## Supplemental Table S1. Different ZF domains used in this study.

| Domain     | Protein Sequence | Target | Target Sequence |
|------------|------------------|--------|-----------------|
| **Zif268** | MHHHHHHHPGEKPYACPVESCDRRFSRSDELTRHIRIHTGKPFQCRICMRNFSRSDHLTTHIRTGKPFACDICGRKFARSDERKRHTKIHTGGS | 5' - GCGTGGCGC-3' | GCGTGGCG-3' |
| **PBSII**  | MHHHHHHHPGEKPYACPECQKSFQRNLXQRTHTGKPYCPECQKSFQRSDHLTTHQRTHTGKPYCPECQKSFQRSDVLVRHQRTHGGS | 5' - GTGGAAA-3' | GTGGAAA-3' |
| **ZFa**    | TSAAAAPGERPFQCRCICMRNFSRSDPSLRRHRRTRHTGKPFQCRICMRNFSVHRNLTRHLRTHGGEKPFQCRICMRNFSRSDRTSRLKTHYPYDVPDYA | 5' - GTCGATGCC-3' | GTCGATGCC-3' |
| **ZFb**    | TSAAAAPGERPFQCRCICMRNFSKKDHLHRHRRTRHTGKPFQCRICMRNFSLQSLKLKHLRTHGGEKPFQCICMRNFSRSDMNSRLKTHYPYDVPDYA | 5' - CGGCTGGGG-3' | CGGCTGGGG-3' |
| **ZFc**    | TSAAAAPGERPFQCRCICMRNFSPSKSLLHRHRRTRHTGKPFQCRICMRNFSGNSLARHLRTHGGEKPFQCICMRNFSRVDNLPRHLKTHYPYDVPDYA | 5' - GAGGACGCG-3' | GAGGACGCG-3' |

**GGGSGGGSGGS** – C-terminal polypeptide linker  
**TSAAA** – N-terminal polypeptide linker  
**HHHHHH** – His-6x tag  
**YPYDVPDYA** – HA tag
Supplemental Figure S1. HPLC chromatograms and MS analysis of resveratrol production. (a) HPLC chromatogram of p-coumaric acid and trans-resveratrol standards that were used to identify and quantify metabolites in culture broth extracts. Representative samples of 10-fold diluted culture broth extracts from cells transformed with plasmids encoding for resveratrol pathway fusion enzymes and either the random scaffold control or the [1:1] 16, 2 bp scaffold. (b) MS analysis of trans-resveratrol standard or diluted culture broth extract. Trans-resveratrol is detected at m/z = 227.
Supplemental Figure S2. Chromatograms for 1,2-PD and mevalonate production. (a) HPLC chromatogram showing representative standards including succinic acid, lactic acid, 1,2-PD and ethanol that were used to identify and quantify metabolites in culture broth extracts. Representative samples of undiluted culture supernatant from cells transformed with a plasmid encoding the 1,2-PD pathway and either the random scaffold control or [1:2:1]$_2$, 12-bp scaffold. (b) GC-MS chromatograms corresponding to mevalonolactone standard and mevalonate extracted from cells with plasmids encoding the mevalonate pathway and either the random scaffold control or the [1:2:2]$_2$, 12-bp scaffold. Samples were selected for the 71 m/z ion for mevalonolactone and the 133 m/z ion for the internal added standard (-)-trans-caryophyllene. Mevalonate standard was acid-treated and extracted identically to experimental sample.
Supplemental Figure S3. Design and evaluation of ZF chimeras. (a) Calibration Western blot using varying amounts of purified MBP-ZFa fusion protein as standard (lanes 2-9) to quantify the level of this protein inside cells after a 12 h fermentation (lane 1). From this analysis, the amount of ZF-chimera present in cells was estimated at approximately 5000 per cell or 8 μM. The plasmid concentration in cells was determined to be approximately 127 per cell or 2 μM. These values were used to design/select ZF domains with appropriate affinity and to design DNA scaffolds with enough binding sites to accommodate all ZF fusion proteins. (b) Western blot analysis comparing the soluble and insoluble protein levels of unmodified MBP with MBP fused to 1, 2, 3 or 4 fingers as indicated. Flow cytometric analysis of cellular fluorescence levels of superfolder green fluorescent protein (sfGFP) fused to increasing number of fingers as indicated. (c) EMSA of purified PBSII-nYFP and cYFP-Zif268 chimeras using 375 mM DNA with PBSII or Zif268 binding sites. MW, molecular weight ladder. Arrow indicates chimera-DNA complexes (d) EMSA of purified MBP-ZFa, MBP-ZFb and MBP-ZFc chimeras using varying amounts of biotinylated DNA containing the ZFa, ZFb or ZFc binding site. Detection of DNA and DNA-protein complexes was with streptavidin-HRP against biotinylated DNA. Arrows indicate chimera-DNA complexes; asterisk indicates MBP-ZFa-DNA dimers.
Supplemental Figure S4. Targeting DNA in vitro and in vivo with ZF domains. (a) DNA-guided reassembly of split YFP fragments, nYFP and cYFP, using ZF domains. Fluorescence spectra of mixtures containing purified PBSII-nYFP and cYFP-Zif268 chimeras in the presence of either buffer, a random DNA scaffold or a DNA scaffold containing specific PBSII/Zif268 sites separated by 2 bp. (b) Binding of 5 μM cYFP-Zif268 alone or a mixture of 5 μM PBSII-nYFP and cYFP-Zif268 to the DNA scaffold containing PBSII and Zif268 binding sites separated by 2 bp. Inset depicts binding of 1 μM cYFP-Zif268 to a specific or random DNA scaffold as indicated. (c) (Left panel) Inhibition of β-gal expression by cells expressing the Zif268 or PBSII ZF domains and the lacZ gene controlled by P SYN. Induction of each ZF was driven from an arabinose inducible promoter. Data was normalized to the β-gal level measured in cells with no ZF induction (0% arabinose). (Right panel) Inhibition of β-gal expression by Zif268 or PBSII in the presence of 1% arabinose, when the P SYN promoter contained either the specific ZF binding site (target DNA) or an unrelated DNA binding site (control DNA; CTCTATCAATGATAGAG). Data was normalized to β-gal levels measured in cells carrying the control DNA binding site. Data are average of three replicate experiments and error bars are the SEM.
Supplemental Figure S5. Impact of different scaffold architectures on 1,2-PD production. 1,2-PD titers from *E. coli* cells expressing the MgsA-ZFa, DkgA-ZFb and GldA-ZFc chimeras in the presence of different [a:b:c]n scaffolds as indicated where the spacing was (a) 12 bp or (b) 4 bp. Cells expressing the ZF-enzyme chimeras in the presence of a random scaffold served as the control to which all data was normalized. The amount of 1,2-PD produced in the random scaffold control cells was 0.13 +/- 0.01 g/L. Data are the average of three replicate experiments and error bars are the SEM.
Supplemental Figure S6. Impact of different scaffold architectures on mevalonate production. Mevalonate titers from *E. coli* cells expressing the AtoB-ZFa, HMGS-ZFb and HMGR-ZFc chimeras in the presence of different [a:b:c]ₙ scaffolds as indicated where the spacing was (a) 12 bp or (b) 4 bp. Cells expressing the ZF-enzyme chimeras in the presence of a random scaffold served as the control to which all data was normalized. The amount of mevalonate produced in the random scaffold control cells was 1.7 +/- 0.07 g/L. Data are the average of three replicate experiments and error bars are the SEM.
Supplemental Methods

Plasmid construction and protein purification. Genes for Zif268 and PBSII were codon optimized for expression in *E. coli* and synthesized by GeneArt. Chimeric PBSII-nYFP and cYFP-Zif268 proteins were assembled according to Biobrick standards into plasmid vector BBa_K245008 (http://partsregistry.org/wiki/index.php?title=Part:BBa_K245008). *E. coli* BL21(DE3) pLysS strain was used for production of PBSII-nYFP and cYFP-Zif268 chimeras. Plasmids encoding fusion proteins under the T7 promoter were transformed in *E. coli* BL21(DE3) pLysS strain using a standard heat shock protocol, plated on LB plates with ampicillin and grown overnight at 37°C to obtain single colonies. Single colonies were further picked for inoculation of 100 ml of LB media with ampicillin and grown at 37°C at 160 rpm overnight. Overnight cultures were used to inoculate 1.2 L of 2xYT media (16 g/l bacto tryptone, 10 g/l bactoyeast extract, 5 g/l NaCl, pH 7) containing 100 µM ZnCl₂ and enriched with 10 g/l glucose to a final \( A_{600} \) of 0.15. Protein production was induced by 1mM IPTG when bacterial cultures reached \( A_{600} \) of 0.7. After 4 h of induction, bacterial cells were pelleted and lysed in 10 mM Tris (pH 7.5), 0.1% deoxycholate, 100 µM ZnCl₂ and 1 mM dithiothreitol (DTT) by sonication. Most of the proteins were expressed as inclusion bodies (IBs; data not shown). IBs were washed three times with the lysis buffer and solubilized in buffer A (10 mM Tris (pH 7.5), 100 mM NaCl, 5 mM DTT, 100 µM ZnCl₂) containing 6M urea. Solubilized IB were either directly dialyzed against buffer A and used in experiments or purified on Ni-NTA agarose beads (Qiagen). Solubilized IB were passed over Ni-NTA agarose beads and eluted with buffer A containing 4 M urea and increasing concentration of imidazole. Most fusion proteins eluted in the 250 mM imidazole fraction. Afterwards fusion proteins were either used in experiments directly or dialyzed against buffer A.
For determining stability of target proteins following the addition of zinc fingers, maltose binding protein (MBP) was cloned with a 6xHis recognition tag between the XbaI and SphI restriction enzymes sites of pBAD18-Kan. Constructs that included up to 4 finger motifs were inserted after MBP, followed by an L5 poly linker (1) connecting a zinc finger triplet with the 6xHis recognition tag at the 3’ end. Sequential fingers were added before the 6xHis tag but without an additional poly linker between zinc finger motifs. For determining activity of target proteins following the addition of zinc fingers, superfolder green fluorescent protein (sfGFP) was cloned into the SacI and SphI restriction enzymes sites of pBAD18-Kan. Constructs that included up to 5 finger motifs were inserted after sfGFP, followed by an L5 poly linker (1) connecting a zinc finger triplet. Sequential zinc fingers appended to the end, but without an additional poly linker between zinc finger motifs. For determination of protein levels in cells and EMSA experiments, MBP was PCR amplified from E. coli MG1665 genomic DNA and cloned into pET28a(+) (Invitrogen). The 3’-end was appended with a codon optimized L5 linker along with the appropriate zinc finger with or without epitope tags. Growth and purification were followed as described (2) with the following exceptions. Cell cultures were induced with 0.3 mM IPTG for 3 h. Cell lysates were applied over amylose resin (NEB) using Poly-Prep chromatography columns (Bio-Rad) with WB1 buffer substituted for column buffers. Elutions were collected in fractions of WB1 buffer supplemented with 10 mM maltose and the resulting protein concentrations were estimated by absorbance at 280 nm.

**Quantification of protein and plasmid concentration.** DH5α cultures expressing MBP-ZFα-HA from pBAD18-Kan were grown in triplicate using the same growth protocol as for 1,2-PD cultures with the exception that cells were harvested at 12 h. At this point, serial dilutions of cells were plated on LB supplemented with kanamycin to determine the number of colony forming
units (CFU) per mL. Another fraction of the culture was reserved for isolation of plasmid DNA using a Qiagen miniprep kit followed by quantification of plasmid DNA using a Nanodrop. The final culture fraction was boiled prior to quantifying the total protein fraction. Western blot analysis was performed on 10 μL of undiluted cell culture along with dilutions of purified MBP-ZFa-HA to concentrations of 625, 1250, 2500, 5000, 10000, 20000, 40000, and 80000 proteins/cell in each lane. Western blots were performed as described in the Materials and Methods.

**Stability and activity of multiple ZFs fused C-terminally to target proteins.** MC4100 cells expressing either MBP-ZFₙ-6xHis or sfGFP-ZFₙ-6xHis (where n = 0-5 fingers tandemly repeated) from pBAD18-Kan were subcultured in LB supplemented with kanamycin. Cells were inoculated to \( A_{600} = 0.05 \) (typically 1:50 to 1:100 dilution) and grown at 37°C, 250 rpm, followed by induction with 0.2% arabinose at \( A_{600} = 0.4 \). For stability analysis, cells were harvested 5 h post induction. The soluble and insoluble fractions were prepared by resuspending cell pellets in PBS followed by sonication at 0°C and then centrifugation at 16,000 g. The supernatant was collected as the soluble cytoplasmic fraction (sol). The resulting pellet was washed twice with 1 mL of Tris-Cl (50 mM) and EDTA (1 mM, pH 8.0) and resuspended in 1x PBS and 2% SDS followed by boiling for 10 min. The insoluble cytoplasmic fraction (insol) was collected as the supernatant following centrifugation for 10 min at 16,000 g. Western blot analysis of soluble and insoluble fractions was as before with the following exceptions: 10 μg of soluble protein was loaded per lane with an equal volume from loaded for the insoluble fraction. Blots were probed with anti-his antibodies. For activity analysis, at 4 h post-induction, 10 μL of cells were diluted into 1 ml of PBS and analyzed by a flow cytometer (FACSCalibur; Becton Dickinson Biosciences). All flow cytometric analysis was performed with 488 nm excitation.
Electrophoretic mobility shift assay. For EMSA with PBSII and Zif268, 1 µg of the purified PBSII-nYFP and cYFP-Zif268 chimeras were incubated with 375 µM scaffold DNA for 3 h. Samples diluted with high-grade laboratory water to 20 µl were loaded on a 2.0% agarose gel with ethidium bromide and run at 70 V for 40 min. Nucleic acid-protein complexes were detected under UV light. Alternatively, for MBP-ZF chimeras, DNA oligos were 5’ biotinylated (IDT) and annealed by slow cooling from 95°C. Binding assays were performed as described (2). Samples were loaded on 5% TBE-PAGE gels (Bio-Rad) at 4°C and then transferred to Biodyne B Nylon Membranes (Pierce). Detection of nucleic acid-protein complexes was performed using a LightShift Chemiluminescent EMSA Kit (Pierce).

Split YFP reassembly assay. Purified proteins (2.5 µM PBSII-nYFP and cYFP-Zif268) were mixed with 0.7 µM of DNA scaffold containing binding sites for PBSII and Zif268, and dialyzed into buffer A over a period of 24 h. Reconstitution experiments were conducted using D-Tube™ Dialyzer Midi, MWCO 3.5 kDa (Novagen). Successful reconstitution of YFP fluorescence was measured by Perkin Elmer LS 55 fluorometer using excitation of 480 nm and measuring emission spectra between 500 and 600 nm.

Surface plasmon resonance. Proteins that were directly dialyzed against buffer A (see above) were used in surface plasmon resonance (SPR) experiments. Proteins were further concentrated using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane. The experiments were conducted using T100 apparatus (GE Healthcare, Biacore) and streptavidin-coated sensor chip (SA). The chip was equilibrated in an SPR buffer (20 mM HEPES, 150 mM NaCl, 2 mM DTT, 0.1 mM ZnCl₂ and 0.005% P20, pH 7.4) and conditioned with three pulses of 1 M NaCl in 50 mM NaOH as suggested by the producer. The biotinylated single-stranded anchor DNA (5’-CGCTCGAGTAGTAAC-3’-Biotin) was immobilized on all four flow-cells. The anchor allowed
capture of the double-stranded DNA molecule with complementary overhang. We used a DNA scaffold (5’- GTTACTACTCGAGCGATCGGAATTCTGAAGGGGAATTGCTGCTG CGGTGGTGGATGGAGGGCGGGGTGGGAAATTGATGCTGACTGATTGACCACCC AAGACGACTGCAGTACA-3’) that contained ZF binding sites for Zif268 and PBSII (underlined), respectively. Control DNA (5’-GTTACTACTCGAGCGAATTCATCTAAGTTA CTAGAGTCCTTATAGTTGACTCTTTGTTCCACATTCTACTGTACACGTCAGTACTCGA GCATACTATCTCCTGCAGTACA-3’) contained a scrambled version of the scaffold and was used to correct the responses for unspecific binding of ZF chimeras. The control DNA was immobilized in the first flow-cell, while the DNA scaffold was used in the second flow cell. Typically, immobilization of control and scaffold DNA used 0.5 µM DNA and was performed for 5 min at 5 µl/min. Protein binding was measured following injection of appropriate concentration of PBSII-nYFP and/or cYFP-Zif268 chimeras in the SPR buffer. The surface of the sensor chip was regenerated by two 30 s injections of 50 mM NaOH that removed the DNA from the anchor.

**β-galactosidase inactivation assay.** To test the binding activity of ZF domains *in vivo*, we designed a lacZ reporter assay on a single low-copy plasmid. The expression of lacZ was driven by a synthetic promoter P<sub>SYN</sub>, which contained different ZF binding sites between the -35 and -10 promoter region (in italics): P<sub>SYN</sub>-Zif268 (5’ *TTGACACATCGCGGCGGTGATTTTT ACC* 3’); P<sub>SYN</sub>-PBSII (5’ *TTGACACATCGGTGGAAATCGATTATAACC* 3’); or the non-specific control P<sub>SYN</sub>-tetO (*TTGACACTCTATCAATGAGTTATTACC*). In addition, the plasmid also carried a gene for expression of the PBSII or Zif268 domains controlled by the arabinose-inducible P<sub>BAD</sub> promoter. All the elements were assembled according to Biobrick standards. β-gal activity was assessed after overnight incubation of *E. coli* DH5α cultures
containing one of the above plasmids at 37°C, 180 rpm, and with or without 1% L-arabinose. 5 µl of each culture was transferred to a 96-well clear bottom microtiter plate in triplicate. 100 µl of Z-buffer with chloroform (Z-buffer: 0.06 M Na₂HPO₄ x 7H₂O, 0.04 M NaH₂PO₄ x H₂O, 0.1M KCl, 0.001 M MgSO₄x7H₂O, pH 7; Z-buffer with chloroform: Z-buffer, 1% β-mercaptoethanol, 10% chloroform) was added and bacterial cells were lysed by addition of 50 µl of Z-buffer with SDS (Z-buffer, 1.6 % SDS) followed by incubation for 10 min at 28°C. 50 µl of 0.4 % ONPG solution in Z-buffer was added to each well and enzyme kinetics were measured by monitoring absorbance at 405 nm over a period of 20 min in 30 sec intervals using a microplate reader. Miller units (MU) were calculated by dividing Vₘₐₓ by the optical density of the corresponding bacterial cultures and multiplied by 1000.

References

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