The tumor suppressor gene p53 is mutated or deleted in over 50% of human tumors. As functional p53 plays a pivotal role in protecting against cancer development, several strategies for restoring wild-type (wt) p53 function have been investigated. In this study, we applied an approach using gene repair with zinc finger nucleases (ZFNs). We adapted a commercially-available yeast one-hybrid (Y1H) selection kit to allow rapid building and optimization of 4-finger constructs from randomized PCR libraries. We thus generated novel functional zinc finger nucleases against two DNA sites in the human p53 gene, near cancer mutation 'hotspots'. The ZFNs were first validated using in vitro cleavage assays and in vivo episomal gene repair assays in HEK293T cells. Subsequently, the ZFNs were used to restore wt-p53 status in the SF268 human cancer cell line, via ZFN-induced homologous recombination. The frequency of gene repair and mutation by non-homologous end-joining was then ascertained in several cancer cell lines, using a deep sequencing strategy. Our Y1H system facilitates the generation and optimisation of novel, sequence-specific four- to six-finger peptides, and the p53-specific ZFN described here can be used to mutate or repair p53 in genomic loci.

Despite p53 being a desirable target, we currently lack the necessary tools to carry out the most direct approach: to modify genomes at will at disease loci like p53. Therefore we set out to develop just such a strategy, using a recently-developed technology: zinc finger nucleases (ZFNs). Since the first seminal publications about ZFN fusions in the late 1990s [10,11,12], these artificial proteins have promised to deliver a wide range of genome engineering tools (reviewed in [13,14]). The beauty of this approach is that zinc fingers are easily re-engineered to bind a wide variety of DNA sequences (reviewed in Ref. [15]). Thus, ZFNs effectively allow a type of ‘genome sculpting’ where externally provided DNA can be recombined precisely into a genome [16], resulting in site-specific gene repair, mutation, insertion or deletion.

ZFN gene targeting was first illustrated in the case of a mutant Drosophila yellow gene [17,18] and has since resulted in a whole field of engineering ZFNs. Examples include targeting disease loci, such as IL2RG (mutated in severe combined immunodeficiency; SCID-X1), where first gene repair [19], and then exogenous gene integration [20] were achieved. ZFNs have also targeted genes in model organisms such as C. elegans and Drosophila [21,22,23], zebrafish [24,25,26], mouse [27], and plants [28,29,30,31,32]. The technology has been extended to mammalian systems such as stem cells [33,34,35], the induction of cellular HIV resistance [36,37,38] and even whole rat knockouts [39,40].

The specificity of ZFNs depends on artificially-engineered DNA-binding domains: multi-zinc finger arrays that recognise...
long DNA sequences. There exists a large body of work on zinc finger engineering (reviewed in [15]). Briefly, the established engineering methods (amongst others) range from rational design [41], modular assembly with pre-made fingers [42,43,44], overlapping finger assembly [45,46] and bacterial-two hybrid [47].

A recent development is the emergence of two publically-available sources of zinc fingers, the academic Zinc Finger Consortium (ZFC; www.zincfingers.org) and the commercially-available CompoZr, offered by Sigma Aldrich [48]. In particular, the ZFC has provided a variety of open source tools for the community to employ [49,50,51,52].

Although both sources facilitate obtaining ZFNs, these have to be tested on a case-by-case basis for in vivo functionality, and suboptimal candidates often have to be abandoned because of a lack of straightforward optimisation protocols. Whereas screening systems exist for 1- to 2-finger libraries (e.g. phage display) and 3-finger mini-libraries of pre-selected modules (B2H [47]), no straightforward system exists to optimize the 4- to 6-finger type libraries of pre-selected modules (B2H [47]), no straightforward system exists to optimize the 4- to 6-finger type scaffolds which are provided by Sigma.

In this study, we set out to target the p53 gene using paired 4-finger ZFN [19], and to develop a platform for optimising polylfinger constructs. Because we found that classical phage display protocol could not handle >3-fingers (4-finger fd phage display always resulted in truncations during selection; data not shown), we modified a commercially-available yeast one-hybrid kit to optimize such libraries. This was used to generate functional ZFNs against two different sites (one exonic, one intronic) located within the human p53 gene, in close proximity to the mutation hotspots of p53 in somatic cancers (Fig. 1).

Results

Yeast one-hybrid selection of zinc fingers against two loci in the human p53 gene

One-hybrid screening in yeast is a powerful method to rapidly identify DNA-binding peptides that can interact with a specific DNA sequence of interest. We therefore developed a yeast one-hybrid (Y1H) selection system for zinc finger peptides, based on the commercially-available Matchmaker Kit (Clontech). The system allowed us to construct semi-randomised zinc finger libraries by PCR (without cloning), and to screen them in one step, by yeast transformation and plating on selective medium (Fig. 2).

We constructed four zinc finger libraries against two sites in the human p53 gene (with two sub-sites L/R each for a functional ZFN configuration; see Fig. 1B, C). The libraries were rationally designed, based on the established protein-DNA recognition code [53] and previously successful library designs [46]. Thus, the libraries were randomized at several base-contacting positions in the alpha-helix domains of the zinc fingers (library designs and randomisation strategy are listed in Methods S1). The four-finger library cassettes were built from degenerate oligonucleotides, via a PCR-based construction [54], and were transformed directly into yeast, together with linearized “prey plasmid” (pGADT7-Rec2) and the “bait plasmids” with the target DNA sequences (Fig. 2B). The system exploits the high rate of recombination in yeast to bypass standard cloning and to allow the PCR library to fuse in frame with the prey vector, in the single selection step.

A further modification to the Y1H, that we found to be essential for selecting ZFP, was that the library cassettes had to be introduced N-terminal to the Gal4-AD, rather than C-terminal as in the Matchmaker kit (Clontech). An example of a PCR cassette, complete with homology arms, ready for transformation into yeast is provided in Methods S1. The new configuration reduces the number of false positive clones arising from truncated peptides binding to the target sites; N-terminal truncations result in frameshifts or deletion of the Gal4-AD, in most cases.

To create the “bait plasmids”, the target DNA sequences for the four ZFNs z771L, z771R, z1166L and z1166R were cloned in single copies, upstream of a minimal promoter controlling the HIS3 reporter gene (Fig. 1C and 2). Successful application of any Y1H system is constrained by requiring low recognition of the target sequence by endogenous transcription factors. However, the basal expression of the His3 protein, in the absence of an activating prey protein, can be repressed by using 3-AT (a competitive inhibitor of the His3 enzyme) [55]. In the presence of increasing amounts of 3-AT, more His3 needs to be expressed to confer growth and so the selection pressure can be fine-tuned. Before screening the libraries, we tested each target site for basal histidine expression in the absence of activating zinc finger proteins. The amount of 3-AT needed to fully suppress basal expression varied between 25mM (z1166L) and 75mM (z771L, z771R, z1166R). Consequently, 100–150 mM 3-AT was used for screening the libraries for DNA-binding ZFP. It is worth noting that, classically, Y1H uses chromosomal integration of the bait plasmid to reduce basal expression. However, the Clontech kit uses low-copy-number plasmids to obviate this need (Clontech Protocol No. PT3289-1).

To interrogate the zinc finger libraries in our Y1H assay, we were co-transformed as PCR products, together with linearized prey plasmids and bait plasmids, into the yeast strain Y187, as described in the manual of the Matchmaker Kit (Clontech). Transformations were plated on selection media containing 3-AT and lacking histidine, tryptophan and leucine. The number of screened clones per library was calculated according to the manual of the Matchmaker Kit (Clontech) and was typically 100 000–160 000 for each experiment. After incubation for 3–5 days at 30°C, library screening revealed many potentially positive colonies of various sizes (200 to 2000 colonies). Ultimately, 96 colonies were picked for each library and were replated in a 96-well pattern, on selection media containing 3-AT.

Although this workflow undersampled both the theoretical library sizes (Methods S1) and potential positives, it was sufficient to obtain potentially-functional ZFP in one transformation step, starting from rational designs that had little or no activity in DNA cleavage assays, such as those shown in Fig. 3. The next step was therefore to test these candidates for ZFN cleavage activity.

In vitro cleavage of p53 target sites

To validate Y1H-based protein-DNA interactions, ZFP genes were recovered from yeast colonies by PCR, while introducing a T7 promoter for subsequent expression. After a PCR-based fusion to the Fold domain, the full length ZFN candidates were expressed in vitro and tested for specific cutting activity in an in vitro cleavage assay (see Methods and Fig. S1). This approach allowed us to identify several 4-finger anti-p53 ZFNs that bind and cleave their palindromic target sites efficiently in vitro (z771L, 4 clones; z771R, 1 clone; z1166R, 3 clones; z1166L, 1 clone).

From these clones, the best ZFN were similarly tested in homo- and heterodimer pairs, for cleaving either palindromic test sites or full DNA target sites (in the configuration shown in Fig. 1C; L = left; R = right). The L/L and R/R-homodimers only cut their respective palindromic target sites but not the unspecific targets (Fig. 3B; left panel). Conversely, the full heterodimer targets (ts/71 and ts/166) were cleaved only by the correct combination of z771L/R or z1166L/R, respectively, showing that a full pair of ZFN are required for cutting (Fig. 3B, right panel).
Episomal gene repair

To evaluate whether our potential p53-specific ZFNs (z771 and z1166) could promote homologous recombination or gene repair in vivo, they were first tested in a plasmid-based EGFP repair assay, developed by the Cathomen lab [56] (Fig. 4A).

In this assay, a promoterless EGFP sequence, with lacZ gene homology arm, is used to repair a 5'-truncated (non-fluorescent) EGFP gene, and the process is stimulated by cleavage with the appropriate nuclease. The target plasmid harbors a 18-bp recognition binding site for the meganuclease I-SceI, which serves as a positive control, in combination with a target site for the anti-p53 ZFNs, z771 or z1166. The system is thus designed to restore EGFP expression by generating a lacZ-EGFP fusion protein upon nuclease-induced homologous recombination. The expression of the red-fluorescent protein DsRed-Express (REx), from a gene cassette located on the repair plasmid, labels transfected cells [56].

To validate the efficiency of our ZFNs, we transfected HEK293T cells with either target plasmid “ts771” or “ts1166”, the repair plasmid and the respective PGK-driven ZFN pairs z771L/R and z1166L/R. 48 hours after transfection, the percentage of green and red cells was assessed by flow cytometry (Fig. 4B). The GFP repair assay revealed that the zinc finger nucleases showed the strongest activity when expressed in appropriate pairs to form heterodimers. The repair efficiencies approached that of the benchmark control, the meganuclease I-SceI (z771L/R, 20.9%; z1166L/R, 24.5%; I-SceI, 20%–25%).

Some gene repair was observed when z771L (12%) and z771R (15%) were expressed alone, probably due to DNA-binding by one ZFN monomer, followed by non-specific FokI dimerisation. This was only seen for the nucleases with stronger activity (e.g. z1166L alone did not considerably activate HR). Recent advances in generating obligate heterodimer ZFN have demonstrated that it is possible to remove this activity [57,58], and we used such mutants in downstream assays. Notably, the EGFP-background level in the absence of a nuclease was relatively high, representing spontaneous homologous recombination events in this episomal system (8.2% for target plasmid ts771 and 6.4% for ts1166). Nonetheless, the nuclease-induced signals were highly reproducible and statistically significant (z771L/R, p<0.002; z1166L/R, p<0.001). Therefore this assay is a good way of validating ZFN for cellular use.

Chromosomal targeting of p53 gene by ZFNs

To determine whether our custom built ZFNs would also work on a genomic level, we transfected HEK293T cells with PGK-driven ZFN expression vectors against the target sites z771 and z1166, together with homology repair plasmids. Because p53 cancer mutations are localised to one region of hotspots (Fig. 1A), repair plasmids covering the majority of hotspots could be
Figure 2. Yeast one-hybrid based selection for ZFNs. (A) The ZF-target site is cloned upstream of a minimal promoter (P_min) and the HIS3 reporter in the bait plasmid. Any interaction between a ZFP:Gal4-AD fusion protein and the target sequence stimulates transcription of HIS3 allowing selection on His-selective medium. (B) The ZF-library:Gal4-AD fusion is generated by yeast recombination of a PCR-generated four-finger library cassette with the linearized prey plasmid; no extra library cloning step is required. Thus, bait plasmid, linearized prey plasmid and library PCR cassette are co-transformed into yeast. After incubation for 3–5 days, expression from the HIS3 reporter is detected in colonies that are able to grow on a selection medium that lacks histidine and contains 3-AT (see Methods). ZFP from positive clones are rescued by colony-PCR, are fused to a FokI domain and are tested for activity by an in vitro cleavage assay. (C) Zinc finger library PCR template (z1166L). The template is based on 262-finger units from F2-F3 of the Zif268 sequence [65]. Each pair of 2-finger units is separated by a longer TGSERP linker [66]. The final linker, (QNNKQLV.KSEL) is compatible with the FokI sequence and is adapted from [16]. DNA-recognition helices are selectively randomised at certain positions (marked “X”). Full sequences and randomisation strategy are in Methods S1.

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synthesised, containing either 1.35 kb or 1.78 kb DNA, homologous to the genomic p53 locus between exons 5–8 or 6–8 (Sequences given in Methods S1).

The donor plasmids contained modified sites in the target sites of the z771- and z1166-ZFNs, to avoid cutting of the donor plasmid by the respective ZFNs, and to allow detection of genomic recombination of the plasmid by PCR analysis (Fig. 5). The latter was achieved using external PCR primers to amplify the p53 genomic regions, followed by semi-nested PCR, with a forward primer specific for the modified DNA sequence (‘‘barcode’’), and an external genomic reverse primer. The modified barcodes in the exonic target z1166 were carefully-chosen silent mutations that do not alter the p53 amino acid sequence.

For analysis of genome editing, genomic DNA was prepared from a pool of treated HEK293T cells, 3–6 days after transfection. Targeted donor recombination at the p53 locus was demonstrated by semi-nested PCR (Fig. 5B). Both ZFN-pairs were able to induce recombination of the donor plasmid with the chromosomal p53 gene, whereas control cells, transfected only with donor plasmid (and an empty PGK expression vector), did not show any sign of donor plasmid recombination. Although ZFN-specific recombination was seen with both repair matrices, the shorter exon 6–8 donor plasmid gave the clearest results because we were able to employ a particularly specific external genomic primer, just at the start of exon 6. Therefore this donor plasmid was mainly used in subsequent assays.

Next, we applied our z1166-ZFNs to induce the restoration of wt-p53 status in the human glioblastoma cancer cell line SF268, which harbors a single missense mutation (cgt\rightarrow cat) at codon 273 in the core domain of p53 [59]. The SF268 cells were also transfected with PGK-driven z1166-ZFN expression vectors and a donor plasmid with wild type p53 sequence at codon 273. The treated cells were analyzed by PCR, as described for the HEK293T cells, and also showed site-specific recombination of the donor plasmid with the p53 gene, only when co-transfected with functional ZFNs (Fig. 5C).

As the point mutation in SF268 cells is located in exon 8, approximately 450 bp downstream of z1166 target site, PCR amplicons obtained with recombination-specific primers were subcloned by Topo-TA cloning; 10 clones were sequenced to check for downstream modification at the mutated R273H codon. All the clones showed a restoration of the p53 wild-type sequence.
This indicated that homology-directed repair occurred. 450 bp downstream of the ZFN-induced double-stranded break (Fig. 5C, bottom). This result is perhaps surprising, because 80% of gene conversion tracts in mammalian cells are expected to be within 100 bp of the double-stranded break [60]. Insertions can occur 400 bp away and further, albeit with much lower frequency [61,62]. It was therefore possible that the PCR amplicons did not reflect independent events, and so we set out to measure the frequency of ZFN-induced homologous recombination.

Measuring gene repair and non-homologous end-joining by deep sequencing

Next generation sequencing is an ideal tool to quantify the effects of ZFN on cells. Reads from genomic PCR-products can routinely give >20 million sequences (~100 bp length) in a single run, and primer barcoding can be used to mix different samples together, allowing subsequent data deconvolution. We therefore developed a Solexa-Illumina method to sequence p53 locus genomic PCRs, at the site targeted by z1166. We thus measured the short insertions and deletions caused by non-homologous end-joining (NHEJ), after a nuclease-induced double-stranded break [25]. We also measured the rate of ‘wt’ sequence insertion from a ‘barcoded’ donor plasmid (with wild-type protein-coding sequence).

First, using 31bp reads, we observed NHEJ from ZFN in HEK293T cells (Fig. 6A). The method involved 2 rounds of PCR: one external genomic PCR and one internal PCR to introduce 3bp sequencing barcodes and an MmeI cleavage site (see Methods S1). MmeI digestion allowed sequencing-adapter ligation as close to the region of interest as possible but, as a result, the method was qualitative rather than quantitative. Nonetheless, the method showed that ZFN treatment was required to observe insertions and deletions around the genomic cutting site; these mutations can be useful for knocking out genes [38,39].

Next, we carried out a series of experiments using an improved protocol: 104bp read length was achieved using a Solexa Genome Analyser IIx. After external genomic PCR, a second internal PCR introduced a 3bp sequencing barcode; the longer read length removed the need for MmeI digestion. Interestingly, we found that the slightly higher error rate of the newer HiSeq Solexa machine was suboptimal for this task, so the GA IIx was preferred. To achieve high-quality long reads with highly-similar PCR products, we found it was necessary to ‘spike’ the samples with random DNA (phiX DNA fragments; 50% of total input DNA). In each flow cell lane, after computationally filtering out phiX sequences, we were able to get around 5 million sequences in the correct orientation (~50%). We were thus able to mix up to 8 sequencing barcodes per lane (each representing a different sample, under different conditions), resulting in around 600 000 reads each.

Processing the data for measuring NHEJ required two steps. First, the different barcoded samples were extracted using filters for any sequences containing a 9bp prefix (with the 3bp unique sequencing barcode) and a 9bp suffix (after the ZFP binding site)(see Methods S1). Second, to reduce random sequencing errors (proportional to read length), we filtered for the short ~30bp region spanning the cutting site (sequences containing a
Methods S1). The percentage of sequences containing insertions or deletions was then calculated (Fig. 6B). By testing different cancer cell lines (SF268, K562 and BT549) we found that ZFN-dependent indels could be detected in all three, but were much more frequent at 30°C (transient cold shock) than at constant 37°C, as was recently reported [63]. Furthermore, we could observe NHEJ with both wt and obligate heterodimer FokI [57]. The increase in NHEJ signal with ZFN was up to 30-fold over background, indicating that next generation sequencing can be used reliably to measure this activity.

ZFN-driven gene repair was quantified in two human cancer cell lines (SF268, and BT-549). The cell lines were either transfected with z1166-ZFNs alone or with both ZFN and donor plasmid (to quantify ZFN-induced homologous recombination). First, the ability of the Solexa system to detect proportions of wt or mutant DNA was tested; plasmid samples were mixed at ratios of 1:100 or 1:1000, and were then processed as if they were genomic PCRs (Fig. 6C). The observed detection rate was indeed similar to that expected, despite the PCR amplification and adapter ligation steps during sample preparation. Next, a variety of constructs with...
different promoter and FokI nuclease variants were tested for their ability to induce homologous recombination (insertion of the donor plasmid sequence) (Fig. 6D,E). The best results were obtained with obligate heterodimer FokI nuclease [57], under a PGK promoter. Collecting the genomic DNA 7 days after the ZFN and donor plasmid transfection also helped to reduce background (Sigma Aldrich; Compo-Zr instructions). Although the absolute rates of homologous recombination are apparently quite low (~0.1%), this is still an ~100-fold improvement over background, indicating that the ZFN are functional at this locus.

In summary, we were able to use Y1H to engineer ZFN against p53 chromosomal targets and were able to show their activity to modify genomes at the selected loci.

Discussion

This study describes the development of a new yeast-based selection tool for the rapid construction and optimization of paired 4-finger ZFN [19]. We developed the Y1H tool because we found that our usual phage display system [46] did not work with more...
the difference between a functional or non-functional ZFN. Measurements were obtained from Y1H. Typically, however, Y1H made
to use PCR libraries directly and does not require a bacterial
classical phage display could not handle more than 3-fingers, we
PCR-gel-purification step in between selection rounds (to recover
different bacterial host strains, selection conditions, and even a
homologous recombination in the bacterial host (data not shown).
spontaneously-truncated variants with fewer fingers, via in-frame
bases beyond the double-stranded break.
indels from NHEJ, because no coding sequence is disrupted.
intronic sequences. Intron sites are likely to be more tolerant of
demonstrable activity (Fig. S2) [56]. Alternatively, the CMV promoter expressing ZFN
could compete with the CMV-driven GFP reporter, decreasing
expression of both. For example, we observed that CMV-HcRed
plasmid, used as a transfection marker, had reduced activity when
co-transfected with other CMV-driven plasmids, but not with
PGK-driven plasmids. After optimisation, i.e. finding the right
promoter for ZFN expression (PGK), and adding a nuclear
localization signal to the ZFN, we were able to get a good
induction of GFP repair by both our anti-p53 ZFNs.
Using the Solexa protocol to quantify ZFN effects on the z1166
chromosomal locus, we confirmed that transient 30°C cold shock
[63] improves the rates of NHEJ. The different cell lines each had
different rates of NHEJ, and K562 had the highest. For gene
repair, the best results were obtained using obligate heterodimer
ZFNs [57], under a PGK promoter, and waiting for 7 days after
transfection to reduce background from left-over donor plasmid.
Although the apparent rates of homologous recombination are
quite low (~0.1%), the percentage of modified cells may actually
be higher, since both alleles will not be modified in all cases.
Moreover, for targeted mutation or repair experiments, the use of
selection genes (e.g. puromycin resistance), combined with these
low but workable recombination frequencies, should help to
establish model cell lines or organisms.
We have demonstrated that our p53-specific ZFNs are functionally
active chromosomally and can be used to mutate or restore wt-p53
status. Overall, this study has provided a Y1H tool to optimise ZFN,
as well as functional anti-p53 ZFN for biotechnological applications.

Materials and Methods

Cell lines and culturing

The wt p53 cell line HEK293T was maintained in DMEM and
the mutant p53 cell line SF268 (kindly provided by A. Camero)
cultured in RPMI1640 at 37°C, in 5% CO2. All media were
supplemented with 10% FCS, 100 units/ml penicillin and
100 µg/ml Streptomycin.

Yeast one-hybrid selection of p53 zinc-fingers

Four-finger library cassettes were constructed from oligonucleo-
tides, using a PCR based construction approach (library designs
are listed in Methods S1). Two-finger units (F1-F2 and F3-F4)
were built from two oligonucleotides by overlapping primer
extension [54]. After amplification by PCR, introducing a BamHI
site at the 3′-end of F1-F2 and a BglII site at the 5′-end of F3-F4,
two-finger units were mixed, cut with BamHI and BglII and
conditionally ligated. The resulting four-finger library cassettes
(F1-F2-F3-F4) were amplified by PCR by using oligonucleotides
which added 5′-and 3′-homology arms for the prey plasmid
sequences in Methods S1).

Target sequences were inserted into pHis2.1 bait plasmid
(Clontech), using 22bp duplex DNA oligomers. Each pair of
oligonucleotides was annealed to form duplex DNA, with EcoRI/
Spel compatible overhangs, and ligated into EcoRI/Spel-cut vector
pHis2.1.

pGADT7-Rec2 prey plasmid (Clontech Matchmaker One-
Hybrid Library Construction and Screening Kit; Ref. 630304)
was modified by removing a second HindIII site at 2351bp by
site directed mutagenesis. The PCR libraries were then
introduced into HindIII-linearized modified prey plasmid, by
in-frame recombination in yeast strain Y187. The yeast were
simultaneously heat-transformed with linearized prey plasmid,
PCR library and bait plasmid, according to the manufacturer’s
instructions.

than three fingers. Selections from 4-finger libraries resulted in
spontaneously-truncated variants with fewer fingers, via in-frame
homologous recombination in the bacterial host (data not shown).
The truncated proteins were likely preferentially encapsidated,
displayed and infected, and so 4-fingers are beyond the size limit
that can be conveniently selected on capsid gene III. Despite trying
different bacterial host strains, selection conditions, and even a
PCR-get-purification step in between selection rounds (to recover
full-length clones), we were unable to overcome these issues. As
classical phage display could not handle more than 3-fingers, we
developed the yeast one-hybrid system, whose main advantage is that it uses PCR libraries directly and does not require a bacterial
cloning step.

Our main motivation was to be able to screen relatively small
libraries (<100 000 variants), with mutations spread over four or
more fingers (the system works equally well for 6-finger proteins;
Fig. S3). Small, targeted libraries can easily be rationally
designed, given the 13 years of data on the zinc finger DNA-
recognition code [15,53]. Moreover, the Y1H system can also be
used for affinity maturation of ZFP; libraries can be made by
error-prone PCR and PCR-shuffling for this purpose, starting
from a single ZFP design.

The use of yeast-based selection has certain advantages over
phage display or prokaryotic expression systems (although the
latter allow larger library sizes). For instance, the strategy allows
the direct selection of peptides that are able to recognize DNA in
vivo, without disrupting an eukaryotic cell. Thus, an element of
screening for eukaryotic specificity and neutrality is added: the
yeast genomic DNA, which is assembled into chromatin, is more
representative of the final environment where the ZFN will be used.
Nonetheless, the selection is for DNA binding and not ZFN
cleavage; future engineering strategies could aim to select for
cleavage activity and specificity directly. For example, the ZFN
could cleave a target-DNA that is contiguous with a conditionally-
lethal gene in yeast, such as Herpes simplex virus-thymidine kinase
(HSV-TK) [64].

It should be noted that the quality of the screening is strongly
dependent on the library design. In three cases (z771L, z1166L and
Ri), the Y1H libraries produced functional ZFN that were much
better than the original rational designs, around which the libraries
were based. Indeed, in these cases, the single rational designs had no
activity in the in vitro cleavage assay, whereas Y1H clones had
demonstrable activity (Fig. 3). The exception was z771R, which
was rationally designed and functioned so well that no improve-
ments were obtained from Y1H. Typically, however, Y1H made
the difference between a functional or non-functional ZFN.

We generated novel functional 4-finger nucleases against two
sites located within the human p53 gene, in close vicinity to the
mutation hotspots of p53 in cancers. The uniqueness of the targets
in the genome was verified by a genome scanning algorithm that
we developed (Table S1). This showed that the z771 and z1166
target sites are unique, and that the z1166 binding site had very
few related targets in the human genome (only one 2bp mismatch
and eight 3bp mismatches, for the full heterodimer site). z771 had
slightly more related targets, but these are mostly in duplicated
intronic sequences. Intron sites are likely to be more tolerant of
indels from NHEJ, because no coding sequence is disrupted.
Moreover, intron sites can still be used for exonic gene repair,
because homologous recombination can extend for hundreds of
bases beyond the double-stranded break.

Initially, expression of our ZFN constructs was driven by a
strong CMV promoter, but this was found to be suboptimal. It is
possible that high expression levels of the nucleases were not well
tolerated by the cells, probably leading to their elimination over
time through accumulation of non-specific double strand breaks
(Fig. S2) [56].
ZFNs were introduced into HEK293T, SF268, single-cuvette format of the Nucleofector Kit V (Lonza), according to the manufacturer’s protocol. HEK293T, SF268, and BT-549 cell lines were transfected in 6-well plates by Lipofectamine 2000 (Invitrogen), with 5 μg repair matrix donor plasmid, and 2 μg of ZFN expression vectors, or empty control vector. An EGFP expression vector (0.5 μg) was cotransfected in all the samples (except K562 cells) to identify transfected cells. Six days after transfection, 1×10⁵ GFP-positive cells per sample were collected by fluorescence activated cell sorting (FACS) and genomic DNA was isolated from cells with the DNA Blood and Tissue Kit (Qiagen). To detect homologous recombination in the p53 gene, we subjected 30–300 ng genomic DNA to PCR with high fidelity enzymes KOD Hot Start (Novagen) or Accuprime Taq DNA Polymerase (Invitrogen). PCR products were column purified with the PCR purification kit (Qiagen) and eluted in 30 μl H₂O. A fraction of the resulting amplicons was amplified by nested PCR with Taq polymerase using primers designed to discriminate between wild-type and integrated modified p53 sequence. Nested PCR amplicons were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. All primers and conditions are indicated in Methods S1.

Quantification of non-homologous end-joining and homologous recombination events

The frequency of targeted gene modification in ZFN-treated pools of cells was determined by Illumina’s Solexa deep sequencing platform. Transfections, genomic DNA preparation and PCR reactions were performed as described above. PCR products were excised from agarose gels, to avoid contamination with transfected donor plasmid, were column purified with a PCR purification kit (Qiagen) and eluted in 30 μl H₂O. 10 ng of the PCR products were amplified by nested PCR with KOD HiFi polymerase, with Solexa primers flanking the 1166 target region (~900bp up- or down-stream). The primers contained 3bp barcodes in order to distinguish between individual samples in a pooled Solexa lane. PCR amplicons were column purified for sequencing adapter ligation with Illumina protocols.

Illumina sequencing and basecalling

PCR products that contained single-read Solexa adapters on either end were mixed 1:1 with a phiX Solexa library (Illumina). Single-read v4 flowcells for the Genome Analyzer were used. After loading DNA at a concentration of 7 pM per flow cell lane, clusters were generated in the Illumina cluster station according to the recommendation of the manufacturer. Sequencing was performed on the Illumina Genome Analyzer Ix with TrueSeq SBS v3 sequencing chemistry, using a 104 cycle recipe. Basecalling was performed using SCS2.8. Primers and conditions and computational filters used for data processing are indicated in Methods S1.
Supporting Information

Figure S1  Experimental scheme to validate yeast one-hybrid clones. Assembly PCR was used to recover zinc finger sequences from positive yeast colonies and to fuse them to a FokI nuclease domain and T7 promoter, for in vitro transcription-translation expression (TnT). Clones marked with an asterisk showed clear cleavage activity of the target DNA (palindromic target site). These positives were subcloned for further verification. (TIF)

Figure S2  ZFN-associated toxicity assay. Flow cytometry data for HEK293T cells transfected with the indicated constructs and stained with antibodies against gH2A.X (top) or unspecific data for HEK293T cells transfected with the indicated constructs (TIF).

Figure S3  Six finger peptides engineered by Y1H for the GFP DNA sequence. (A) Canonical model of ZFP binding, where primary DNA contacts (arrows) are from four positions on each zinc finger alpha helix (circled, –1, 2, 3 and 6). Contacts are shown for the EGFP gene-binding construct, ZFP-GFPb249. The alpha helices are shown aligned to the DNA bases they would contact according to the canonical model. Note that zinc finger proteins (N-C) bind antiparallel to their primary contacting DNA strand (3'–5'). (C) Gel shift assays on ZFPs expressed in vitro from T7-promoter PCR products show that the 2 ZFPs bind their DNA targets. (TIF)

Table S1  Putative off-target sites. The number of occurrences (in the human genome) of sequences related to the target sequence were counted with a computer script written in C. For example, there are 35 sequences with 2 bases different from the full z771 zinc finger binding site (b8771). Overall, the z1166 binding site has fewer related targets in the human genome. bs = binding site, psts = palindromic target site. The different left- and right-ring-finger binding sites are highlighted in bold or normal font, respectively. (DOCX)

Methods S1  Supplementary methods and sequences. This document contains sequence templates and instructions for inserting zinc finger DNA sequences into the yeast 1-hybrid template. Strategies for selective codon randomisation are discussed. The sequences for the zinc finger libraries, final clones and donor plasmid sequences used in this study are also provided. Protocols for Solexa sequencing of ZFN genomic targets are described, as well as protocols for measuring double-stranded DNA breaks. (DOC)

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Author Contributions

Conceived and designed the experiments: FH AM HH ML. Performed the experiments: FH MG-C RB EF-S AM. Analyzed the data: JC. Contributed reagents/materials/analysis tools: JC. Wrote the paper: FH ML.

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