

ARTICLE

Received 4 Jan 2013 | Accepted 28 Mar 2013 | Published 21 May 2013

DOI: 10.1038/ncomms2825

Stimuli-responsive selection of target DNA sequences by synthetic bZIP peptides

Jesús Mosquera¹, Adrián Jiménez-Balsa¹, Verónica I. Dodero¹, M. Eugenio Vázquez¹ & José L. Mascareñas¹

One of the strategies used by nature to regulate gene expression relies on the stimuli-controlled combination of DNA-binding proteins. This in turn determines the target-binding site within the genome, and thereby whether a particular gene is activated or repressed. Here we demonstrate how a designed basic region leucine zipper-based peptide can be directed towards two different DNA sequences depending on its dimerization arrangement. While the monomeric peptide is non-functional, a C-terminal metallo-dimer recognizes the natural ATF/CREB-binding site (5'-ATGA cg TCAT-3'), and a N-terminal disulphide dimer binds preferentially to the swapped sequence (5'-TCAT cg ATGA-3'). As the dimerization mode can be efficiently controlled by appropriate external reagents, it is possible to reversibly drive the peptide to either DNA site in response to such specific inputs. This represents the first example of a designed molecule that can bind to more than one specific DNA sequence depending on changes in its environment.

¹ Departamento de Química Orgánica and Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CIQUS), Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain. Correspondence and requests for materials should be addressed to J.L.M. (email: joseluis.mascarenas@usc.es).

The pattern of gene expression in a multicellular organism is determined by the action of defined extracellular cues and/or environmental signals that ensure the production of specific proteins only when and where needed. In most of the cases, these signals work by switching the activity of transcription factors (TFs), key gene regulatory proteins that promote the expression of selected genes by binding to relatively short stretches of specific regions of the DNA^{1,2}. Among the different mechanisms used by nature to activate or inactivate TFs, those that affect their DNA-binding properties are particularly prominent^{3–5}. In this context, it is well known that many TFs are unable to bind to their DNA targets by themselves and need to form cooperative multimeric complexes with other partners. This is a clever regulatory mechanism used by nature that allows the combinatorial interaction with a large number of sites using a relatively small set of monomeric transcription regulators^{6–8}. For instance, the basic region leucine zipper (bZIP) family of DNA-binding proteins can bind DNA as homo- or heterodimers, and in this way recognize different DNA sites depending on the regulated combination of dimerization partners^{9,10}.

Over the last years there have been many reports on the design and preparation of short synthetic peptides capable of reproducing the basic DNA recognition properties of natural TFs^{11–13}. Particularly relevant are those based on dimeric bZIP basic regions, in which the natural C/C-terminal leucine zipper is replaced by artificial dimerizers^{14–17}. Some of these designs have gone even further and incorporate switchable elements that allow conditional off/on DNA binding by the application of external stimuli such as light or metal ions^{18–22}. Despite advances in these and other switchable DNA binders^{23,24}, designed systems capable of interacting with alternative DNA sites in an externally regulated manner are unknown. Herein we prove for the first time the concept of ‘site switchability’ in the DNA binding of synthetic systems, and demonstrate how a single bZIP-based peptide can be directed towards two different DNA sequences depending on its dimerization arrangement. As the dimerization can be controlled by the application of selected reagents, the methodology allows remote control of the DNA site selectivity. In addition to providing a simplified model of the natural mechanism of combinatorial DNA recognition, our work set the bases for future achievements on the stimuli-controlled differential expression of selected genes.

The bZIP family of TFs is characterized by a C-terminal coiled-coil leucine zipper dimerization domain and a highly charged, N-terminal basic region, that mediates DNA binding^{25–27}. In addition to the straightforward substitutions of the dimerization domain described before, the GCN4 bZIP-binding domain has

inspired the development of a variety of synthetic, sequence-selective DNA-binding peptides^{28–34}. Interestingly, although in all naturally occurring bZIP proteins the basic region is positioned N-terminal to the leucine zipper, Oakley and coworkers³⁵ showed that model ‘reverse’ bZIP peptides, in which the basic region of the bZIP factor GCN4 is placed C-terminal to its leucine zipper, can specifically bind to inverted DNA sites. Thus, while natural GCN4 C/C-terminal dimers specifically bind to ATF/CREB (5′-ATGA(c/g)TCAT-3′) or AP1 (5′-ATGA(c)TCAT-3′) sites, reversed N/N-terminal dimers bind to inverted 5′-TCAT(...)ATGA-3′ sites. Additionally, Morii *et al.*³⁶ had also previously described synthetic N-terminal dimers of GCN4 with selectivity towards the swapped-binding site.

On these grounds, we envisioned that an appropriate dimerization of a GCN4 basic region through either its C- or the N-terminal side might allow to target two different sites by the same peptide unit. In addition, if the peptide is equipped with orthogonally responsive elements so that either dimerization can be triggered in response to different external stimuli, we would be in position to couple site selectivity to external inputs (Fig. 1). The resulting system would, therefore, represent a simplified model of the combinatorial DNA recognition in natural TFs. Reversibility would be an additional and highly desirable property as it might endow site switchability.

Results

Peptide design and synthesis. Based on earlier reports by Schepartz and co-workers^{17,37} on the use of metal-terpyridine complexes as dimerization elements for bZIP basic regions, we chose a terpyridine chelating ligand for the C-terminal end of the GCN4 basic region. This would allow promoting the dimerization by addition of a metal ion and formation of a stable metal complex with two terpyridine-tagged basic regions, while providing for reversibility. For the N-terminal dimerization, we selected a disulphide bond, as it could be easily implemented by introduction of a N-terminal cysteine residue. The disulphide bridge is sensitive to oxidizing or reducing conditions, and should be chemically orthogonal to the metal-mediated C-terminal dimerization.

The starting point for our design is the truncated Asp226-Gln248 GCN4 basic region, which had been identified as the minimum length required for specific DNA binding¹⁴. This core sequence was extended with a cysteine residue at the C-terminus (introduced as trityl derivative) that would act as a nucleophilic handle for attachment of the terpyridine ligand. The N-terminal

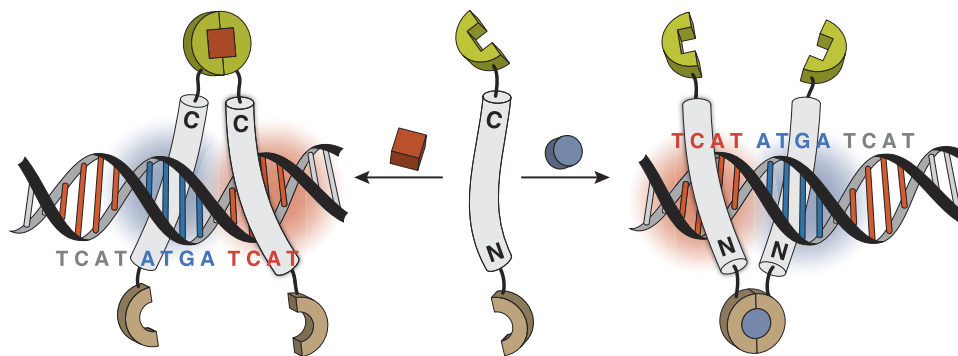


Figure 1 | Representation of a stimuli-responsive dimerization strategy for specific binding to two DNA sites. A modified peptide derived from the GCN4 TF contains orthogonal functionalities at both termini that in response to different stimuli (represented as a cube or a cylinder) result in alternative dimerization states with different DNA-binding preferences: 5′-ATGA(...)TCAT-3′ for the natural C/C-terminal dimer (left), or reversed 5′-TCAT(...)ATGA-3′ for the non-natural N/N-terminal dimer (right).

connection was engineered by adding a Cys–Gly linker to the Lys231 side chain, which was introduced as an Alloc-protected derivative to allow its selective deprotection on the solid-phase using palladium catalysis. The resulting linker is geometrically equivalent to the linker described by Morii *et al.*³⁶ in their studies of N-terminal covalent dimers of GCN4 basic regions (Supplementary Fig. S1). In order to differentiate both thiols, the N-terminal Cys was introduced protected as Cys(tButhio), which is stable to trifluoroacetic acid (TFA) but can be selectively deprotected using thiols, such as dithiothreitol or dithioerythritol. Assembly of the peptide chain in solid-phase, followed by a standard TFA deprotection/resin cleavage step, gave the desired Cys(tButhio)-protected basic region (**1**) in good yield (Fig. 2). Alkylation of the free thiol with 4'-methylbromo-2,2':6',2''-terpyridine, followed by treatment of the resulting peptide with dithioerythritol produced the desired thiol **3a**. In addition, a control peptide (**3b**), in which the terpyridine is replaced by a 4'-nitroacetophenone chromophore (NAP), was also synthesized.

Monomeric peptides **3a** and **3b** were dimerized by treatment with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), Ellman's reagent) in phosphate buffer at pH 7.5. On the other hand, addition of 5 equiv. of Ni(ClO₄)₂ to **3a** in neutral water promotes the formation of the expected nickel-bis-terpyridine complex, as deduced from liquid chromatography (LC)-mass spectrometry (MS) (Supplementary Fig. S5).

DNA-binding studies. The DNA-binding properties of the synthetic peptides were studied by electrophoretic mobility shift assays (EMSA) under non-denaturing conditions, and using SYBR-gold for DNA staining. As expected, while monomeric peptide **3a** does not bind to short double-stranded (ds)DNAs containing the direct ATF/CREB consensus DNA-binding sequence (*dir*: 5'-...ATGA cg TCAT...-3'), addition of 5 equiv. of Ni(ClO₄)₂ results in a new slow-migrating band, consistent with the formation of the specific complex between the (3a)₂Ni dimer and the DNA (Fig. 3a, lanes 1–3).

Importantly, the dsDNA *inv*, which features an inverted target site (5'-...TCAT cg ATGA...-3'), as well as a control dsDNA containing a single consensus half-site (*hs*: 5'-...ATGA cg TTCG...-3'), did not lead to any new bands in the EMSA assays (Fig. 3a, lanes 4–9). These results demonstrate that the metal-promoted dimers of **3a** show a clear preference for binding to the consensus *direct* site over the *inverted* alternative. With regard to the N-terminal disulphide dimer (3a)₂SS, we observed the formation of a DNA complex with its target inverted DNA (*inv*, Fig. 3b, lanes 4–6) but, curiously, it also produces retarded bands with the other oligos *dir* and *hs* (Fig. 3b, lanes 1–3, and 7–9). These bands with the non-consensus dsDNAs are more diffuse than those observed with the direct DNA, and most probably arise from a relatively loose complex in which one of the basic regions is interacting specifically in the major groove of the consensus site while the other makes nonspecific contacts³⁸. Curiously, the control disulphide (3b)₂SS, in which the terpyridine was replaced by an inert chromophore, did not elicit such interactions with the non-consensus DNAs (Fig. 3c), which suggests that the terpyridine unit might be contributing to the formation of the undesired EMSA bands.

Changing the dimerization state. Despite the selectivity problem of the N-terminal disulphide dimer, peptide **3a** seemed appropriate to explore the viability of controlling site-interactions by altering its dimerization state in the presence of DNA. As expected, reduction of the disulphide bond of (3a)₂SS by treatment with the reducing agent TCEP (tris(2-carboxyethyl)phosphine) resulted in the formation of monomeric species and hence complete loss of DNA binding to either the direct or the inverse DNA sequence (Fig. 4, compare lanes 2 and 6 versus lanes 3 and 7, respectively). Importantly, addition of Ni(ClO₄)₂ to the solution obtained after the reduction step generates the C-terminal (3a)₂Ni dimer, as deduced by the appearance of the corresponding retarded DNA band when incubated with the dsDNA

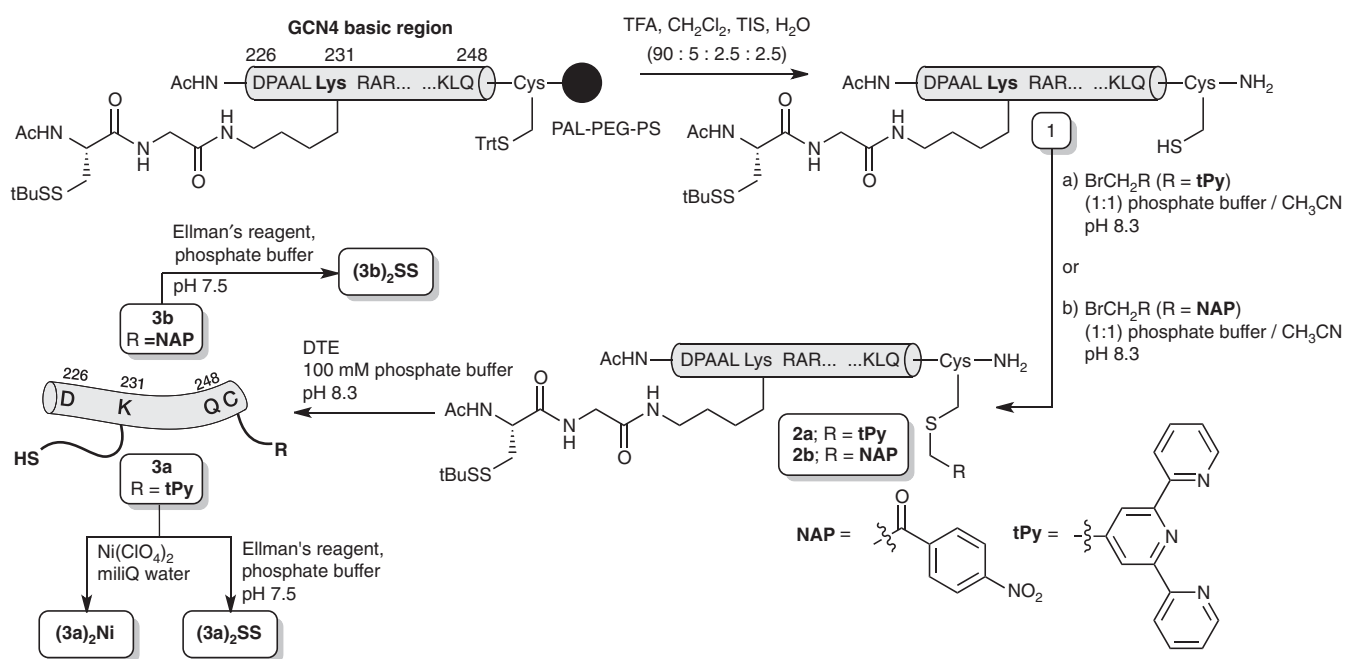


Figure 2 | Synthesis of the modified monomeric peptides and dimerization reactions. Standard solid-phase peptide synthesis yields the fully protected peptide containing an unprotected C-terminal Cys (**1**), which is modified in solution to yield the terpyridine derivative (**2a**) or the nitroacetophenone (NAP) control peptide (**2b**). Final deprotection of the N-terminal Cys side chain gives the desired bifunctional peptides **3a** and **3b**, which can be dimerized as described. The full sequence of the GCN4 basic region peptide is D²²⁶PAALKRARNTAARRSRARKLQ²⁴⁸.

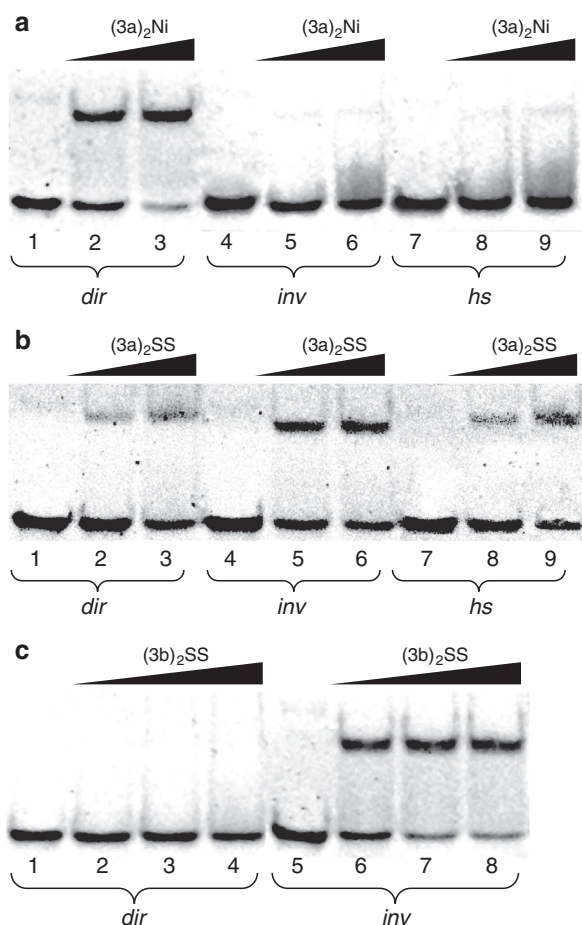


Figure 3 | EMSA analysis of DNA binding. (a) Lanes 1–3: dsDNA *dir* (which contains the target ATF/CREB-binding site), lanes 4–6: dsDNA *inv* (which contains an exchanged binding site), lanes 7–9: dsDNA *hs* (which contains a consensus half-site). Lanes 2–3, 5–6 and 8–9: 600 and 1200 nM of **3a** and 5 equiv. of $\text{Ni}(\text{ClO}_4)_2$, as well as excess of TCEP to prevent the oxidation of the thiols. (b) Lanes 1–3: dsDNA *dir*; lanes 4–6: dsDNA *inv*; lanes 7–9: dsDNA *hs*. Lanes 2–3, 5–6 and 8–9: 300 and 600 nM of **(3a)₂SS**. (c) Lanes 1–4: dsDNA *dir*; lanes 5–8: dsDNA *inv*. Lanes 2–4 and 6–8: 400, 600, 800 nM of **(3b)₂SS**. The protocol involved addition of peptide solutions in 20 mM Tris-HCl, pH 7.5 to 18 mM Tris-HCl, pH 7.5, 90 mM KCl, 1.8 mM MgCl_2 , 1.8 mM EDTA, 9% glycerol, 0.11 mg ml^{-1} BSA, 2.25% NP-40 and 100 nM of DNA (4 °C, 30 min) and loading into the gel. DNA was detected by fluorescent staining with SYBR-gold. Oligonucleotide sequences (binding sites underlined, only one strand shown): *dir*: 5'-CGG ATGA CG TCAT TTTTTC-3'; *inv*: 5'-CGG TCAT CG ATGA TTTTTC-3'; *hs*: 5'-CGG ATGA CG TTCG TTTTTC-3'.

dir (Fig. 4, lane 8). This result, which confirms the viability of switching from the disulphide N-terminal dimer to the metal-promoted C-terminal dimer, encouraged us to further refine the design in order to achieve better sequence selectivities.

Optimization of the design. As previously shown, in contrast to the result with the terpyridine containing disulphide **(3a)₂SS**, the dimer **(3b)₂SS** showed very low affinities for the non-consensus DNAs *dir* and *hs*, which suggest that the terpyridine group is actively contributing to the weaker, undesired binding mode, probably by providing DNA intercalating capability. Therefore, we decided to use a terpyridine unit featuring two methyl substituents, which might hinder that interaction. To facilitate the

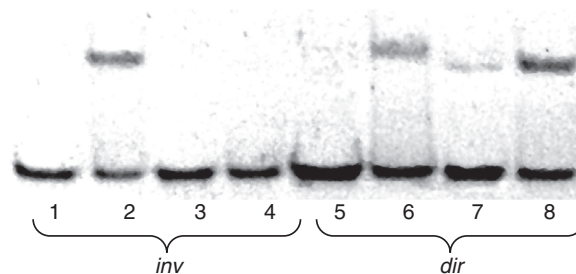


Figure 4 | EMSA analysis of DNA binding after changing the dimerization state. Two stock solutions of dimeric peptide **(3a)₂SS** (3 μM) in 20 mM Tris-HCl pH 7.5 in the presence 500 nM of either dsDNAs *inv* (lanes 1–4) or *dir* (lanes 5–8) were successively treated at room temperature with TCEP (40 mM), 5 min, and $\text{Ni}(\text{ClO}_4)_2$ (15 μM) another 5 min. Aliquots of the different solutions, each containing over 50 nM DNA, were mixed with the loading buffer and run under non-denaturing conditions. Lanes 2 and 6: **(3a)₂SS**; lane 3 and 7: same mixture after treatment with TCEP; lanes 4 and 8: previous mixture after treatment with $\text{Ni}(\text{ClO}_4)_2$. DNA was detected by fluorescent staining with SYBR-gold.

synthetic procedures, and minimize solution chemistry, the terpyridine group was introduced on solid-phase on the side chain of a lysine residue engineered at the C-terminal position of the peptide, which also provided for further isolating the terpyridine from the DNA-binding basic region.

As shown in Fig. 5, the synthetic protocol involved removal of the Mtt (4-methyltrityl) protecting from the first Lys side chain under mild acidic conditions³⁹, and coupling of 5,5'-dimethyl-[2,2':6',2''-terpyridine]-4'-carboxylic acid to the resulting amine, followed by introduction of the rest of the peptide sequence. The lysine at position 231 was inserted as Lys(alloc), which allowed the selective deprotection of its side chain by Pd catalysis after acetylation of the N-terminal Asp226 (ref. 40). Finally, the Gly-Cys linker was assembled onto the Lys side chain and acetylated to give the full terpyridine-modified peptide attached to the resin. Standard deprotection and cleavage, and high-performance liquid chromatography (HPLC) purification afforded the desired peptide **4**, in an overall 30% yield. This peptide can be readily dimerized upon oxidation with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in a slightly basic phosphate buffer. On the other hand, addition of Ni^{2+} or Fe^{2+} salts to peptide **4** led to the formation of the expected bis-terpyridine complexes **(4)₂Ni** and **(4)₂Fe**, which could be isolated and identified by MS (Supplementary Figs S10 and S11).

DNA-binding studies with the peptide 4. As shown in the Fig. 6a, the dimer **(4)₂Ni**, made *in situ* by addition of $\text{Ni}(\text{ClO}_4)_2$ to the peptide **4**, binds the consensus direct DNA *dir*, but does not give rise to retarded bands with the inverse DNA *inv*. A titration using a P^{32} -labelled ds-oligonucleotide allowed us to calculate an apparent binding constant of ≈ 299 nM at 4 °C (Supplementary Fig. S14). More importantly, in contrast to the former disulphide **(3a)₂SS**, the new N-terminal dimer **(4)₂SS** displayed good selectivity for its target sequence (DNA *inv*) against the non-consensus *dir* dsDNA (Fig. 6b), although, curiously, in order to observe neat EMSA retarded bands we found that it is important that the binding mixture contains Ni^{2+} or Co^{2+} metal ions. It is probable that coordination of the metals to either terpyridine, complex observed by MS (Supplementary Fig. S12), might prevent unproductive folding or nonspecific DNA binding, and hence favour the formation of the desired DNA complexes. Remarkably, addition of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ to **(4)₂SS** suppresses the formation of complexes with the target DNA *inv*, most probably because of the intrinsic tendency of Fe^{2+} to simultaneously bind

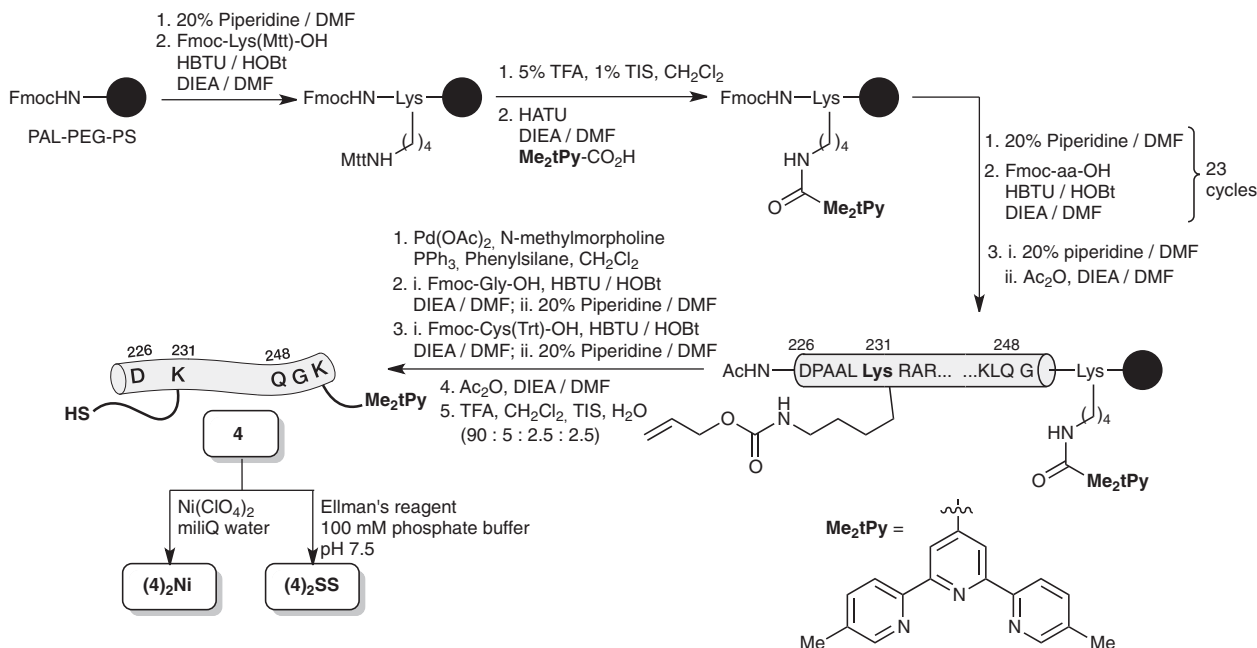


Figure 5 | Synthesis of peptide 4 and its N and C-terminal dimers. Incorporation of the terpyridine moiety at the C-terminal lysine side chain while the peptide is still bound to the resin. The tether on the lysine 231 is similar to that used in the peptide **1** (Gly-Cys, Fig. 2).

both terpyridines, thus forming a stable macrocyclic species unable to bind DNA. In the presence of 5 equiv. of Ni(ClO₄)₂, and using radioactive EMSA experiments, we calculated a K_D of ≈ 375 nM for the binding of (4)₂Ni to its consensus inverted DNA *inv* (Supplementary Fig. S13).

To get further insights on the performance of the C- and N-terminal dimers, we measured the binding preferences in solution, and in the presence of excess of a competitor DNA (calf-thymus), using fluorescence anisotropy titrations with rhodamine-labelled oligonucleotides (Fig. 7). Thus, a solution of Rhodamine-labelled oligonucleotide *dir* (*Rho-dir*) was incubated with increasing concentrations of (4)₂Ni in the presence of a competitive nonspecific calf-thymus DNA (50 μ M in base pairs) at 10 °C, and the anisotropy at 585 nm was measured after each addition. We obtained a typical titration profile, which could be fitted to a simple 1:1 binding model with an apparent K_D of ≈ 670 nM. In contrast, incubation of the non-target rhodamine-labelled *Rho-inv* oligonucleotide under the same conditions with increasing concentrations of this C-terminal dimer resulted in a less pronounced titration curve with a much weaker K_D of ≈ 25 μ M. Using similar experiments, we found that (4)₂SS displays a higher affinity for the consensus rhodamine-labelled *inv* oligo ($K_D \approx 758$ nM) than for the non-target *Rho-dir* oligonucleotide ($K_D \approx 5.3$ μ M). These values are in agreement with those obtained using radioactive EMSA titrations, and further support the differential binding of the two dimers. The observation of a more intense anisotropy with the two non-target DNAs ((4)₂Ni/*Rho-inv* and (4)₂SS/*Rho-dir*), than with the specific complexes ((4)₂Ni/*Rho-dir* and (4)₂SS/*Rho-inv*) is consistent with the formation of more compact species in the case of the specific interactions, and larger complexes with the non-target oligonucleotides, which therefore display slower dynamics.

Finally, circular dichroism experiments were consistent with the behaviour observed in the EMSA experiments. Thus, incubation of the N-terminal disulphide dimer (4)₂SS with its target sequence (DNA *inv*) resulted in a significant increase in the negative ellipticity at 222 nm, consistent with the α -helical folding expected for an specific interaction^{14,41}. This increase is

not observed with the natural ATF/CREB sequence contained in the *dir* oligo; likewise, the C-terminal dimer (4)₂Ni only showed an increase in the negative ellipticity in the presence of its target *dir* DNA, but not with the swapped *inv* DNA (see Supplementary Fig. S17).

Switching between sites. Once we confirmed that peptide **4** exhibits the desired orthogonal DNA binding, we were interested in exploring the viability of a sequence-selective recognition in a situation where both target sites are simultaneously available for binding. Thus, a stock solution containing peptide **4** was added to a 50 nM equimolar mixture of both dsDNAs, containing the direct and the inverse sequences *dir1* and *inv*. Note that in this case we use ds-oligonucleotides of different lengths in order to distinguish the binding to either of them. As anticipated, the monomer by itself is unable to elicit retarded shifts (Fig. 8a, lane 3); addition of 1 equiv. of Ellman's reagent (DTNB) and 5 equiv. of Ni(ClO₄)₂ to that solution resulted and in the appearance of a new band consistent with the formation of a specific complex with the inverse DNA *inv* (Fig. 8a, band a in lane 4, and control lane 1). Interestingly, addition of TCEP to the mixture led to the disappearance of this complex and the formation of a new retarded band involving the oligo *dir1*, consistent with the DNA binding of the *in situ* formed dimer (4)₂Ni (Fig. 8a, band b in lane 5, and control lane 2). A new addition of DTNB dismounts this complex and regenerates binding to the inverse DNA. Despite the excess of reagents in the mixture, it is yet possible to induce a new switch and regenerate binding to other DNA. These results represent the first proof of concept on the viability of making molecules that can be induced to jump between two different DNA sites, at will, in response to specific external stimuli.

Discussion

Recent years have witnessed important advances in the design and preparation of switchable *on-off* DNA binders. However, molecules that target different DNA sites in response to specific signals, or that can switch between specific DNA sites, have not

been developed. Inspired by the concept of combinatorial gene regulation, which is particularly common for bZIP-type of TFs, we have made designed derivatives of bZIP basic regions that can be induced to bind to different DNA sites depending on their dimerization mode. In particular, our results show that installing a suitable terpyridine moiety at the C-terminus of the basic region of GCN4, and a cysteine residue at its N-terminus, it is possible to induce either a C- or N-terminal dimerization by using appropriate triggers. The C-terminal dimerization can be induced by metals like Ni^{+2} , while the addition of an oxidant, such as Ellman's reagent, promotes a disulphide dimerization through the N-terminus. The resulting dimers show a more than remarkable selectivity for their cognate-binding sites. Therefore, whereas the C-terminal metallo-dimer binds the natural ATF/CREB-binding site (5'-ATGA cg TCAT-3') >40 times better than the swapped sequence (5'-TCAT cg ATGA-3'), the N-terminal disulphide dimer binds the inverted sequence over 10 times better than the

ATF/CREB site. The high DNA-binding selectivity of each dimeric state allows the precise targeting of each site in mixtures containing both oligonucleotides or in the presence of an excess of calf-thymus DNA. Importantly, our results indicate that the dimerization can be also triggered *in situ*, in the presence of DNA. Therefore, the peptide can be driven to either DNA target site by using specific external signals that code its dimerization mode. It is even possible to induce a reversible 'hopping between sites' in a stimuli-responsive manner (Fig. 8b).

This relatively simple design shows many of the features found in natural TFs, namely cooperativity, reversibility, high selectivity and response to specific environmental signals, and thus represent a starting point for the design of more complex DNA combinatorial interaction networks and for gaining a more precise control on the artificial regulation of genes.

Methods

General. All reagents were acquired from commercial sources: dimethyl formamide (DMF) and TFA were purchased from Scharlau, CH_2Cl_2 from Panreac, CH_3CN from Merck, [2,2':6',2''-terpyridin]-4'-yl-methanol was purchased from HetCat. The rest of reagents were acquired from Sigma-Aldrich. All peptide synthesis reagents and amino-acid derivatives were purchased from GL Biochem (Shanghai) Ltd and Novobiochem. Amino acids were purchased as protected Fmoc amino acids with the standard side-chain protecting scheme, except for the orthogonally protected Fmoc-Lys(Alloc)-OH and Fmoc-Lys(Mtt)-OH.

Reactions were followed by analytical reversed phase (RP)-HPLC with an Agilent 1100 series LC/MS using an Eclipse XDB-C8 analytical column (4.6 × 150 mm, 5 μm). Electrospray Ionization Mass Spectrometry was performed with an Agilent 1100 Series LC/MSD model in positive scan mode using direct injection of the purified peptide solution into the MS. Standard conditions for analytical RP-HPLC consisted on an isocratic regime during the first 5 min, followed by a linear gradient from 5 to 75% of solvent B for 30 min at a flow rate of 1 ml min⁻¹ (A: water with 0.1% TFA, B: acetonitrile with 0.1% TFA). Compounds were detected by ultraviolet absorption at 220, 270, 304 and 330 nm. Purification was performed by semipreparative RP-HPLC with an Agilent 1100 series LC using a Luna 5u C18(2) 100A (5 μm , 10 × 250 mm) reverse-phase column from Phenomenex. Matrix-assisted laser desorption/ionization mass spectrometry (MS) was performed with a Bruker Autoflex matrix-assisted laser desorption/time-of-flight (TOF) model in positive scan mode by direct irradiation of the matrix-absorbed peptide. Concentrations were measured using the listed extinction coefficients.

Calf-thymus DNA was acquired from Sigma-Aldrich. Oligonucleotides were purchased from Thermo Fisher Scientific GmbH on a 0.2 mmol scale as freeze-dried solids. After solving in H₂O milliQ their concentrations were measured by ultraviolet absorption at 260 nm with a BioRad SmartSpec Plus Spectrophotometer. Absorbance was measured twice and concentrations were calculated applying Lambert-Beer's equation. The molar extinction coefficients of single-strand oligonucleotides were calculated by using the following formula⁴², $\epsilon(260 \text{ nm}) = \{(8.8 \times \#T) + (7.3 \times \#C) + (11.7 \times \#G) + (15.4 \times \#A)\} \times 0.9 \bullet 103 \text{ M}^{-1} \text{ cm}^{-1}$, where #A, #T, #C, #G stand for the number of each type of bases in the DNA

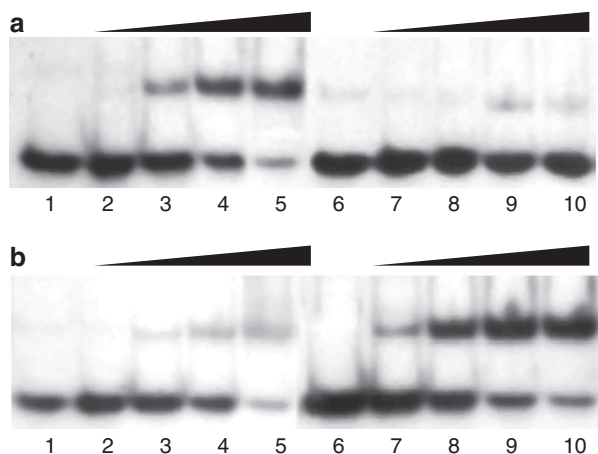


Figure 6 | Radioactive EMSA analysis of the DNA-binding properties of peptide 4 and its dimeric derivatives. (a) and (b) Lanes 1–5: dsDNA *dir* (100 nM); lanes 6–10: dsDNA *inv* (100 nM); a small proportion (~0.1%) of the oligonucleotides are labelled with ³²P for radioactive detection. (a) Lanes 2–5 and lanes 7–10: 200, 600, 1,000, 1,600 nM of peptide 4 with 5 equiv. of $\text{Ni}(\text{ClO}_4)_2$, and excess of TCEP. (b) Lanes 2–5 and lanes 7–10: 100, 300, 500, 800 nM of peptide (4)₂SS in the presence of 5 equiv. of $\text{Ni}(\text{ClO}_4)_2$.

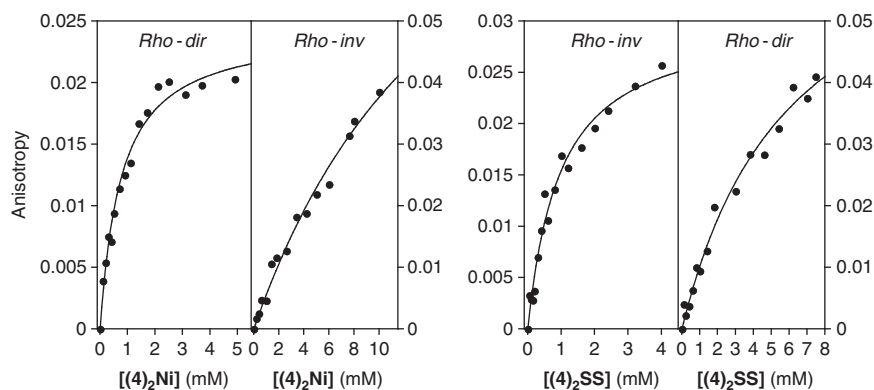


Figure 7 | Fluorescence anisotropy analysis of the DNA-binding properties of peptide 4 and its dimeric derivatives. Fluorescence anisotropy titrations of the C- and N-terminal dimers with DNA oligonucleotides in 20 mM Tris-HCl, pH 6.5, 100 mM NaCl at 10 °C. Left: 50 nM of rhodamine-labelled *Rho-dir* and *Rho-inv* oligonucleotides in the presence of 50 μM concentration (in base pairs) of nonspecific calf-thymus DNA, with increasing concentrations of C-terminal (4)₂Ni dimer (stock solution: 400 μM of 4, 600 μM $\text{Ni}(\text{ClO}_4)_2$ and 5 mM of TCEP). Right: 50 nM of rhodamine-labelled *Rho-dir* and *Rho-inv* oligonucleotides in the presence of nonspecific calf-thymus DNA (50 μM base pairs) with increasing concentrations of N-terminal (4)₂SS dimer (stock solution: 200 μM of (4)₂SS, 600 μM $\text{Ni}(\text{ClO}_4)_2$). Lines represent best fits to a 1:1 binding model. *Rho-inv*: Rho-TGGAG TCAT CG ATGA CTCGT-3'; *Rho-dir*: Rho-TGGAG ATGA CG TCAT CTCGT-3'; excitation at 559 nm and anisotropy measured at 585 nm.

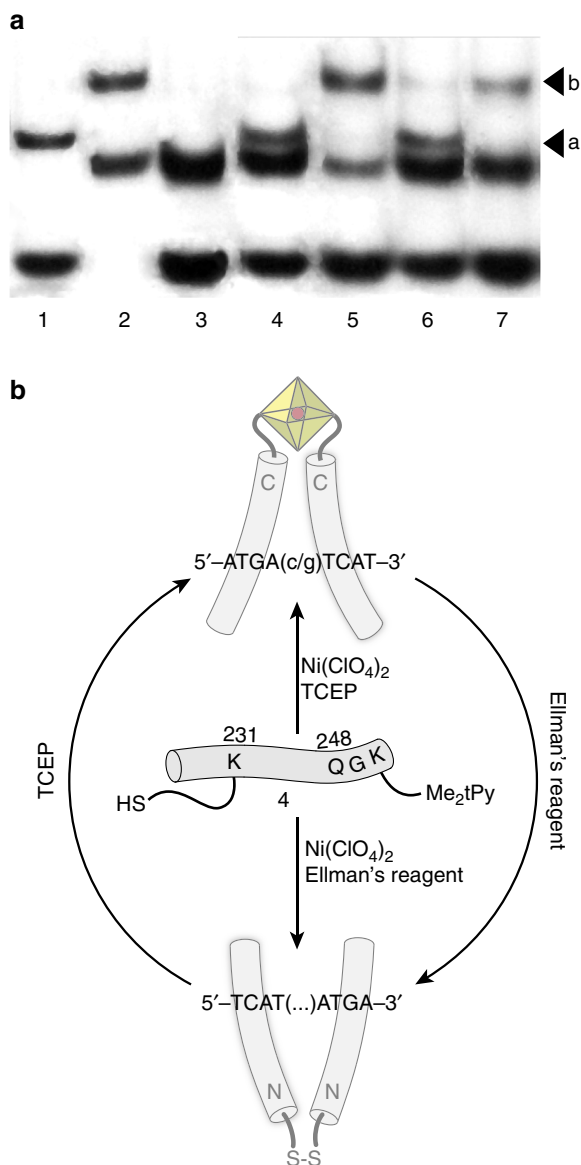


Figure 8 | Dynamic target DNA selection by peptide 4. (a) Radioactive EMSA experiments: A stock solution containing an equimolar concentration of ds-oligonucleotides *dir1* and *inv* (3 μ M, a small proportion of the oligonucleotides are labelled with 32 P) in the presence of peptide **4** (36 μ M) was successively treated at room temperature with the oxidant (DTNB (0.5 equiv.) and Ni(ClO₄)₂ (5 equiv.)), the reducing agent (TCEP, 40 mM), and again DTNB (40 mM) and TCEP (80 mM); after 2–3 min of reaction, aliquots of the solutions resulting after each step, were mixed with the loading buffer and run under non-denaturing conditions. Lane 1: DNA *inv* (50 nM) in the presence of 300 nM of (**4**)₂SS; lane 2: DNA *dir1* (50 nM) in the presence of 600 nM peptide **4** and Ni(ClO₄)₂ (5 equiv.). Lanes 3–7, aliquots of the above reaction mixtures containing both dsDNAs (~50 nM of each) and peptide **4**. Lane 3: starting mixture; lane 4: mixture after the first addition of DTNB (and Ni(ClO₄)₂) lane 5: mixture after the addition of TCEP; lane 6: mixture after the second addition of DTNB; lane 7: mixture after the last addition of TCEP. *dir1*: 5'-CGG ATGA CG TCAT TTTTTCG TTACGAAGCC-3'. (b) Cartoon representation of the switching process.

strand. The concentration of Rhodamine-labelled oligonucleotides was calculated using the molar extinction of rhodamine. Oligonucleotides were hybridized by mixing complementary sequences at equal molar concentration (50 μ M), heating at 90 °C for 10 min and then slowly cooling the mixture to room temperature over 1 h.

The synthesis of the terpyridine moieties and their coupling to the peptides is detailed in the Supplementary Methods.

Electrophoretic mobility shift assay. EMSA were performed with a BioRad Mini Protean gel system, powered by an electrophoresis power supplies PowerPac Basic model, maximum power 150 V, frequency 50.60 Hz at 140 V (constant V). The binding reactions were performed over 30 min in 18 mM Tris (pH 7.0), 50 mM KCl, 1.2 mM MgCl₂, 0.5 mM EDTA, 9% glycerol, 0.11 mg ml⁻¹ BSA and 4.2% NP-40 at 4 °C. In the experiments analysed by fluorescent staining we used 100 nM of the unlabelled dsDNAs and a total incubation volume of 20 μ l. In the experiments with 32 P-labelled DNAs we used ~100 pM of labelled dsDNAs and 100 nM of the same unlabelled dsDNAs in a 20- μ l binding mixture. Use of lower amounts of DNA gave less clear gels because of precipitation. In order to prevent the oxidation of peptides with free thiols, 2 mM of TCEP is present in the incubation solution.

Products were resolved by polyacrylamide gel electrophoresis using a 10% non-denaturing polyacrylamide gel and 0.5 \times TBE buffer, and analysed by autoradiography (when radioactivity was used) or by staining with SyBrGold (Molecular Probes: 5 μ l in 50 ml of 1 \times TBE) for 10 min and visualized by fluorescence.

The labelling of oligonucleotides with 32 P was accomplished in the following manner: 1 μ l of the single-strand oligonucleotide which will be labelled (10 μ M), 1 μ l kinase buffer 10 \times (40 mM, Tris-HCl pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol), 2 μ l ATP (ATP (γ - 32 P) (5,000 Ci mmol⁻¹)) and 0.5 μ l of the kinase (10 units μ l⁻¹); after shaking, the mixture is heated for 1 h at 37 °C, and then quenched by heating for 10 min at 95–100 °C.

Peptide synthesis. C-terminal amide peptides were synthesized following standard peptide protocols (Fmoc/tBu strategy) on a 0.1 mmol scale using a 0.19 mmol g⁻¹ loading Fmoc-PAL-PEG-PS resin from Applied Biosystems, using a PS3 automatic peptide synthesizer from Protein Technologies. The amino acids were coupled in fourfold excess using HBTU (O-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate) as activating agent. Each amino acid was activated for 30 s in DMF before being added onto the resin, and couplings were conducted for 30 min. Deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF for 10 min. Manual couplings were monitored using the TNBS (trinitrobenzene sulphonate) test⁴³. The resin cleavage-deprotection was accomplished by shaking the resin-bound peptides for 2 h (~0.025 mmol), in 3 ml of the cleavage cocktail (25 μ l of H₂O, 25 μ l of triisopropylsilane, 50 μ l of CH₂Cl₂ and 900 μ l of TFA). The resin is filtered, and the TFA filtrate is added to ice-cold diethyl ether (30 ml). After 10 min, the precipitate is centrifuged and washed again with 20 ml of ice-cold ether. The solid residue is dried under argon, dissolved in CH₃CN/water 1:1 (2 ml) and purified by semipreparative RP-HPLC. The collected fractions are lyophilized and stored at -20 °C. Acetylation of peptides was carried using mixtures of Ac₂O/DMF (1:2) in the presence of 2 equiv. of DIEA (*N,N*-Diisopropylethylamine).

Deprotection of Lys(Alloc) side chain. The resin-bound peptide (~0.05 mmol) was treated overnight at room temperature with a deoxygenated mixture of methylmorpholine (55 μ l, 10 equiv.), triphenylphosphine (20 mg, 1.5 equiv.), phenylsilane (62 μ l, 10 equiv.) and Pd(OAc)₂ (3.4 mg, 0.3 equiv.) in CH₂Cl₂ (2.7 ml). The resin was filtered and washed with DMF (2 \times 1.5 ml \times 2 min), diethyldithiocarbamate (25 mg in 5 ml of DMF, 2 \times 1.5 ml \times 5 min), DMF (2 \times 1.5 ml \times 2 min) and CH₂Cl₂ (2 \times 5 ml \times 2 min).

Deprotection of Lys(Mtt) side chain. The resin-bound peptide (~0.05 mmol) was treated twice with a mixture of TFA (250 μ l), triisopropylsilane (50 μ l) and CH₂Cl₂ (4.7 ml) for 5 min. The resin was then filtered and washed with CH₂Cl₂ (2 \times 5 ml \times 2 min).

General procedure for the obtention of the disulphide dimers. The monomeric thiol-containing peptide was dissolved in 100 mM buffer phosphate (pH 7.5) to give an approximate peptide concentration of 2 mM Ellman's reagent⁴⁴ (0.8 mM in acetonitrile, 0.4 equiv.) was added, and the resulting mixture was stirred for 20 min at room temperature. The reaction was quenched using 0.1% aqueous TFA, and the crude purified by RP-HPLC. The collected fractions were lyophilized and stored at -20 °C, after characterization. The dimeric peptides were obtained in yields over 70%.

Fluorescence anisotropy. Steady-state fluorescence anisotropy measurements were made in a Jobin-Yvon Fluoromax-3 (DataMax 2.20), coupled to a temperature controller Wavelength Electronics LFI-3751, and using a standard Hellma semi-micro cuvette (114F-QS, 10 mm light path). Titrations were carried out at 10 °C using the following parameters: λ_{ex} = 559 nm (bandwidth 2.0 nm), λ_{em} = 585 nm (bandwidth 12.0 nm), 1 s integration time, and each measure was taken in triplicate. Titrations were made by adding increasing amounts of the desired peptide to a solution containing a rhodamine-labelled oligonucleotide

(50 nM) in 20 mM Tris-HCl pH 6.5 100 mM NaCl and 50 μ M concentration (in base pairs) of nonspecific calf-thymus DNA.

Circular dichroism spectroscopy. CD measurements were made in a 2-mm cell at 4 °C. Samples contained 5 μ M of corresponding dsDNA (when present) and 5 μ M of peptides in 10 mM phosphate buffer (pH 7.5) and 100 mM of NaCl. The CD spectra of the peptides (when measured in the presence of DNA) were calculated as the difference between the spectrum of the peptide/DNA mixture and the measured spectrum of a sample of the DNA oligonucleotide.

References

- Latchman, D. S. *Eukaryotic Transcription Factors*, 4th edn, 77–134 (Elsevier, Academic Press, 2004).
- Müller, C. W. Transcription factors: global and detailed views. *Curr. Opin. Struct. Biol.* **11**, 26–32 (2001).
- Arndt, H. -D. Small molecule modulators of transcription. *Angew. Chem. Int. Ed.* **45**, 4552–4560 (2006).
- Marmorstein, R. & Fitzgerald, M. X. Modulation of DNA-binding domains for sequence-specific DNA recognition. *Gene* **304**, 1–12 (2003).
- Latchman, D. S. *Gene Control* (Garland Science, 2010).
- Chen, L. Combinatorial gene regulation by eukaryotic transcription factors. *Curr. Opin. Struct. Biol.* **9**, 48–55 (1999).
- Reményi, A., Schöler, H. R. & Wilmanns, M. Combinatorial control of gene expression. *Nat. Struct. Mol. Biol.* **11**, 812–815 (2004).
- Amoutzias, G. D., Robertson, D. L., Van de Peer, Y. & Oliver, S. G. Choose your partners: dimerization in eukaryotic transcription factors. *Trends in Biochem. Sci.* **33**, 220–229 (2008).
- Garvie, C. W. & Wolberger, C. Recognition of specific DNA sequences. *Mol. Cell.* **8**, 937–946 (2001).
- Fujii, Y., Shimizu, T., Toda, T., Yanagida, M. & Hakoshima, T. Structural basis for the diversity of DNA recognition by bZIP transcription factors. *Nat. Struct. Mol. Biol.* **7**, 889–893 (2000).
- Mapp, A. K. Regulating transcription: a chemical perspective. *Org. Biomol. Chem.* **1**, 2217–2220 (2003).
- Pazos, E., Mosquera, J., Vázquez Sentís, M. E. & Mascareñas, J. L. DNA recognition by synthetic constructs. *Chem. Bio. Chem.* **12**, 1958–1973 (2011).
- Vázquez Sentís, M. E., Caamaño, A. M. & Mascareñas, J. L. From transcription factors to designed sequence-specific DNA-binding peptides. *Chem. Soc. Rev.* **32**, 338–349 (2003).
- Talanian, R. V., McKnight, C. J. & Kim, P. S. Sequence-specific DNA binding by a short peptide dimer. *Science* **249**, 769–771 (1990).
- Talanian, R. V., McKnight, C. J., Rutkowski, R. & Kim, P. S. Minimum length of a sequence-specific DNA binding peptide. *Biochemistry* **31**, 6871–6875 (1992).
- Ueno, M., Murakami, A., Makino, K. & Morii, T. Arranging quaternary structure of peptides by cyclodextrin-guest inclusion complex: sequence-specific DNA binding by a peptide dimer with artificial dimerization module. *J. Am. Chem. Soc.* **115**, 12575–12576 (1993).
- Cuenoud, B. & Schepartz, A. Altered specificity of DNA-binding proteins with transition metal dimerization domains. *Science* **259**, 510–513 (1993).
- Caamaño, A. M., Vázquez, M. E., Martínez-Costas, J., Castedo, L. & Mascareñas, J. L. A light-modulated sequence-specific DNA-binding peptide. *Angew. Chem. Int. Ed.* **39**, 3104–3107 (2000).
- Woolley, G. A. *et al.* Reversible photocontrol of DNA binding by a designed GCN4-bZIP protein. *Biochemistry* **45**, 6075–6084 (2006).
- Azuma, Y., Imanishi, M., Yoshimura, T., Kawabata, T. & Futaki, S. Cobalt(II)-responsive DNA binding of a GCN4-bZIP protein containing cysteine residues functionalized with iminodiacetic acid. *Angew. Chem. Int. Ed.* **48**, 6853–6856 (2009).
- Zhang, F., Timm, K. A., Arndt, K. M. & Woolley, G. A. Photocontrol of coiled-coil proteins in living cells. *Angew. Chem. Int. Ed.* **49**, 3943–3946 (2010).
- Jimenez, A., Pazos, E., Martínez-Albaronedo, B., Mascareñas, J. L. & Vázquez, M. E. Temporary electrostatic impairment of DNA recognition: light-driven DNA binding of peptide dimers. *Angew. Chem. Int. Ed.* **51**, 8825–8829 (2012).
- Sánchez, M. I. *et al.* *In vivo* light-driven DNA binding and cellular uptake of nucleic acid stains. *ACS Chem. Biol.* **7**, 1276–1280 (2012).
- Sánchez, M. I., Vázquez, O., Vázquez, M. E. & Mascareñas, J. L. Light-controlled DNA binding of bisbenzamidines. *Chem. Commun.* **47**, 11107–11109 (2011).
- Ellenberger, T. E., Brandl, C. J., Struhl, K. & Harrison, S. C. The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted α helices: crystal structure of the protein-DNA complex. *Cell* **71**, 1223–1237 (1992).
- König, P. & Richmond, T. J. The X-ray structure of the GCN4-bZIP bound to ATF/CREB site DNA shows the complex depends on DNA flexibility. *J. Mol. Biol.* **233**, 139–154 (1993).
- Keller, W., König, P. & Richmond, T. J. Crystal structure of a bZIP/DNA complex at 2.2 Å: determinants of DNA specific recognition. *J. Mol. Biol.* **254**, 657–667 (1995).
- Blanco, J. B., Vázquez, M. E., Martínez-Costas, J. M., Castedo, L. & Mascareñas, J. L. A synthetic miniprotein that binds specific DNA sequences by contacting both the major and the minor groove. *Chem. Biol.* **10**, 713–722 (2003).
- Vázquez, M. E., Caamaño, A. M., Martínez-Costas, J., Castedo, L. & Mascareñas, J. L. Design and synthesis of a peptide that binds specific DNA sequences through simultaneous interaction in the major and in the minor groove. *Angew. Chem. Int. Ed.* **40**, 4723–4725 (2001).
- Zondlo, N. J. & Schepartz, A. Highly specific DNA recognition by a designed miniature protein. *J. Am. Chem. Soc.* **121**, 6938–6939 (1999).
- Bird, G. H., Lajmi, A. R. & Shin, J. A. Sequence-specific recognition of DNA by hydrophobic, alanine-rich mutants of the basic region/leucine zipper motif investigated by fluorescence anisotropy. *Biopolymers* **65**, 10–20 (2002).
- Pellegrini, M. & Ebright, R. Artificial sequence-specific DNA binding peptides: branched-chain basic regions. *J. Am. Chem. Soc.* **118**, 5831–5835 (1996).
- Blanco, J. B. *et al.* Sequence-specific DNA binding by noncovalent peptide-tripyrrole conjugates. *Angew. Chem. Int. Ed.* **45**, 8210–8214 (2006).
- Vázquez, O., Vázquez, M. E., Blanco-Canosa, J. B., Castedo, L. & Mascareñas, J. L. Specific DNA recognition by a synthetic, monomeric Cys2His2 zinc-finger peptide conjugated to a minor-groove binder. *Angew. Chem. Int. Ed.* **46**, 6886–6890 (2007).
- Hollenbeck, J. J., Gurnon, D. G., Fazio, G. C., Carlson, J. J. & Oakley, M. G. A GCN4 variant with a c-terminal basic region binds to DNA with wild-type affinity. *Biochemistry* **40**, 13833–13839 (2001).
- Morii, T., Saimei, Y., Okagami, M., Makino, K. & Sugiura, Y. Factors governing the sequence-selective DNA binding of geometrically constrained peptide dimers. *J. Am. Chem. Soc.* **119**, 3649–3655 (1997).
- Metallo, S. J. & Schepartz, A. Distribution of labor among bZIP segments in the control of DNA affinity and specificity. *Chem. Biol.* **1**, 143–151 (1994).
- Hollenbeck, J. J. & Oakley, M. G. GCN4 binds with high affinity to DNA sequences containing a single consensus half-site. *Biochemistry* **39**, 6380–6389 (2000).
- Aletras, A., Barlos, K., Gatos, D., Koutsogianni, S. & Mamos, P. Preparation of the very acid-sensitive Fmoc-Lys(Mtt)-OH. Application in the synthesis of side-chain to side-chain cyclic peptides and oligolysine cores suitable for the solid-phase assembly of MAPs and TASP. *Int. J. Pept. Protein Res.* **45**, 488–496 (1995).
- Thieriet, N., Alsina, J., Giralt, E., Guibe, F. & Albericio, F. Use of alloc-amino acids in solid-phase peptide synthesis. Tandem deprotection-coupling reactions using neutral conditions. *Tetrahedron Lett.* **38**, 7275–7278 (1997).
- Greenfield, N. J. & Fasman, G. D. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8**, 4108–4116 (1969).
- Kallansrud, G. B. & Ward, B. A comparison of measured and calculated single- and double-stranded oligodeoxynucleotide extinction coefficients. *Anal. Biochem.* **236**, 134–138 (1996).
- Hancock, W. & Battersby, J. A new micro-test for the detection of incomplete coupling reactions in solid-phase peptide synthesis using 2,4,6-trinitrobenzenesulphonic acid. *Anal. Biochem.* **71**, 260–264 (1976).
- Arpicco, S., Dosio, F., Brusa, P., Crosasso, P. & Cattel, L. New coupling reagents for the preparation of disulfide cross-linked conjugates with increased stability. *Bioconjugate Chem.* **8**, 327–337 (1997).

Acknowledgements

We thank the support given by the Spanish grants SAF2010-20822-C02, CTQ2012-31341, CSD2007-00006, Consolider Ingenio 2010, and the Xunta de Galicia INCITE09 209 084PR, GRC2010/12, PGIDIT08CSA-047209PR. J.M. and A.J.-B. thank the Spanish Ministry of Education for their PhD fellowships. We also thank Manuel Marcos from the University of Vigo for his support and critical help with MS experiments.

Author contributions

J.L.M. conceived the idea and designed the project. J.M., A.J.-B., and V.D. performed the experiments. J.L.M. and M.E.V. analysed the data and wrote the paper.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>

How to cite this article: Mosquera, J. *et al.* Stimuli-responsive selection of target DNA sequences by synthetic bZIP peptides. *Nat. Commun.* 4:1874 doi: 10.1038/ncomms2825 (2013).