

Phenotypic alteration of eukaryotic cells using randomized libraries of artificial transcription factors

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We have developed a method in which randomized libraries of zinc finger-containing artificial transcription factors are used to induce phenotypic variations in yeast and mammalian cells. By linking multiple zinc-finger domains together, we constructed more than 100,000 zinc-finger proteins with diverse DNA-binding specificities and fused each of them to either a transcription activation or repression domain. The resulting transcriptional regulatory proteins were expressed individually in cells, and the transfected cells were screened for various phenotypic changes, such as drug resistance, thermotolerance or osmotolerance in yeast, and differentiation in mammalian cells. Genes associated with the selected phenotypes were also identified. Our results show that randomized libraries of artificial transcription factors are useful tools for functional genomics and phenotypic engineering.

In the course of differentiation, development and response to environmental challenges, cells and organisms display myriad phenotypes by altering the expression of specific genes in a timed manner. To facilitate the identification of genes and gene products responsible for phenotypes of interest, we sought to develop a method by which researchers can modify the phenotypes of cells and organisms at will for further study. Our approach mimics the method whereby cells and organisms achieve new phenotypes, namely, selective regulation of gene expression on a genome-wide scale. In our method, a large collection of artificial transcription factors is introduced into a population of cells and the population is then screened for the desired phenotype.

Zinc fingers are well-characterized, highly specific DNA-binding domains found in a wide variety of transcriptional regulatory proteins. Each individual finger recognizes a specific 3-base-pair (bp) DNA sequence¹, and a single transcription factor can contain multiple zinc fingers. Together, these fingers determine the DNA-binding specificity of the protein and, in part, its target genes. Because of their diversity and modular structure, zinc-finger DNA-binding domains are ideal building blocks with which to construct large numbers of transcription factors with diverse DNA-binding specificities^{2,3}.

Here we describe an approach for altering phenotypes of eukaryotic cells with combinatorial libraries of artificial transcription factors. We used a pool of individual zinc fingers with diverse DNA-binding specificities and randomly shuffled them to construct a collection of multifinger proteins. These proteins were then fused to either a transcription activation or repression domain. We used these libraries of artificial transcription factors to induce various phenotypes, such as drug resistance, thermotolerance or osmotolerance in Baker's yeast, and differentiation in mammalian cells.

RESULTS

Construction of randomized libraries of zinc-finger proteins

A typical zinc finger protein (ZFP) consists of three or more zinc fingers, and, as a first approximation, most zinc fingers recognize 3-bp subsites (Fig. 1a). Thus, a three-finger protein recognizes and binds to a 9-bp DNA sequence, and a four-finger protein binds to a 12-bp sequence. Scores of zinc fingers with diverse DNA-binding specificities have been identified in many naturally occurring transcription factors or created artificially by protein engineering techniques such as phage display and site-directed mutagenesis^{4–11}.

To produce libraries of ZFPs, we cloned DNA segments that encode zinc fingers with diverse DNA-binding specificities and shuffled them randomly to make composite DNA segments that encode three-finger or four-finger proteins (Fig. 1b). These assembled DNA segments were then linked to DNA segments that encode either a transcription activation or repression domain to produce libraries of transcription activators or repressors, respectively. Because a ZFP that does not contain an activation or repression domain can function as an efficient transcription repressor when it competes with endogenous transcription factors for binding to DNA sequences near the site of transcription initiation^{12,13}, we also included in our transcription factor library assembled zinc-finger domains that had not been fused to any additional domains. We chose 25 zinc fingers with which to build four-finger proteins and 40 zinc fingers with which to build three-finger proteins^{4,8,9,14–20}. The zinc fingers used to generate these two sets of multifinger ZFPs recognize 25 3-bp subsites out of 64 ($= 4 \times 4 \times 4$) possible subsites (Supplementary Table 1 online). Therefore, we estimate that our ZFP transcription factor (ZFP-TF) libraries contain, on average, 358 three-finger proteins ($= (25/64)^3 \times 2,000 \text{ (bp)} \times 3 \text{ (TFs)}$) or

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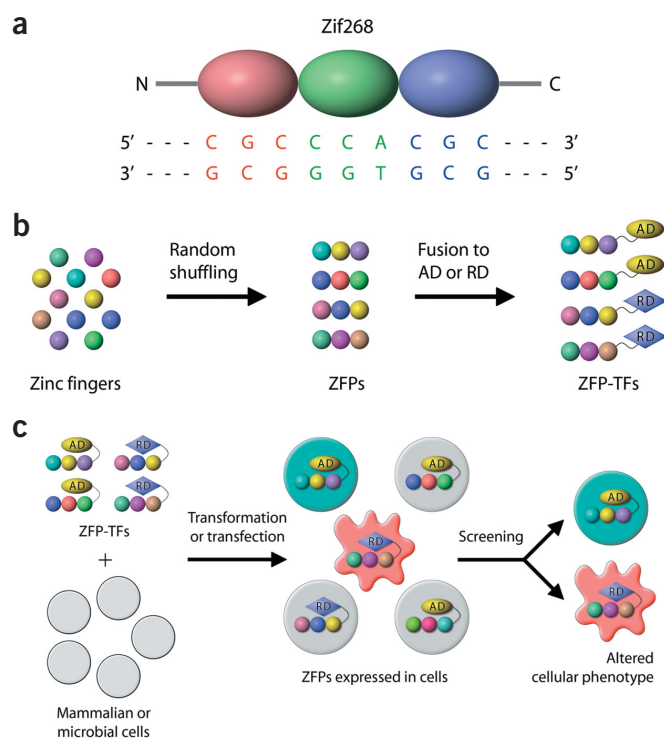


Figure 1 Schematic representation of the ZFP library approach. **(a)** Mode of target-site recognition by the Zif268 zinc-finger protein¹. Each zinc-finger domain recognizes a 3-bp subsite, and the Zif268 protein, which is composed of three zinc fingers, binds to a 9-bp target DNA sequence. **(b)** Construction of randomized libraries of ZFP-TFs. DNA segments that encode zinc fingers are randomly shuffled to generate composite DNA segments that encode three-finger ZFPs. These assembled DNA segments are then linked to DNA sequences that encode a transcription activation domain or repression domain to produce libraries of artificial transcription factors. We used the Gal4 activation domain and the Ume6 repression domain in yeast and the p65 activation domain and the KRAB repression domain in mammalian cells. **(c)** Steps of the ZFP library approach. Plasmid libraries encoding ZFP-TFs are used to transform or transfect cells. In each cell, a different ZFP-TF is expressed and regulates unknown target genes in an unbiased manner. This leads to phenotypic variation among transformed cells. Cells that display an altered phenotype are selected by screening. ZFP, zinc-finger protein; AD, activation domain; RD, repression domain.

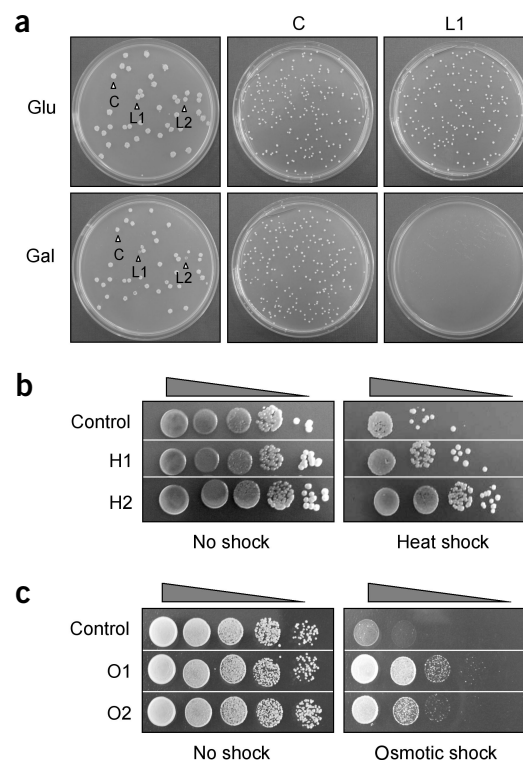


Figure 2 Various phenotypic changes in yeast induced by artificial ZFP-TFs. **(a)** Growth-defective cells are observed upon expression of ZFP-TFs. Colonies that grew normally on glucose but became growth defective on galactose were identified. Arrowheads marked L1 and L2 indicate positive clones that display a growth-defective phenotype, whereas an arrowhead marked C indicates control cells. **(b)** Thermotolerant phenotype induced by ZFP-TFs. Growth of two selected thermotolerant clones, H1 and H2, was monitored after incubation at 30 °C (no shock) or 52 °C (heat shock). **(c)** Resistance to osmotic shock induced by ZFP-TFs. The growth of osmotolerant mutants (O1 and O2) and control cells was monitored on galactose plates containing 100 mM LiCl. After the first round of phenotypic screening, all the phenotypic changes were confirmed by plasmid rescue, sequence analysis and retransformation. In **b** and **c**, triangles above each panel indicate tenfold serial dilutions (1:1 to 1:10,000, left to right) of spotted yeast cells.

140 four-finger proteins per a given 1 kilobase pair (kbp) stretch of DNA. (Note that the probability that at least one ZFP exists in the three-finger protein library that matches a given 9-bp stretch of DNA is $(25/64)^3$. In a given 1-kbp stretch of DNA—the typical size of a eukaryotic promoter—, there are approximately 2,000 overlapping 9-bp sites on both strands. Finally, in our libraries, there are three different types of transcription factors: isolated ZFPs, ZFP–activation domain fusions and ZFP–repression domain fusions.) However, it is unlikely that all of these transcription factors will actually bind to a given stretch of DNA in the genome if one considers the local chromatin structure. Still, these numbers seem large enough that one could expect to find transcription factors that regulate most, if not all, of the genes in virtually any eukaryotic genome.

Phenotypic changes in yeast

First, we chose baker's yeast, *Saccharomyces cerevisiae*, as a model organism in which to test our approach. In all subsequent experiments in yeast, cells were transformed with plasmid libraries encoding ZFP-

TFs, grown under selective or stressful conditions, and screened for several desired phenotypes (Fig. 1c). Plasmids encoding ZFP-TFs were rescued from cells selected in the screen and were used again to transform yeast cells to confirm the phenotypic alteration. The identities of the ZFPs were determined by sequencing.

The first phenotype that we screened for was growth arrest, and a number of cells showed growth arrest only in the presence of galactose (Fig. 2a). Because expression of the ZFP-TFs in yeast had been put under the control of a galactose-inducible promoter, we concluded that the ZFP-TFs expressed in the yeast cells were responsible for the growth arrest phenotype.

We also screened for ZFPs that induced industrially important traits in yeast. During fermentation, osmotic pressure is increased and excessive heat is generated. These stressful conditions often limit the productivity of yeast culture systems. We screened for cells that became resistant to heat treatment or osmotic pressure upon expression of the ZFP-TFs in our libraries. For example, more than 99.6% of wild-type cells died upon heat treatment at 52 °C for 2 h (Fig. 2b). In

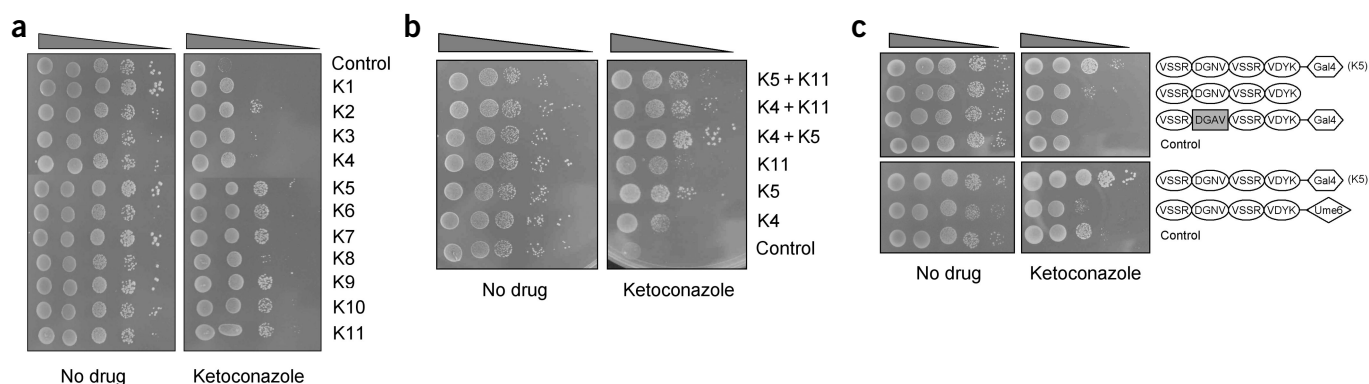


Figure 3 Characterization of ketoconazole-resistant transformants. **(a)** Eleven ketoconazole-resistant ZFP-TF transformants were selected on the basis of their growth on agar plates containing ketoconazole (35 μ M). **(b)** Coexpression of ZFP-TFs in yeast cells leads to phenotypic enhancement. Various combinations of plasmids, each of which encodes the K4, K5 or K11 ZFP-TFs, were used to transform yeast cells. The increased drug-resistance phenotype of cotransformants was analyzed by growing them on plates containing ketoconazole (50 μ M). **(c)** Selective mutagenesis of the K5 ZFP-TF (VSSR-DGNV-VSSR-VDYK-Gal4). Three different mutants were prepared. In one, the activation domain was removed by inserting a stop codon after the fourth zinc finger (VSSR-DGNV-VSSR-VDYK). In another, an asparagine residue in the DGNV zinc finger was mutated to alanine (VSSR-DGAV-VSSR-VDYK-Gal4). Yeast cells expressing each of these mutated ZFPs were tested for drug resistance by growth on medium containing ketoconazole (35 μ M). In the third mutant, the Gal4 activation domain was replaced with the Ume6 repression domain (VSSR-DGNV-VSSR-VDYK-UME6), and cells expressing this protein were tested by growth on medium containing ketoconazole (25 μ M). Control, wild-type yeast cells transformed with an empty plasmid. Triangles above each panel indicate tenfold serial dilutions (1:1 to 1:10,000, from left to right) of spotted yeast cells.

contrast, 10% of cells transformed with certain ZFP-TFs survived under these extreme conditions, a 25-fold increase in the thermotolerance phenotype (that is, the percentage of cells expressing ZFP-TFs that survived under heat-stress conditions (10%) divided by the percentage of control wild-type cells that survived under the same conditions (0.4%)). These phenotypic improvements were observed only when galactose was present in the growth medium (data not shown).

Next, we used lithium chloride (LiCl) to induce osmotic stress²¹. The presence of 100 mM LiCl in the culture medium almost completely inhibited the growth of wild-type yeast cells. However, we were able to isolate surviving cells from the pool of ZFP-TF transformants grown under the same conditions (Fig. 2c). Various levels of osmotolerance were observed in the selected cells, some displaying up to a 100-fold increase in the phenotype. Again, these phenotypic improvements were observed only in the presence of galactose. These two examples clearly demonstrate that screening ZFP-TF libraries can rapidly yield improvements in the traits of industrially important organisms.

We then applied our approach to a drug resistance phenotype in fungi. Although ketoconazole is a widely used antifungal drug, strains that have developed resistance to this drug limit its utility²². An understanding of the mechanisms of drug resistance and the identification of genes associated with this phenomenon are of interest to the biomedical community. Thus, we screened for ZFP-TFs that conferred ketoconazole resistance upon yeast cells. Of 10^7 transformants screened, 120 gave rise to colonies on agar that contained 35 μ M ketoconazole. We determined the DNA sequences that encoded the ZFP-TFs from 23 randomly chosen ketoconazole-resistant colonies. This led to the identification of 11 different ZFP-TFs (Supplementary Table 2 online). The degree of drug resistance varied among these ZFP transformants (Fig. 3a).

Expression of two selected ZFPs often resulted in an enhancement of the drug resistance phenotype. For example, each of the ZFP-TFs, K4, K5 and K11, alone conferred only partial (10-fold to 100-fold) ketoconazole resistance upon yeast cells. When two of these ZFP-TFs were expressed together, yeast cells became completely resistant to

ketoconazole; this was an $\sim$ 1,000-fold enhancement in the resistance phenotype (Fig. 3b).

If the various phenotypic changes observed in these selected cells resulted from the activity of ZFPs as transcription factors, then DNA-binding activities of ZFPs and the presence of appropriate functional domains would be essential. To study this, we generated ZFP mutants with one of the two following alterations: (i) a key amino acid residue that was expected to function in DNA base recognition was mutated, or (ii) an activation domain was either removed or replaced with a repression domain. In one mutant, an asparagine residue in the second zinc finger was mutated to alanine. Electrophoretic mobility shift assays demonstrated that the mutated ZFP exhibited at least a tenfold decrease in affinity for its cognate DNA-binding site (data not shown). As expected, this mutant protein did not confer the ketoconazole resistance phenotype upon yeast cells (Fig. 3c). In another mutant, the Gal4 transcription activation domain present in the K5 ZFP-TF was deleted by inserting a stop codon in front of the DNA sequence that encodes the activation domain. This protein also failed to render cells resistant to ketoconazole (Fig. 3c). When the activation domain fused to the K5 ZFP-TF was replaced with the Ume6 repression domain²³, the transformant showed a reversed phenotype (that is, the cells became sensitive to ketoconazole) (Fig. 3c). This result implies that, at least in some cases, it is possible to induce the desired phenotype by first selecting ZFP-TFs that confer the opposite phenotype and then replacing the effector domain with another domain with the opposite function. This is useful for cases where the opposite phenotype is more amenable to screening than the desired phenotype.

From these mutagenesis analyses, we conclude that our selected ZFP-TFs do indeed function as transcription regulators *in vivo*. However, we note that some naturally occurring ZFPs exert their effects by binding to RNA or proteins^{24,25}. Therefore, it is possible that some of our ZFP-TFs induce the selected phenotypes by these alternative mechanisms.

Identification of genes associated with drug resistance

We performed DNA microarray experiments to identify genes that are associated with the ketoconazole resistance phenotype. Three ZFP-TFs,

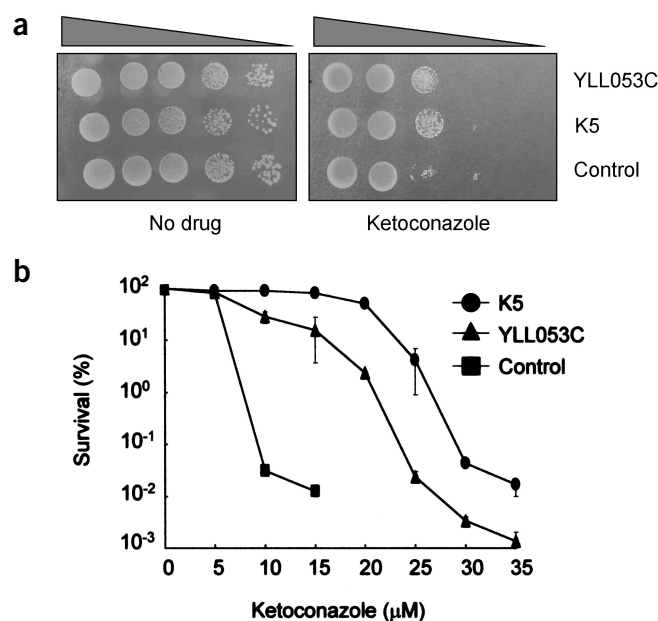


Figure 4 Identification of target gene associated with ketoconazole resistance. (a) Induction of ketoconazole resistance by overexpression of yeast ORF *YLL053C*. Cells were grown on medium containing ketoconazole (35 μM). K5, K5 ZFP-expressing yeast cells; control, wild-type yeast cells. Triangles above each panel indicate tenfold serial dilutions (1:1 to 1:10,000, left to right) of spotted yeast cells. (b) Cell viability test on medium containing variable concentrations of ketoconazole (0, 5, 10, 15, 20, 25, 30 or 35 μM). Control, cells harboring an empty cloning vector; K5, K5 ZFP-expressing yeast cells; YLL053C, yeast cells overexpressing *YLL053C*.

K5, K6 and K7, were chosen for genome-scale expression profiling analyses. All of these transcription factors contained the Gal4 activation domain (Supplementary Table 2 online). These ZFP-TFs recognize 12-bp DNA-binding sites. We analyzed intergenic sequences in the yeast genome to search for sequence elements that matched the binding sites for the ZFP-TFs. The number of open reading frames (ORFs) that have 12-bp binding sites in their upstream intergenic regions that perfectly match binding sites for the K5, K6 or K7 ZFP-TFs was 2, 0 or 14, respectively. Expression of these ORFs was not shown to be activated or repressed more than twofold. It still is possible that some of the genes that are modulated less than twofold are direct targets of the ZFP-TFs. Alternatively, ZFP-TFs may also bind to sites in which there is some degree of mismatch. However, because our intention was to identify genes associated with the drug resistance phenotype, and not necessarily genes directly regulated by our ZFP-TFs, we shifted our focus to genes that are differentially coregulated by at least two ZFP-TFs.

We reasoned that different ZFP-TFs that confer the identical phenotype might regulate similar sets of genes whose differential expression is associated with the phenotype. Indeed, out of 6,400 yeast ORFs, ten ORFs were activated more than twofold by at least two of the ZFP-TFs tested, and four ORFs were activated by all three ZFP-TFs (Supplementary Table 3 online). First, we noticed that *PDR5*, a gene known to pump ketoconazole out of the cell²⁶, was activated by two ZFPs, K6 and K7, but not by K5. Thus it appears that at least two different biological pathways, one of which involves the activation of *PDR5*, participate in conferring ketoconazole resistance in yeast. To identify new genes associated with the drug resistance phenotype, we overexpressed each of the four activated genes and tested for drug

resistance. One of the genes induced ketoconazole resistance when overexpressed in yeast cells (Fig. 4). This gene sequence was identified as a hypothetical ORF, *YLL053C* (NCBI accession number NC_001144), that is highly homologous to plasma membrane and water channel proteins in another yeast, the pathogen *Candida albicans*. It is not yet clear whether the product of *YLL053C* gives rise to the observed phenotype by pumping out ketoconazole, as seen with the *PDR5* gene product.

Phenotypic changes in mammalian cells

We next demonstrated that the ZFP-TF approach is useful for conducting screens in mammalian cell culture systems. To synthesize ZFP-TFs that are active in mammalian cells, we created a library using ZFPs that had been fused to the p65 transcription activation domain^{20,27} or the Krüppel-associated box (KRAB) repression domain^{28,29}.

Using these libraries, we first screened for factors that can induce neurogenesis of the mouse neuroblastoma cell line Neuro2A, which is capable of differentiating into a neuronal cell type. Cells were transiently transfected with randomly chosen ZFP-TFs from the library, along with a β-galactosidase (*lacZ*) reporter plasmid to follow the morphological changes of the transfected cells. Five days after transfection, the cells were fixed and stained for *lacZ* enzyme activity, and cells were surveyed for morphologies characteristic of neuronal differentiation, such as increased neurite length and thickness. From a screen of about 1,000 randomly chosen ZFP-TFs, we identified several that induced neurogenesis. Of these, we chose one ZFP activator, named Neuro1-p65, for further study. More than 40% of cells transfected with Neuro1-p65 underwent neuronal differentiation, as judged by neural morphologies of the *lacZ*-positive cells (Fig. 5a). Retinoic acid has been shown to induce neuronal differentiation in neuroblastoma cells³⁰, and we observed neurites in about 15% of cells treated with 10 μM retinoic acid (Fig. 5b). Thus, in terms of the percentage of cells affected, Neuro1-p65 induces neuronal differentiation more efficiently than does retinoic acid at the tested concentrations. Site-directed mutagenesis of an amino acid residue critical for DNA binding by Neuro1 ZFP (Neuro1mut-p65) abolished the ability of the ZFP-TF to induce neurogenesis (Fig. 5b). The capability of Neuro1-p65 to induce neurogenesis was also dependent on the transcription activation efficiency of the p65 domain, as the Neuro1 ZFP DNA-binding domain either alone (Neuro1 alone) or fused to the KRAB transcription repression domain (Neuro1-KRAB) failed to induce neurogenesis (Fig. 5b).

Next, we screened for ZFP-TFs that induced another differentiation process, transdifferentiation of murine myoblasts to osteoblasts. The C2C12 cell line is of the myoblast lineage, but can differentiate into osteoblasts upon the addition of bone morphogenic protein-2 (BMP-2)³¹. We transiently transfected C2C12 cells with randomly chosen ZFP-TFs and looked for cells that underwent transdifferentiation in the absence of BMP-2. Seven days after transfection, cells were stained for alkaline phosphatase (ALP), a marker of osteoblasts³¹. As a positive control, cells were treated with BMP-2. Nearly 100% of cells stained strongly for ALP upon BMP-2 treatment (Fig. 5c, left). Without BMP-2, transfection of cells with the empty vector gave only background staining (Fig. 5c, middle). From a screen of about 2,000 ZFP-TFs, we identified one activator, named Osteo1-p65, that induced strong ALP staining in about 30% of the cells (Fig. 5c, right). The percentage of cells that stained positive for ALP was similar to the percentage of transfected cells, as judged by *lacZ* staining of cells transfected in parallel (data not shown).

Finally, we used the well-established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)³² assay to screen for a

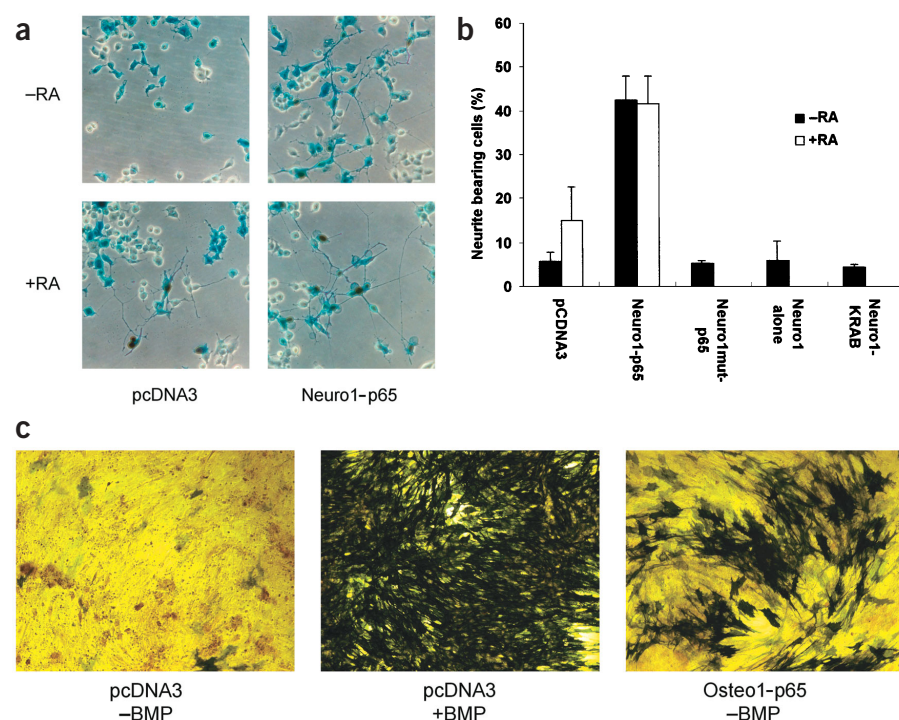


Figure 5 Phenotypic changes induced by ZFP-TFs in mammalian cell culture systems. (a) Neuro2A cells were transiently transfected with either an empty vector (pcDNA3) or a vector encoding Neuro1-p65, a ZFP-TF activator selected by library screening, in the presence (+RA) or absence (–RA) of 10 μ M retinoic acid. A vector expressing *lacZ* was used to cotransfect cells. Four days after transfection, cells were stained with LacZ and cellular morphology was surveyed. (b) Quantitative analysis of neurogenesis. LacZ-positive cells whose neurite lengths were at least twice that of the cell body were counted. These numbers were divided by the total number of LacZ-positive cells to calculate the percentage of neurite-bearing cells. Data shown here are the averages of two independent experiments, and for each experiment at least 250 cells were counted. (c) A ZFP-TF triggers transdifferentiation of C2C12 from myoblasts to osteoblasts. C2C12 cells were transiently transfected with either an empty vector (pcDNA3) or a vector that encoded the Osteo1-p65 activator in the presence or absence of BMP-2. Seven days after transfection, cells were stained for alkaline phosphatase activity. The identities of ZFP-TFs screened in the experiments summarized in this figure are shown in **Supplementary Table 4**.

change (positive or negative) in the growth rate of cells. We selected two ZFP-TF clones, one in which growth was inhibited and another in which cell proliferation was stimulated (**Supplementary Fig. 1** online). Taken together, these results demonstrate the utility of ZFP-TF library screening in mammalian cell culture systems.

DISCUSSION

We conclude that it is possible to generate phenotypic variation both in microbes and mammalian cells by randomly regulating gene expression using combinatorial libraries of artificial transcription factors. This ZFP library approach is reminiscent of the chemical genetics approach³³. Both of these approaches use combinatorial libraries of small molecules or transcription factors in phenotype-driven, cell-based assays. Whereas small molecules target proteins and modulate their functions, ZFPs target genes and regulate their expression.

We suggest that the ZFP library approach provides a useful tool for functional genomics and cell engineering and has several advantages over current techniques such as random mutagenesis, comprehensive gene overexpression, gene knockout and screening of antisense libraries, ribozyme libraries or siRNA libraries^{34–38}.

First, because we have shown that ZFP-TFs function in two evolutionarily distant systems (yeast and mammalian cells), we predict that this technology will be applicable in many, if not all, organisms of commercial and scientific interest. In this regard, we note that ZFP-TFs have been successfully tested in plants^{39,40} and eubacteria⁴¹. Second, ZFP-TF libraries encode both transcription activators and repressors, increasing the possibility that highly diverse phenotypic changes will be observed. Third, ZFP-TFs can cause subtle changes in gene expression. In cases where overexpression or knockout of a gene is lethal to the organism, subtle changes in gene expression might allow one to achieve the desired phenotypes. Fourth, our method requires substantially fewer rounds of screening than do most other approaches. Conventional mutagenesis typically requires screening of 10^7 – 10^9 clones⁴². The diversity of a ribozyme library must be at least

on the order of 10^7 (refs. 35,37). We have isolated various ‘mutants’ with the desired phenotypes by screening only 10^5 – 10^6 ZFP-TFs in yeast and only a few thousand ZFP-TFs in mammalian systems. Fifth, the genes that are associated with the selected phenotypes can be identified. And finally, phenotypic transfer from one strain to another strain of the same species is straightforward with our approach.

Recently, Blancafort *et al.* described the use of combinatorial libraries of ZFPs in the regulation of surface antigen expression in mammalian cells⁴³. They used flow cytometry to select cells in which a target gene product was overexpressed as a result of the action of a ZFP-TF. Thus, this paper described a method for using randomized libraries of ZFP-TFs in the expression of predetermined target genes. In contrast, we have used libraries of ZFP-TFs in the selection of phenotypes with no predetermined target genes.

Zinc fingers constitute the most abundant DNA-binding motif encoded in the human genome⁴⁴. Various genetic events, such as DNA duplication, translocation, deletion or addition, that took place during evolution are believed to have contributed to the proliferation of ZFPs and the phenotypic diversity they engender. Similarly, our approach represents a form of ‘mutagenesis’ that should facilitate the generation of phenotypes useful in diverse areas of biomedical research and biotechnology.

METHODS

Yeast strains and plasmids. The *S. cerevisiae* strain used for all our experiments except the osmotolerance test was YPH499a (*MATa*, *ade2-101*, *ura3052*, *lys2-801*, *try1- Δ 63*, *his3- Δ 200*, *leu2- Δ 1*, *GAL⁺*). In the osmotolerance experiments, EGY48 (*MAT α* , *his3*, *trp1*, *ura3*, *LexA_{op}(X6)-LEU2*) was used. Transformation of yeast cells was carried out with the lithium acetate method. The pcDNA3 vector (Invitrogen) was modified to have restriction enzyme sites necessary for the initial cloning of zinc fingers and the subsequent construction of the ZFP-TF library. pYESTrp2 (Invitrogen), with the following modifications, was used for expression of the ZFP library in yeast. The two origins of replication in the pYESTrp2 vector were replaced with CEN-ARS, and the B42 transcription activation domain was removed. A V5 epitope tag and a nuclear localization signal

were inserted between the initiation codon and the cloning site of the zinc-finger protein. The constructed vector was termed pYTC-Lib.

Construction of ZFP-TF libraries for use in yeast. Plasmid libraries encoding three-finger or four-finger transcription factors were constructed with 40 or 25 zinc fingers, respectively (Supplementary Table 1 online). First, nucleic acids that encode each zinc-finger domain were cloned into pYTC-Lib to form 'single-finger' vectors. Equal amounts of each 'single-finger' vector were combined to form a pool. The pool was separately digested with two sets of enzymes: *AgeI* and *XhoI*, and *XmaI* and *XhoI*. After incubation with a phosphatase enzyme for 30 min, the vector fragments from the *AgeI*- and *XhoI*-digested pool were ligated to the insert fragment, which encoded a single zinc-finger domain released from the vector by the digestion with *XmaI* and *XhoI*. The ligation of the insert fragments to the vector fragments yielded new plasmids that encoded two zinc-finger domains. After transformation of *Escherichia coli* by the plasmids, the ligation products yielded approximately 1.4×10^4 colonies. Plasmids were purified from these transformants, which constituted a two-finger plasmid library.

Subsequently, the two-finger plasmid library was digested with *AgeI* and *XhoI*. Vector fragments that encoded two zinc-finger domains were ligated to the pool of one-finger or two-finger fragments prepared by digestion of the single-finger library or the two-finger library, respectively, with *XmaI* and *XhoI*. The products of these ligation reactions were used to transform *E. coli* to yield about 2.4×10^5 independent transformants. Plasmids were purified from these transformants, which constituted a three-finger or four-finger library. The three-finger and four-finger ZFPs encoded by these libraries were then fused to either the Gal4 transcription activation domain or the Ume6 transcription repression domain²¹.

Construction of ZFP-TF libraries for use in mammalian systems. For expression of ZFP-TFs in mammalian cells, DNA fragments encoding ZFPs in the four-finger library described above were fused to either the p65 activation domain (NCBI accession number NP_068810; amino acids 275–535) or the KRAB repression domain (NCBI accession number AAB07673; amino acids 12–74). The individual plasmids in the library were isolated separately from several thousands of individual *E. coli* transformants.

Screening for various phenotypic changes in yeast. Yeast cells transformed by the randomized libraries described above were analyzed for their conditional lethality, thermotolerance, osmotolerance and antifungal drug resistance upon ZFP-TF expression. For the screening of conditional lethal mutants, transformants of ZFP-TFs were plated on synthetic defined (SD) minimal media (Clontech) with 2% (wt/vol) glucose and then replica-plated to both glucose and galactose (2%, wt/vol) SD plates. Comparison of the glucose and galactose plates to the original glucose plates allowed the identification of clones that were completely unable to grow on galactose. For the screening of thermotolerant mutants, yeast transformants were cultured on SD minimal liquid medium with 2% (wt/vol) galactose for 3 h at 30 °C to induce ZFP-TF expression. Cells were then incubated at air temperatures of 30 °C (no shock) or 52 °C (heat shock) with slow gyration for 2 h. After heat treatment, cells were plated on galactose medium, the plate was incubated for 5 d at 30 °C and cell growth was assessed. The osmotolerance phenotype was analyzed by plating yeast transformants on galactose medium with 100 mM LiCl. After 5 d of incubation at 30 °C, growing colonies were selected and individually analyzed for their osmotolerance. For the screening of the antifungal drug resistance phenotype, yeast transformants were cultured on SD synthetic liquid medium with 2% (wt/vol) galactose for 3 h at 30 °C and then cells were plated onto SD galactose agar plates containing 35 µM ketoconazole (ICN Biomedicals). After 4 d of incubation at 30 °C, growing colonies were selected and analyzed for their antifungal drug resistance. Plasmids that encoded ZFP-TFs were rescued from cells that were selected in these screens, retransformed back into yeast cells to confirm the phenotypic alteration, and sequenced to reveal the identities of the ZFPs.

DNA microarray analysis. Yeast cells transformed with plasmids encoding the K5, K6 or K7 ZFP-TFs or the pYTC-Lib empty vector were shifted, in the exponential growth phase, from glucose to galactose minimal medium (2% (wt/vol) sugar, 0.67% (wt/vol) yeast nitrogen base without amino acids, supplemented with histidine, leucine and uracil). Total RNA was isolated with the Qiagen

RNeasy extraction kit 12 h after the medium change, and the RNA was subjected to reverse transcription. Purified double-stranded cDNA was transcribed *in vitro* in the presence of biotinylated dUTP and dCTP. Fragmented cRNA was hybridized to the Yeast Genome-S98 array (Affymetrix), which was then stained with streptavidin-phycoerythrin. Arrays were scanned and analyzed using GeneChip3.1 software (Affymetrix).

Mammalian cell culture and phenotype screening. Mouse neuroblastoma (Neuro2A) cells were maintained in MEM- α with 10% (vol/vol) FBS and antibiotics at 37 °C, in a humidified atmosphere containing 95% air and 5% CO₂. Cells were seeded at 8.0×10^3 cells per 96-well culture plate and then were transfected with 50 ng of the ZFPs and 20 ng of the *lacZ* reporter plasmids using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. *In vitro* differentiation was induced with retinoic acid (10 µM), and 24 h after transfection, cells were treated with G418 (1 mg/ml) to reduce the number of untransfected cells. Cells were then cultured for an additional 96 h, fixed, stained for β -galactosidase activity and photographed. Within the β -galactosidase-positive cell population, cells were regarded as differentiated if the length of the neurite extension was at least two times the diameter of the cell body. The mouse myoblast cell line, C2C12, was maintained in DMEM with 4.5 g/l glucose, 10% (vol/vol) FBS, and antibiotics, at 37 °C, in a humidified atmosphere containing 95% air and 5% CO₂. Cells were seeded at 1.0×10^4 cells per 96-well culture plates and then were transfected with 50 ng of ZFP expression vector using Lipofectamine Plus reagent according to the manufacturer's protocol. Twenty-four hours after transfection, the growth medium was replaced with DMEM containing 2% (vol/vol) FBS, and the cells were cultured for an additional 6 d. To generate the Neuro1mut, a defective form of the Neuro1 ZFP, the amino acid arginine in the C-terminal finger was changed to alanine (QSNR to QSNA) by PCR-mediated mutagenesis. To examine the differentiation of C2C12 cells to osteoblasts, ALP staining was performed as described previously²⁷.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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Erratum: Drugs in crops—the unpalatable truth

Editorial

Nat. Biotechnol. 22, 133 (2004)

On line 16 of the editorial, both Samyang Genex (Daejeon, Korea) and Maxygen (Redwood City, CA, USA) were wrongly indicated to be exploring the expression of biopharmaceuticals in corn. This erroneous information was obtained from a report *A Strategic Evaluation of Transgenic Plant and Animal Biomanufacturing Systems* (Revelogic, Ft. Collins, CO, USA, 2003). In fact, Samyang Genex has a corn processing plant and uses plant cell culture to produce paclitaxel, whereas Maxygen has no program focus on expressing biopharmaceuticals in corn.

Erratum: Make or break for costimulatory blockers

Ken Garber

Nat. Biotechnol. 22, 145–147 (2004)

In Box 1 on p. 147, column 1, line 13, the sentence “Antigen-presenting cell (APC) B7 signaling induces T cells to express the enzyme indoleamine 2,3 dioxygenase (IDO), which catabolizes the amino acid tryptophan, presumably starving T cells and causing proliferation arrest” should have read, “B7 signaling in antigen-presenting cells (APCs) leads them to express the enzyme indoleamine 2,3 dioxygenase (IDO), which catabolizes the amino acid tryptophan, presumably starving T cells and causing proliferation arrest.”

Corrigendum: Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins

Yong A Yu, Shahrokh Shabahang, Tatyana M Timiryasova, Qian Zhang, Richard Beltz, Ivaylo Gentshev, Werner Goebel & Aladar A Szalay

Nat. Biotechnol. 22, 313–320 (2003)

On page 319, column 1, line 9 from bottom, the phrase “subcutaneous colon fibrosarcoma” should have read “subcutaneous fibrosarcoma.”

Corrigendum: Phenotypic alteration of eukaryotic cells using randomized libraries of artificial transcription factorsKyung-Soon Park^{1,2}, Dong-ki Lee^{1,2}, Horim Lee¹, Yangsoon Lee¹, Young-Soon Jang¹, Yong Ha Kim¹, Hyo-Young Yang¹, Sung-Il Lee¹, Wongi Seol¹ & Jin-Soo Kim¹*Nat. Biotechnol.* 21, 1208–1214 (2003)

In the author list, the name of author Seong-il Lee was misspelled as Sung-Il Lee.