^9 geneticin-resistant colonies resulted in the identification of 20 positive clones, a targeting efficiency of 18%. This was 10-fold higher than the targeting efficiency of this construct in HCT116 as shown in Table 2. This result suggests that the SEPT targeting approach will be generally useful in a variety of human cell lines.

To restore the general architecture of the CTNNB1 gene and thereby facilitate expression of a full length β-catenin transcript, we removed the SEPT cassette with the use of a Cre-expressing recombinant adenovirus. Subclones in which neo had been excised were identified functionally, by virtue of their sensitivity to geneticin. Correct targeting and removal of the SEPT cassette was confirmed by Southern blot (Figure 3A, B). The observed bands were consistent with the homologous integration of the SEPT cassette and its subsequent removal by Cre (Figure 3B).

Expression of the introduced, mutant form of β-catenin was demonstrated by RT–PCR amplification followed by DNA sequencing. PCR primers annealed to sequences encoded by exons 2 and 7, the latter was downstream of the homology region. A total of 1072 bp of β-catenin encoding cDNA was amplified. All PCR products were consistent with full-length transcripts (data not shown). These were gel isolated and sequenced. DLD1 cells expressed a homogenous population of wild-type transcripts, as expected (Figure 3C). In contrast, the knockin clones showed a homogeneous sequence between the primer annealing site and the serine 45 codon, after which the sequence became heterogeneous. Inspection of the sequence electropherograms showed the presence of two species with sequences offset by 3 bp. The height of the electropherogram peaks indicated the presence of roughly the same sequence elements or aspects of local chromosome structure that are not understood. Further studies at additional loci are required to determine whether there is a direct relationship between repeats and targeting efficiency.
number of wild-type and mutant transcripts, indicating that the knockin allele was expressed at roughly the same level as the untargeted, wild-type allele.

In summary, we have amplified an oncogenic mutation from one cell line and, by incorporating this mutation into a targeting construct, transferred this mutation into another cell line that is wild-type for this gene. It would have been equally straightforward to introduce this mutation, or any other short alteration, into a targeting construct by site-directed mutagenesis.

A key attribute of the SEPT approach is its versatility. The SEPT cassette is generic and can be used without modification. To create a targeting construct, the SEPT cassette is simply ligated between two regions of homology to the gene of interest, which are generated by PCR. The simplicity of this scheme contrasts with other promoter trap approaches, in which the junction between the endogenous exon to be targeted and the selectable marker gene must be laboriously customized for each gene. Recently, a novel method has been described that employs a fusion PCR technique to streamline the construction of rAAV for gene targeting (11). Fusion PCR could be similarly employed to generate SEPT-containing constructs with only minor modifications to the published protocol, further streamlining this method.

In this study, we show that the combined use of rAAV and the SEPT approach can significantly improve the efficiency of gene targeting in human cells. This method does not require the disruption of an endogenous exon and can therefore be used for the generation of human gene knockin cell lines. We anticipate that human cell knockins will be powerful new tools for studying how gene mutants and variants contribute to disease and affect responses to therapy.

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REFERENCES

1. Bunz,F. (2002) Human Cell Knockouts. Curr. Opin. Oncol., 14, 73–78.
2. Yanez,R.J. and Porter,A.C. (1999) Influence of DNA delivery method on gene targeting frequencies in human cells. Somat. Cell Mol. Genet., 25, 27–31.
3. Sedivy,J.M. and Dutriaux,A. (1999) Gene targeting and somatic cell genetics—a rebirth or a coming of age?. Trends Genet., 15, 88–90.
4. Porteus,M.H. and Baltimore,D. (2003) Chimeric nucleases stimulate gene targeting in human cells. Science, 300, 763.
5. Hirata,R., Chamberlain,J., Dong,R. and Russell,D.W. (2002) Targeted transgene insertion into human chromosomes by adeno-associated virus vectors. Nat. Biotechnol., 20, 735–738.
6. Hanson,K.D. and Sedivy,J.M. (1995) Analysis of biological selections for high-efficiency gene targeting. Mol. Cell. Biol., 15, 45–51.
7. Inoue,N., Dong,R., Hirata,R.K. and Russell,D.W. (2001) Introduction of single base substitutions at homologous chromosomal sequences by adeno-associated virus vectors. Mol. Ther., 3, 526–530.
8. Huang,M.T. and Gorman,C.M. (1990) Intervening sequences increase efficiency of RNA 3’ processing and accumulation of cytoplasmic RNA. Nucleic Acids Res., 18, 937–947.
9. Hellen,C.U. and Sarnow,P. (2001) Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev., 15, 1593–1612.
10. Jallepalli,P.V., Lengauer,C., Vogelstein,B. and Bunz,F. (2003) The Chk2 tumor suppressor is not required for p53 responses in human cancer cells. J. Biol. Chem., 278, 20475–20479.
11. Kohl,M., Rago,C., Lengauer,C., Kinzler,K.W. and Vogelstein,B. (2004) Facile methods for generating human somatic cell gene knockouts using recombinant adeno-associated viruses. Nucleic Acids Res., 32, e3.
12. Yanez,R.J. and Porter,A.C. (1998) Therapeutic gene targeting. Gene Ther., 5, 149–159.
13. Sedivy,J.M., Vogelstein,B., Liber,H.L., Hendrickson,E.A. and Rosmarin,A. (1999) Gene targeting in human cells without isogenic DNA. Science, 283, 9.
14. Porteus, M.H., Cathomen, T., Weitzman, M.D. and Baltimore, D. (2003) Efficient gene targeting mediated by adeno-associated virus and DNA double-strand breaks. *Mol. Cell. Biol.*, 23, 3558–3565.

15. Kim, J.S., Crooks, H., Dracheva, T., Nishanian, T.G., Singh, B., Jen, J. and Waldman, T. (2002) Oncogenic beta-catenin is required for bone morphogenetic protein 4 expression in human cancer cells. *Cancer Res.*, 62, 2744–2748.

16. Cortez, D., Guntuku, S., Qin, J. and Elledge, S.J. (2001) ATR and ATRIP: partners in checkpoint signaling. *Science*, 294, 1713–1716.

17. Chen, Y.T., Liu, P. and Bradley, A. (2004) Inducible gene trapping with drug-selectable markers and Cre/loxP to identify developmentally regulated genes. *Mol. Cell. Biol.*, 24, 9930–9941.

18. Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K.W. (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science*, 275, 1787–1790.

19. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W. and Vogelstein, B. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*, 282, 1497–1501.

20. Traverso, G., Bettegowda, C., Kraus, J., Speicher, M.R., Kinzler, K.W., Vogelstein, B. and Lengauer, C. (2003) Hyper-recombination and genetic instability in BLM-deficient epithelial cells. *Cancer Res.*, 63, 8578–8581.