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An arrayed human genomic library constructed in the PAC shuttle vector pJCPAC-Mam2 for genome-wide association studies and gene therapy

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Abstract

The various iterations of the HapMap Project and many genome-wide association studies (GWAS) have identified hundreds of potential genes involved in monogenic and multifactorial traits. We constructed an arrayed 115,000-member human genomic library in the PAC shuttle vector pJCPAC-Mam2 that can be propagated in both bacterial and human cells. The library appears to represent a two-fold coverage of the human genome. Transient transfection of a p53-containing PAC clone into p53-null Saos-2 human osteosarcoma cells demonstrated that both p53 mRNA and protein were produced. Additionally, expression of the p53 protein triggered apoptosis in a subset of the Saos-2 cells. This library should serve as a valuable resource to validate potential disease genes identified by GWAS in human cell lines and in animal models. Also, individual library members could potentially be used for gene therapy trials for a variety of recessive disorders.

Keywords

Genomic library; Genome-wide association studies; Functional genomics; Gene therapy

1. Introduction

The Human Genome Project has ushered in the era of big science. The International HapMap Project's original goal was to catalog the millions of single nucleotide polymorphisms (SNPs) present in many haplotypes in an attempt to establish links between certain variants and specific diseases. This project led to the identification of dense maps of human genetic variation. The International HapMap Project Phase 2 catalogued over 3 million polymorphisms (Sabeti et al., 2007). These investigations have uncovered over 150 risk loci for more than 60 common diseases and traits (Manolio and Collins, 2009). Genome-wide association studies (GWAS) are underway to identify candidate genes for many complex traits such as Alzheimer's disease, autism, bipolar disorder, Type I diabetes, multiple sclerosis, Parkinson's disease, obesity, rheumatoid arthritis and schizophrenia (Cichon et al., 2011; Menon and Farina, 2011; Stefansson et al., 2008a, 2008b; Thorliefsson et al., 2009; Weiss et al., 2008). As candidate genes are identified using a GWAS approach, investigators still need to demonstrate which if any genes have a causative role in the various diseases.

Genomic libraries constructed in the original BAC and PAC cloning systems were very useful for completion of the Human Genome Project (HGP). Libraries constructed in these vector systems were used to generate physical maps of all twenty three chromosome pairs and served as the templates for DNA sequencing (Ioannou et al., 1994; Kim et al., 1996; Shizuya et al., 1992). Construction of next generation BAC and PAC shuttle vectors that could be propagated in both bacterial and human cells allow scientists to study the functions of individual genes in tissue culture and in animal models. Inclusion of the Epstein Barr Virus latent origin of replication *oriP* allows for the episomal replication of these vectors in a broad range of mammalian cells including human, monkey, dog, murine, rodent and porcine cell lines (Black and Vos, 2002; Min et al., 2003; Miziguchi et al., 2000; Tomiyasu et al., 1998; Wolfgang et al., 2002; Yates et al., 1985). Libraries housed in the vectors pPAC4 and pBACe4 contained the EBV *oriP* required for propagation in mammalian cells, but both vectors lacked the EBNA1 gene that is necessary for episomal propagation of clones in mammalian cells. Therefore, investigators had to establish an EBNA1-expressing cell line before introducing a desired clone into human cells (Frengen et al., 2000). The shuttle vector pEBAC190G that was constructed later does contain the EBNA1 gene, but no human genomic libraries constructed in this vector have been reported (Al-Hasani et al., 2003). Additionally, none of these vector systems have the ability to activate a multi-copy origin of replication during propagation in *Escherichia coli* to increase the yield of individual library members to recover ample DNA for subsequent transfections into mammalian cells.

The shuttle vector pJCPAC-Mam1 contains two P1-derived origins of replication, the P1 single-copy replicon for low copy expression and a multi-copy lytic replicon under the control of the *lac* repressor for high copy expression upon the addition of IPTG in *E. coli*. This vector also contains the EBV latent replication origin *oriP*, the EBNA1 gene, and a puromycin-resistance gene for propagation of all library members in mammalian cells (Coren and Sternberg, 2001).

To increase the utility of this cloning vector a mutant *loxP*5171 element was inserted into the unique NotI site of pJCPAC-Mam1 to generate pJCPAC-Mam2. Highly specific nested deletions could then be generated efficiently from both ends of any genomic DNA of interest using the mutant and wild-type *loxP* elements flanking the genomic insert (Chatterjee et al., 2004; Gundersen and Coren, 2007; Lee and Saito, 1998). This technology enables one to determine *cis*-acting sequences such as enhancers and repressors as well as to determine gene boundaries.

We constructed an arrayed human genomic library containing 115,000 clones in the PAC shuttle vector pJCPAC-Mam2. We digested a small subset of clones with the restriction enzyme NotI and then used FIGE to determine insert size. Additionally PCR screening of the final pools with nine genes was used to obtain an estimate of library coverage. A p53-containing PAC clone was transiently transfected into the p53-null Saos-2 human osteosarcoma cell line to demonstrate the utility of individual library members in human cell culture models. Members from this library can be used to validate candidate genes identified by GWAS in human cells and in animal models.

2. Results

2.1. Insert size analysis and size distribution of random PAC clones

For the analysis of average genomic DNA insert size, 473 clones were randomly chosen from the 115,000-member library. Miniprep DNA was prepared from each clone, the DNA was digested with NotI and restriction fragment sizes were determined using FIGE. Since the vector contributes 23 kb, this value was subtracted from the size of each fragment(s) to

obtain the insert size. The range of insert size was from 1 kb to 210 kb. Among all of the clones analyzed, 81.4% contained inserts, whereas 18.6% represented vector alone or a deleted version of the vector. The clones with the insert sizes ranging from 60 kb to 210 kb constituted 39.9% of the examined insert-containing clones (Table 1). The average insert size was determined to be 57 kb for those clones containing genomic DNA.

2.2. PCR screening of the final pools

Next we screened the final pools using PCR primers for nine genes (AP Endonuclease, β -Polymerase, BRCA2, *c-myc*, DNA Ligase, HPRT, HSA, PAX6 and p53) in an attempt to obtain an estimate of library coverage. The plasmid DNA's prepared from all 19 final pools were used with each primer set to determine how many final pools amplified the desired fragment of interest. Every set of PCR primers, with the exception of the PAX6 primers, amplified the desired band in at least one final pool (Table 2). Since twenty final pools were amplified for the nine genes screened, these results suggest that the library contains 2.2-fold coverage of the human genome.

2.3. Functional studies with a p53-containing PAC clone in Saos-2 cells

We identified one full-length p53 clone in final pool I using PCR primer pairs upstream of exon 1, within exon 4 and downstream of exon 11 (data not shown). Unfortunately, we were not able to identify this clone as we screened up the library. Therefore, the p53 gene containing 19 kb of upstream sequences was subcloned from a fosmid (a gift of Jon Jarvik, SpectraGenetics) into pJCPAC-Mam1 at its unique NotI site to demonstrate the utility of this PAC vector for functional studies in human cells (Coren and Sternberg, 2001).

The Saos-2 osteosarcoma cell line has a homozygous deletion of exons 2 through 11 in the p53 gene (Diller et al., 1990). We did observe that the 5' UTR PCR primers and the exon 1 PCR primers amplified the expected PCR products of 539 bp and 819 bp respectively (Fig. 1A, lanes 1 and 4). We also determined that the remaining exons were missing in this cell line using PCR primer sets that spanned the entire gene (Fig. 1A, lanes 7, 10, 14, 17, 20, 23). Human foreskin fibroblast (HFF) genomic DNA was used as the positive control. The brighter intensity of this band in every primer set is due to the higher concentration of HFF genomic DNA in the reaction (Figs. 1A and B). The Saos-2 cell line was then transiently transfected with this p53-containing PAC clone using a polyethyleneimine procedure, and the genotyping was repeated. PCR amplification across the gene demonstrated that all of the exons and introns were now present (Fig. 1B, lanes 1, 4, 7, 10, 14, 17, 20, 23). The presence of intron 1 was not analyzed in either experiment since it is very large (>10.7 kb).

2.4. Studying transcription and translation of the p53 gene

To test for the expression of p53 mRNA, total RNA was recovered from the original Saos-2 cell line, the transiently transfected Saos-2 cell line and the HEK 293T cell line (p53^{+/+}) (Pear et al., 1993). PCR primers were designed such that the forward primer spanned the end of exon 6 and the beginning of exon 7 so that spliced mRNA would be amplified but genomic DNA and pre-mRNA could not be amplified. One Step RT-PCR was used to generate a cDNA product. The desired 120 bp cDNA product was present in both the transiently transfected Saos-2 cell line and in the HEK 293T cell line but not in the parental Saos-2 cell line (Fig. 2). This experiment demonstrated that p53 mRNA was being produced and properly spliced.

Total protein was also recovered from Saos-2 cells, Saos-2 cells that had been transiently transfected for 1 to 7 days with the p53-containing PAC clone and the HEK 293T positive control so that Western blotting could be performed using the monoclonal anti-p53 DO-1 antibody. The protein concentration of each sample was determined with a Bradford assay.

Equal amounts of total protein were loaded into each well. Weak positive signals were seen after two days in the transiently transfected cells (Fig. 3, lanes 2,4, 6 and 7) and a strong signal was seen in the positive control (lane 8). As expected there was no p53 protein present in the parental Saos-2 cell line.

2.5. Assessing pJCPAC-Mam1-p53-induced apoptosis in Saos-2 cells

Reintroduction of p53 into Saos-2 cells has been demonstrated to trigger apoptosis (Benchimol, 2001; Wang et al., 1995). In an attempt to determine if the transient transfection of this p53-containing PAC clone was causing apoptosis in some of the Saos-2 cells, an annexin V-FITC apoptotic assay was conducted. In apoptosis plasma membrane phospholipid bilayer asymmetry is altered. Phosphatidylserine phospholipids (PS) that are normally present in the cytoplasmic leaflet of the plasma membrane invert to the extracellular leaflet. This change was detected using annexin V-FITC conjugates, as annexin V has high affinity for PS (Engeland et al., 1998). The shuttle vector pJCPAC-Mam1 or this vector containing the p53 gene was transiently transfected into Saos-2 cells. Three days post-transfection, transiently transfected cells were stained with annexin V-FITC conjugates. A significant increase in FITC fluorescence was detected in pJCPAC-Mam1-p53-transfected Saos-2 cells ($p < 0.001$), suggesting increased apoptotic induction relative to pJCPAC-Mam1 transfected cells (Fig. 4).

Saos-2 cells were also transfected with pJCPAC-Mam1-p53GFP, and the p53GFP fusion protein was found to localize predominantly to the nuclei of Saos-2 cells as expected (Fig. 5A). Additionally, three days post-transfection, membrane blebbing, a hallmark characteristic of apoptosis (Wang et al., 1995) occurred in a subset of transfected Saos-2 cells (Fig. 5B), whereas the GFP-expressing plasmid pmaxGFP (Amara) did not induce blebbing (data not shown). These data suggest that both untagged p53 and GFP-tagged p53 pJCPAC-Mam1 clones induced apoptosis in a subset of Saos-2 cells.

3. Discussion

In this study an arrayed 115,000-member human genomic library was constructed in the PAC shuttle vector pJCPAC-Mam2. Investigators have previously constructed the RPCI-6 human genomic library in the PAC shuttle vector pPAC4 (Osoegawa et al, 2001). Unfortunately this vector lacks a multi-copy bacterial origin of replication that would significantly increase the yield of a given clone in *E. coli*. Also, pPAC4 lacks the EBNA1 gene needed to activate the EBV latent replication origin *oriP*. Therefore, clones contained in this library must be transfected into human cell lines that have previously incorporated EBNA1. pJCPAC-Mam2 contains origins of replication for both bacterial and eukaryotic propagation, namely the P1 single-copy replicon and P1 multi-copy lytic replicon under the control of the *lac* repressor and the EBV latent replication origin *oriP*, respectively. It also possesses selectable markers, kanamycin and puromycin-resistance genes, for bacterial and eukaryotic propagation and the EBNA1 gene for transactivation of *oriP*. Therefore, pJCPAC-Mam2 can serve as a versatile shuttle vector that can be utilized for a variety of studies of genes in mammalian cells.

During the construction of the library randomly chosen clones were analyzed for their insert sizes in order to obtain an estimate of average insert size and genome coverage. Substantial variation in insert size was observed (Table 1). Twenty six percent of the insert-containing clones were 80 kb or larger. The average insert size determined was lower than expected based on the results from the generation of a previous library in pJCPAC-Mam1 (Coren and Sternberg, 2001). But since the average size of a human gene is 20–25 kb, we anticipate that several thousand full-length genes are present in this library.

The importance of the genome coverage is that maximized representation of the genome in a library is crucial in successful genome-wide studies. The estimated coverage of the library is 1.8-fold based on the average insert size and 2.2-fold based on the PCR screening meaning that any locus of interest is, on average, theoretically represented 2 times, with some loci over-represented and others underrepresented. The PCR screening of the final pools cannot determine if more than one PAC clone representing the gene of interest is present amongst the 6,144 clones in that pool. This calculation probably under-represents library coverage since when screening up the pools for several genes, we identified more than one cryovial containing the gene of interest (data not shown). Being able to screen high-density nylon filters of all of the library members for the same nine genes would generate a more accurate estimation of library coverage since each individual library member representing any given gene would be identified. It is important to understand that random cloning cannot be expected in reality due to two main reasons: non-uniform distribution of MboI restriction sites for cloning and innately poor maintenance of some genomic regions such as centromeres, telomeres and highly repetitive DNA by host bacteria (Dunham et al., 1999).

The lowered level of p53 protein in the transiently transfected Saos-2 cells as compared to the HEK-293 cell line probably resulted from several factors. First, we observed a transfection efficiency of ~60–70% with a small vector (<10 kb) that expresses GFP in Saos-2 cells (data not shown). The transfection efficiency of the p53-containing PAC clone was probably lower since this plasmid is 58 kb in size. Every cell contains two copies of the p53 gene for the positive control HEK 293T cell line, and the p53 protein is stabilized by the SV40 large T antigen, which explains the much higher intensity of this band (Pear et al., 1993). Also, some of the transfected Saos-2 cells could have undergone apoptosis before total protein was recovered. One way to potentially increase the amount of p53 protein would be to generate a Saos-2 cell line containing a plasmid that overexpresses Bcl-2 before transfecting with the p53-containing PAC clone. This should prevent apoptosis from occurring (Marcellus et al., 1996).

Because the p53 gene was transcribed and translated in the Saos-2 cells and caused the cells to undergo apoptosis demonstrates that any PAC clone in this library could be used for functional studies in tissue culture. We could also quantitate the amount of apoptosis by using FACS to detect annexin V-FITC positive cells.

Several investigators have shown that BACs containing the CFTR gene and the *frda* gene could rescue knockout mice lacking the cystic fibrosis transmembrane regulator receptor protein and the frataxin protein (Manson et al., 1997; Sarsero et al., 2004). We plan to microinject a p53-GFP-tagged PAC clone into the tumors of p53 null rats to determine if *in vivo* p53 protein expression and regression in tumor size occurs. A positive result would indicate that individual library members could be used for gene therapy studies, but a more efficient method of delivery would have to be established before these vector systems are used in clinical trials.

Many investigators have been using GWAS to identify candidate genes for several multifactorial traits. Potential candidate genes for any of these disorders can be isolated from this library and then be used to test proof of principle in the appropriate human cell lines and animal models. Therefore, this library should be a valuable resource for the scientific community.

4. Materials and methods

4.1. Construction of the PAC library in pJCPAC-Mam2

A human genomic library was constructed in the PAC vector pJCPAC-Mam2 (version LoxP^{*}-1) (Gundersen and Coren, 2007). This vector was digested with BamHI and prepared as previously described (Coren and Sternberg, 2001). High molecular weight genomic DNA (Viomed passage six cells) was prepared from primary foreskin fibroblasts as previously described (Shepherd et al., 1994). Genomic DNA was partially digested with MboI and size fractionated through a 10–40% linear sucrose gradient. DNA in the 80–130 kb range was recovered and ligated to the vector as previously described (Coren and Sternberg, 2001). The ligations were electroporated into Electromax DH10B cells (3 µl DNA plus 25 µl cells) using a BTX ECM 600 electroporator with the following conditions: 2.5 kV, 40 µF capacitance and a resistance of 129 Ohms. Insert-containing clones were selected on LB media containing 5% sucrose and 25 µg/ml kanamycin. Bacterial colonies were picked with sterile toothpicks to individual wells of a 96 well plate (Nunc) that contained 300 µl of LB + 25 µg/ml kanamycin + 10% glycerol. The plates were incubated overnight at 37 °C. Each plate was covered with aluminum foil and stored in a –80 °C freezer.

4.2. Pooling of the arrayed library

The pooling of the arrayed library was accomplished in a columns and rows fashion, similar to a pooling method described earlier (Shepherd et al., 1994). First, a hundred plate series B library was constructed by pooling each twelve-member row of a series A library to a single well of a new plate. Cultures from one series A plate (one clone/well) were ultimately represented in one column of eight wells in one series B plate (12 clones/well), where each well contained clones from twelve wells of the series A library. Next entire series A dishes were pooled to generate 1200 cryovials. Two additional rounds of pooling generated 19 cryovials containing final pools A through S that contained 6144 PAC clones/cryovial with the exception of final pool S that contained 4608 PAC clones.

4.3. Analysis of insert size of randomly selected PAC clones

Clones were randomly selected, usually one clone from each of the original series A plates. Five µl of culture from the incubated plates were removed and incubated overnight in 2.5 ml of LB broth containing 25 µg/ml kanamycin. The PAC plasmid DNA's were isolated using a standard alkaline lysis miniprep procedure (Sambrook et al., 1989). The isolated plasmids were digested with NotI for linearization. Field Inversion Gel Electrophoresis (FIGE) was performed using the BIO RAD FIGE Mapper (Program 4) along with MidRange Pulse Field Gel size standards I and II (New England Biolabs). An image of the gel was captured using the Kodak EDAS 290 imaging system. A standard curve based on the mobility of the marker bands was drawn on semi-log paper for each gel and used for insert size calculation. The insert size was obtained by subtracting 23 kb, the size of the original vector, from the total size of the fragment(s).

4.4. Screening the library using PCR

PCR primers were prepared using DNA sequence information from GenBank (Shepherd et al., 1994). Primers were synthesized at the Core Facility of the Penn State Milton Hershey Medical Center. The sequence of the primer pairs for each gene is as follows:

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
AP endonuclease	GTGCAGATACGGCGTTGCTG	CATTCCCGTTACGAACGCC
β -polymerase	GAGCTGGGTTGCTCCTGCTC	AGCATGTCGGTGATTCCCC
BRCA2	GTGGCGCGAGCTTCTGAAAC	CCAACGCCGCTGGTAATTC
<i>c-myc</i>	GGGGAGAGGTTCCGGACTG	CTCGTCGTCCGGGTGCAATG
DNA ligase	GGATAGTGACAAGGGCATCT	GTAGGTATCTTCAGGGTCAG
HPRT	GGATTCTGAACGTCTTGCTCGAGATG	ACATCGAACCGATGTAGCTCAAGAGAGAGG
HSA	ATGAAGTGGGTAACCTTTATTTCCC	AACCGATGAGCAACCTCACTCTTGT
PAX6	CGGGAAACTTTTATGGTG	GGAATCTAATTTACTGGGC
p53	AATGGATGATTGTAGTGTCTGCC	CGTGCAAGTCACAGACTTGGC

4.5. Generating PAC DNA for PCR screening

One hundred microliter aliquots of each final pool were diluted in 5ml of LB + 25 μ g/ml kanamycin and grown overnight at 37 °C. Plasmid DNA was generated using 1.5 ml volumes from each final pool culture using a standard alkaline lysis miniprep procedure (Sambrook et al., 1989). The DNA was resuspended in 40 μ l of TE (pH 8.0) containing 5 μ g/ml RNase A and incubated at room temperature for 2 h. Five microliters of each final pool was mixed with the appropriate primers (5 μ M final concentration), 25 μ l of BioMix Red (BIOLINE) and H₂O to a final volume of 50 μ l. Five microliters of HFF genomic DNA was used in the standard PCR reaction as the positive control, and 5 μ l of sterile H₂O was used as the negative control. Each sample was subjected to 30 cycles of amplification using the following steps: 1 min at 94 °C, 1 min at the appropriate annealing temperature (available upon request) and 1 min at 72 °C. Next, a final 7 min 72 °C polymerization step and a 4 °C soak were carried out. Individual PCR reactions (10 μ l) were loaded into a 2% NuSieve agarose/0.5% agarose gel in 0.5 $\times$ TBE and electrophoresed at 60 V for ~2–3 h.

4.6. Cell culture conditions

Saos-2 (Diller et al., 1990) and HEK-293T (Pear et al., 1993) cells were cultured in D-MEM-F12 (ATCC) supplemented with 10% FBS, 0.03% L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37 °C with 5% CO₂. Saos-2 cells were passaged at a 1:3 subculture ratio and HEK-293 and HEK-293T cells were passaged at a 1:8 subculture ratio.

4.7. Polyethyleneimine (PEI) and lipofectamine 2000 transfection of Saos-2 cells

Saos-2 cells (4.5×10^5) were seeded in 60 mm cell culture dishes in 2 ml antibiotic-free D-MEM. Next 200 μ l serum-free and antibiotic-free D-MEM, 2 μ l of p53-containing PAC DNA (1 μ g/ μ l) and 12 μ l PEI (1 mg/ml solution) per dish were added to sterile polypropylene tubes, vortexed briefly and incubated at room temperature for 15 min. Aliquots of the DNA:PEI complexes were pipetted into media of culture dishes. Dishes were then incubated as described above in the absence of any selection, and the transfection media was replaced with complete media 12 h post-transfection. For Lipofectamine 2000 transfections used only in the Annexin V-FITC apoptotic assays, 5×10^4 Saos-2 cells were seeded in 500 μ l antibiotic-free D-MEM per well of a 24-well plate. Transfections were performed in accordance with the manufacturer's specifications by complexing 0.8 μ l of the Lipofectamine 2000 reagent with 0.8 μ g DNA per well. Transfection media was replaced with antibiotic-free D-MEM 4 h post-transfection.

4.8. PCR amplification of p53 loci from various cell lines

PCR primers were prepared using DNA sequence information of the p53 gene from GenBank. Genomic and episomal DNA was extracted from cell lines using the Puregene DNA Purification Kit (Gentra Systems). Five microliters of DNA was mixed with the appropriate primers (5 μ M final concentration), 25 μ l of BioMix Red (BIOLINE) and H₂O to a final volume of 50 μ l and PCR was performed as described earlier.

4.9. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was harvested from the Saos-2 cells, the transiently transfected Saos-2 cells and the HEK 293T cells using the RNeasy mini kit (Qiagen) and quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific). One Step RT-PCR (Qiagen) was then performed on 500 ng extracted RNA from pJCPAC-Mam1-p53 transiently transfected Saos-2 cells, untransfected Saos-2 cells and HEK-293 cells in accordance with the manufacturer's protocol. The forward primer was 5'-TATGAGCCGCCTGAGGTTGG-3' which spans the end of exon 6 and the beginning of exon 7. The reverse primer was 5'-GTCTTCCAGTGTGATGATGGTG-3'. An annealing temperature of 56 °C in the PCR step was used. RT-PCR products were resolved on composite 2% NuSieve agarose/0.5% agarose gels.

4.10. SDS-PAGE/western blotting

Transfected cells were harvested one through seven days post-transfection, washed twice in PBS and lysed in an appropriate volume of EBC cell lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl and 0.5% Nonidet P-40). HEK-293T cells and Saos-2 cells were harvested the same way. Protein concentration was quantified via a Bradford assay and equal amounts of protein supplemented with an equal volume of 2 $\times$ SDS sample buffer were loaded onto a 4–20% gradient SDS-PAGE gel. The gel was electrophoresed at 80 V until adequate band separation was achieved and proteins were transferred to a nitrocellulose membrane using the Invitrogen iBlot. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline Tween 20 (TBS-T) for 1 h and were probed with monoclonal DO-1 p53 antibody (Santa Cruz Biotechnology) diluted 1:2000 in 5% BSA in TBS-T overnight. Membranes were washed with TBS-T and probed with GE Healthcare Protein A-HRP diluted 1:10,000 in TBS-T for 30 min. After final washes in TBS-T, blots were treated with the SuperSignal West Femto Chemiluminescent Substrate (Pierce) for five min and visualized using the GeneGnome bioimaging system.

4.11. Detection of apoptosis in Saos-2 cells

Saos-2 cells were seeded and transfected as described above in 60 mm dishes containing four glass coverslips each. At the desired time point post-transfection, cells on the coverslips were subjected to the ApoAlert Annexin V apoptotic detection kit (Clontech). Following the 10 min incubation with Annexin V-FITC and propidiumiodide, the coverslips were washed once and then incubated for 30 min in 200 μ l 2% formaldehyde to fix the cells. Following sample preparation and image capture, 20 images of empty vector and pJCPAC-Mam1-p53 transfected Saos-2 cells stained with Annexin V-FITC were analyzed by ImageJ for mean FITC fluorescence. An unpaired student's *t* test was then performed to compare the two means and establish a *p* value. For the graphical representation, mean fluorescence was normalized by setting the empty vector fluorescence to one arbitrary fluorescence unit (a.u.).

A p53-GFP cassette (Gift of Jon Jarvik, SpectraGenetics) was subcloned into the unique NotI site of pJCPAC-Mam1 and Saos-2 cells were also transfected with this p53-GFP fusion protein-expressing construct using PEI as previously described. Three days post-

transfection, photographs were taken of cells using white light and UV light, and the resulting images were merged.

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Abbreviations

BAC	bacterial artificial chromosome
PAC	P1 artificial chromosome
GWAS	genome-wide association studies
SNP	single nucleotide polymorphism
HGP	Human Genome Project
EBV	Epstein Barr virus
EBNA1	Epstein Barr Nuclear Antigen 1
IPTG	isopropyl β -D-1-thiogalactopyranoside
<i>oriP</i>	origin of replication P1
<i>loxP</i>	locus of crossover P1
kb	kilobase
PCR	polymerase chain reaction
UTR	untranslated region
HFF	human foreskin fibroblast
RT-PCR	reverse transcriptase polymerase chain reaction
mRNA	messenger RNA
FITC	fluorescein isothiocyanate
PS	phosphatidylserine phospholipids
HEK	human embryonic kidney
SV40	simian virus 40
FACS	Fluorescence-activated Cell Sorting
LB	Luria Bertani
FIGE	field inversion gel electrophoresis
min	minute
ml	milliliter
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
TBS-T	Tris-buffered saline Tween 20

References

- Al-Hasani K, et al. Development of a novel bacterial artificial chromosome cloning system for functional studies. *Plasmid*. 2003; 49:184–187. [PubMed: 12726772]
- Benchimol S. p53-dependent pathways of apoptosis. *Cell Death Differ*. 2001; 8:1049–1051. [PubMed: 11687883]
- Black J, Vos JM. Establishment of an oriP/EBNA1-based episomal vector transcribing human genomic beta-globin in cultured murine fibroblasts. *Gene Ther*. 2002; 9:1447–1454. [PubMed: 12378407]
- Chatterjee PK, et al. Mutually exclusive recombination of wild-type and mutant loxP sites in vivo facilitates transposon-mediated deletions from both ends of genomic DNA in PACs. *Nucleic Acids Res*. 2004; 32:5668–5676. [PubMed: 15494454]
- Cichon S, et al. Genome-wide association study identifies genetic variation in neurocan as a susceptibility factor for bipolar disorder. *Am. J. Hum. Genet*. 2011; 88:372–381. [PubMed: 21353194]
- Coren JS, Sternberg N. Construction of a PAC vector system for the propagation of genomic DNA in bacterial and mammalian cells and subsequent generation of nested deletions in individual library members. *Gene*. 2001; 264:11–18. [PubMed: 11245973]
- Diller L, et al. P53 functions as a cell cycle control protein in osteosarcoma. *Mol. Cell. Biol*. 1990; 10:5772–5781. [PubMed: 2233717]
- Dunham, I.; Dewar, K.; Kim, U-J.; Ross, MT. Bacterial cloning systems. In: Birren, B.; Green, ED.; Klapholz, S.; Myers, RM.; Riethman, H.; Roskams, J., editors. *Genome Analysis: A Laboratory Manual Volume 3/Cloning Systems*. New York: Cold Spring Harbor Laboratory Press; 1999. p. 5-6.
- Engeland MV, Nieland LJW, Frans CS, Ramaekers FCS, Schutte B, Reutelingsperger CPM. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry*. 1998; 31:1–9. [PubMed: 9450519]
- Frengen E, et al. Modular bacterial artificial chromosome vectors for transfer of large inserts into mammalian cells. *Genomics*. 2000; 68:118–126. [PubMed: 10964509]
- Gundersen E, Coren J. Generating nested deletions in PAC clones constructed in pJCPAC-Mam2. *BIOS*. 2007; 78:87–94.
- Ioannou PA, et al. A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nat. Genet*. 1994; 6:84–89. [PubMed: 8136839]
- Kim UJ, et al. Construction and characterization of a human bacterial artificial chromosome library. *Genomics*. 1996; 34:213–218. [PubMed: 8661051]
- Lee G, Saito I. Role of nucleotide sequence of *loxP* spacer region in Cre-mediated recombination. *Gene*. 1998; 216:55–65. [PubMed: 9714735]
- Manolio TA, Collins F. The HapMap and genome-wide association studies in diagnosis and therapy. *Annu. Rev. Med*. 2009; 60:443–456. [PubMed: 19630580]
- Manson AL, et al. Complementation of null CF mice with a human CFTR YAC transgene. *EMBO J*. 1997; 16:4238–4249. [PubMed: 9250667]
- Marcellus RC, Teodoro JG, Charbonneau R, Shore GC, Branton PE. Expression of p53 in Saos-2 osteosarcoma cells induces apoptosis which can be inhibited by Bcl-2 or the adenovirus E1B-55 kDa protein. *Cell Growth Differ*. 1996; 7:1643–1650. [PubMed: 8959332]
- Menon R, Farina C. Shared molecular and functional frameworks among five complex human disorders: a comparative study on interactomes linked to susceptibility genes. *PLoS One*. 2011; 6:e18660. [PubMed: 21533026]
- Min KA, Oh ST, Yoon KH, Kim CK, Lee SK. Prolonged gene expression in primary porcine pancreatic cells using an Epstein–Barr virus-based episomal vector. *Biochem. Biophys. Res. Commun*. 2003; 305:108–115. [PubMed: 12732203]
- Miziguchi H, Hosono T, Hayakawa T. Long-term replication of Epstein–Barr virus-derived episomal vectors in the rodent cells. *FEBS Lett*. 2000; 472:173–178. [PubMed: 10788606]
- Osoegawa, K.; de Jong, PJ.; Frengen, E.; Ioannou, PA. Construction of bacterial artificial chromosome (BAC/PAC) libraries. Chapter 5:Unit 5.15. In: Haines, JL.; Korf, BR.; Morton, CC.; Seidman,

- CE.; Seidman, JG.; Smith, DR.; Sharer, JD., editors. Current Protocols in Human Genetics. New York: John Wiley & Sons, Inc; 2001.
- Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. U. S. A.* 1993; 90:8392–8396. [PubMed: 7690960]
- Sabeti PC, et al. Genome-wide detection and characterization of positive selection in human populations. *Nature.* 2007; 449:913–918. [PubMed: 17943131]
- Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
- Sarsero JP, et al. Human BAC-mediated rescue of the Friedreich ataxia knockout mutation in transgenic mice. *Mamm Genome.* 2004; 15:370–382. [PubMed: 15170226]
- Shepherd NS, et al. Preparation and screening of an arrayed human genomic library generated with the P1 cloning system. *Proc. Natl. Acad. Sci. U. S. A.* 1994; 91:2629–2633. [PubMed: 8146166]
- Shizuya H, et al. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci. U. S. A.* 1992; 89:8794–8797. [PubMed: 1528894]
- Stefansson H, et al. Common variants conferring risk of schizophrenia, large recurrent microdeletions associated with schizophrenia. *Nature.* 2008a; 455:232–236. [PubMed: 18668039]
- Stefansson H, et al. *Nature.* 2008b; 460:744–747. [PubMed: 19571808]
- Thorliefsson G, et al. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. *Nat. Genet.* 2009; 4:18–24.
- Tomiyasu K, Satoh E, Nishizaki K, Kondo M, Imanishi J, Mazda O. Gene transfer in vitro and in vivo with Epstein–Barr virus-based episomal vector results in markedly high transient expression in rodent cells. *Biochem. Biophys. Res. Commun.* 1998; 253:733–738. [PubMed: 9918796]
- Wang J, Bucana CD, Roth JA, Zhang W-W. Apoptosis induced in human osteosarcoma cells in one of the mechanisms for the cytotoxic effect of Ad5CMV-p53. *Cancer Gene Ther.* 1995; 2:9–17. [PubMed: 7621257]
- Weiss LA, et al. Association between microdeletion and microduplication at 16p11.2 and autism. *N. Engl. J. Med.* 2008; 358:667–675. [PubMed: 18184952]
- Wolfgang MJ, Marshall VS, Eisele SG, Schotzko ML, Thomson JA, Golos TG. Efficient method for expressing transgenes in nonhuman primate embryos using a stable episomal vector. *Mol. Reprod. Dev.* 2002; 62:69–73. [PubMed: 11933162]
- Yates JL, Warren N, Sugden B. Stable replication of plasmids derived from Epstein–Barr virus in various mammalian cells. *Nature.* 1985; 313:812–815. [PubMed: 2983224]

Fig. 1.

PCR amplification across the p53 gene. (A). Genomic DNA from the Saos-2 cell line was amplified using PCR primers that spanned from upstream of exon 1 to the end of exon 11. Each group of three samples contained Saos-2 genomic DNA, H₂O and HFF genomic DNA respectively. Lanes 1–3 were amplified with the 5' upstream primers; lanes 4–6 used the exon 1 primers; lanes 7–9 used the exon 2–4 primers; lanes 10–12 used the intron 4 primers; lanes 14–16 used the intron 4–6 primers; lanes 17–19 used the exon 7 primers; lanes 20–22 used the exon 8–10 primers; lanes 23–25 used the exon 11 primers; lanes 13 and 26 contain a New England Biolabs 100 bp ladder. (B). Episomal DNA from the Saos-2 cell line that was transiently transfected with the p53-containing PAC clone was amplified using PCR primers that spanned from upstream of exon 1 to the end of exon 11. The lane identifications are identical to those in Fig. 2A for the negative and positive controls (H₂O and HFF genomic DNA).

Fig. 2.
RT-PCR of various cell lines. Lane 1: pJCPAC-Mam1-p53-transiently transfected Saos-2 cells 4 days post-transfection, lane 2: HEK-293T cells, lane 3: Saos-2 cells, lane 4: H₂O, lane 5: New England Biolabs 100 bp ladder.

Fig. 3.

Western blotting of the various cell lines. Lanes 1–7 contain total protein recovered one to seven days post-transfection of the Saos-2 cell line with the p53-containing PAC clone, lane 8 is the HEK 293T positive control and lane 9 is the Saos-2 negative control. A mouse monoclonal p53 antibody was used to probe the membrane.

Fig. 4.

Annexin V-FITC staining of transfected Saos-2 cells. Saos-2 cells were analyzed three days post-transfection for apoptotic induction using annexin V-FITC conjugates of the ApoAlert Annexin V apoptotic detection kit. (A and B) Representative images of FITC fluorescence of Saos-2 cells transfected with pJCPAC-Mam1 or pJCPAC-Mam1-p53, respectively. (C). Graphical representation of mean FITC fluorescence of 20 images of pJCPAC-Mam1 or pJCPAC-Mam1-p53-transfected Saos-2 cells as a readout of apoptotic induction. a.u. equals arbitrary fluorescence unit. Error bars indicate SD, $n = 20$ images. * $p < 0.001$ as compared to pJCPAC-Mam1.

Fig. 5.

Live-cell imaging of p53-GFP-expressing Saos-2 cells. Saos-2 cells were transfected as described previously with pJCPAC-Mam1-p53-GFP. Three days post-transfection, Saos-2 cells were photographed and UV and white light images were merged. (A). Exogenous p53-GFP localizes predominantly to the nuclei of Saos-2 cells. (B). Membrane blebbing, a hallmark event in apoptosis occurs in a subset of p53-GFP-expressing Saos-2 cells.

Table 1

Analysis of the insert size of random PAC clones.

Size ranges (in kb)	# of clones	Percentage
1–9	39	9.9%
10–19	26	6.6%
20–29	40	10.2%
30–39	28	7.1%
40–49	49	12.6%
50–59	50	12.7%
60–69	29	7.4%
70–79	28	7.1%
80–89	41	10.4%
90–99	28	7.1%
100–109	19	4.8%
110–119	7	1.8%
120–129	1	0.3%
130+	8	2%

Table 2

PCR screening of the final pools.

Gene screened	Clones identified	Final pool locations
AP endonuclease	One	D
BRCA2	One	M
DNA ligase	Three	H, P, Q
HPRT	One	P
HSA	Four	F, K, J, O
<i>c-myc</i>	Seven	C, J, O, P, Q, R, S
p53	Two	B, I
PAX6	None	
β -Polymerase	One	Q