Coupling Computational and Intracellular Screening and Selection Towards Co-compatible cJun and cFos Antagonists

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ABSTRACT

Basic leucine-zipper (bZIP) proteins represent difficult yet compelling oncogenic targets, since numerous cell-signalling cascades converge upon them where they function to modulate transcription of specific gene targets. bZIPS are widely recognised as important regulators of cellular processes that include cell proliferation, apoptosis and differentiation. Once such validated transcriptional regulator, Activator Protein-1 is typically comprised of heterodimers of Fos and Jun family members, with cFos-cJun being the best described, and demonstrated to be key in the progression and development of a number of different diseases. As a proof-of-principle for our approach, we describe the first use of a novel combined in silico/in cellulo peptide-library screening platform that facilitates the derivation of a sequence which displays high selectivity for cJun relative to cFos, while also avoiding homodimerisation. In particular, >60 million peptide peptides were computationally screened and all potential on/off targets ranked according to predicted stability, leading to a reduced size library that was further refined by intracellular selection. The derived sequence is predicted to have limited cross-talk with a second previously-derived peptide antagonist that is selective for cFos in the presence of cJun. The study provides new insight into the use of multi-state screening with the ability to combine computational and intracellular approaches in evolving multiple co-compatible peptides that are capable of satisfying conflicting design requirements.
INTRODUCTION

Basic leucine zipper (bZIP) transcription factors are a diverse family of DNA-binding proteins, consisting of a transactivation domain, a basic domain for binding to specific recognition elements within gene promotor regions, and a leucine zipper (LZ) region that mediates bZIP dimerisation required for activity. The transcription factor activator protein 1 (AP-1) can be composed from a wide range of dimeric complexes containing proteins which have physiologically varied roles linked to control of cellular proliferation, gene regulation, and more. Overexpression of AP-1 leads to oncogenic effects, such as dysregulation of the proliferation and differentiation of cells\textsuperscript{1–3}, making it and other related transcription factors compelling, if challenging, drug targets. Their dysregulation as part of oncogenic signalling pathways highlight the importance of specific targeting while maintaining the ability to successfully modulate their activity in cellular transformation\textsuperscript{4–7}. The dimerisation of bZIPS is mediated by the LZ domain, which contains a seven amino acid repeat (a heptad) which displays property specific positions to drive the requisite interaction patterns\textsuperscript{8}. The coiled coil (CC) within bZIPS is comprised of two right-handed parallel α-helices that interact to form a left-handed supercoil structure, allowing key residues to periodically align in forming a heptad repeat every two turns of the helix. Despite the apparent simplicity of the CC, the relationship between its primary structure and the specificity found in its quaternary structure are not fully understood. Given the diversity and breadth of human bZIPS alone, there has been a focus on engineering highly selective peptide-peptide interactions\textsuperscript{9–12}. The creation of tools that can guide the user from the primary sequence to quaternary structure, along with quantitative information relating to interaction stability is an ongoing effort\textsuperscript{9,10,12,13}. The role of AP-1 in cancer has made it a target of particular interest within therapeutic research and multiple methods of inhibition have been explored\textsuperscript{14–16}. Previous work has explored designing antagonists based on Jun or Fos family peptides. A recent focus has been on the use of in silico techniques to simulate in cellulo screening techniques, with a view to either predicting one peptide that satisfies the design requirements\textsuperscript{17}. Here we take this a step further by using the computational screens as a mechanism to reduce large libraries (~10\textsuperscript{7–9}) to smaller higher quality libraries (~10\textsuperscript{5}) which are then more readily accessible to intracellular screening approaches and predicted to contain many members with the desired properties, thus increasing the chances of success. This approach has made extensive use of the bZIP Coiled Coil Prediction Algorithm (bCIPA)\textsuperscript{13,18} engine and focuses on creating in silico tools that mimic both the Protein-fragment Complementary Assay (PCA) and Competitive And Negative Design Initiative (CANDI) using bCIPA as an underlying algorithm to generate in silico PCA (isPCA)\textsuperscript{19} and in silico CANDI (isCAN) equivalents\textsuperscript{17}. The use of CANDI as a framework for competitor mimicry promotes not only stability of a library-derived peptide in complex with its target but also the
specificity required to avoid defined off-target states (i.e. more stable than predicted affinities of a target—target or library member—library member interactions, or user defined off-target—library-member interactions). Here, we describe a novel combination of both isPCA and isCAN from a large library followed by intracellular PCA on the refined library – with the aim of utilising sequential screening capable of fulfilling the design constraints imposed by the need for specificity. In practice, this is twofold – the stability of the antagonist-target complex must be improved while also engineering a system in which the stability of the undesired complexes is decreased; thereby maximising the difference between the multiple potential complexes. Additionally, we explore the capacity of the derived antagonist to complement a previously characterised antagonist that is selective for cFos in the presence of cJun $\text{(JunW}_{\text{CANDI}})$. Importantly the design of the library described is such that it has the potential to target cJun while disfavouring binding to not only cFos but also potential library member homodimers. Moreover, the sequence and $\text{JunW}_{\text{CANDI}}$ preferentially target their cognate binding partners over each other. Taken together, this raises the possibility to intentionally avoid cross-talk between either antagonist, library member-library member interactions (i.e. as homodimers), or the targets to which they bind. A key goal therefore is to offer bespoke co-compatible peptides with the potential for synergy in applying two peptides to simultaneously target a cJun-cFos AP-1 heterodimer remains.

**Figure 1. Overview of in the combined silico / in cellulo workflow.** **Left:** A 60,466,176 member cFos-based library was generated and screened via isPCA and isCAN using parameters and templates taken from previous work. The top 25 sequences from both were used to create a library (34 unique sequences due to overlap). This 31,104 (23328 without the addition of 1 His at α') member library was expressed and the winner sequence (cFosULib) was selected. **Middle:** Complexes that can be formed via the combination of library/target peptides (L/T). Negative and desired complexes are found within PCA, in considering the undesired homodimeric complexes (i.e. LL/TT). **Right:** isCAN incorporates the competitor complexes (i.e the addition of LC/TC). Within isCAN, specificity is driven by the desired delta ($\Delta$) value as specified by the user. The library member is only successful if it is able to form the desired complex with predicted $T_d$ values greater the $\Delta$. In this cFos-based system, the competitor sequences included are from the FOS family (cFos, Fra1, Fra2, and FosB) for a total of 11 predictions for every library member simulation ($2n+3$ where $n =$ number of competitors).
Here we present our first efforts in this area, where we seek to address the following: i) Does the combination of \textit{in silico} and \textit{in cellulo} screening result in a peptide able to successfully target cJun? ii) Does the peptide chosen via a combined isCAN→PCA approach have increased target-specificity relative to previous \textit{in cellulo} techniques? iii) Can this peptide retain specificity in the presence of JunW\textsubscript{CANDI}—an exemplar cFos antagonist that the library has not been screened against?

**MATERIALS AND METHODS**

**In silico PCA/CANDI (isPCA/isCAN)** – The isPCA/isCAN computational screening techniques have been described elsewhere\textsuperscript{17}. Briefly, the approach mimics \textit{in cellulo} PCA/CANDI screening techniques and simulates a defined library screened against a specific target sequence. Alongside the desired interaction with the target, the software considers homodimeric stability of both target and individual library members and, in the case of isCAN, a wide number of user-defined off-target stabilities. It identifies the predicted highest affinity binder (the desired threshold is set by the user) which also meets parameters for differences between predicted desired heterodimer T\textsubscript{m} and that of all other complexes. Utilising the underlying bCIPA algorithm, this pairwise analysis incorporates helical propensity (HP), core (C), and electrostatic interactions (ES) to provide quantitatively estimated values relating to the interaction affinities as thermal melting data (T\textsubscript{m})\textsuperscript{11,13,20,21}.

**Library Design and Cloning** - Library design and cloning has been described elsewhere\textsuperscript{22}. Briefly mega-primers were synthesized including relevant semi-randomised codons for library residue options, and a fill-in reaction performed, resulting in 140 bp double stranded oligonucleotides. These were digested and cloned via NheI and Ascl sites into a pQE16 derivative (Qiagen) containing a G/S linker tagged to fragment 1 (p230d; Fos library; ampicillin resistance) or fragment 2 (p300d; cJun; chloramphenicol resistance) of murine dihydrofolate reductase (mDHFR) respectively. All proteins were under control of a lac promoter, and expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). Library plasmids were transformed into BL21 cells (Stratagene) containing target plasmid and pREP4 (Qiagen; for lac repression; kanamycin resistance). To assess library quality, pools were sequenced collectively as well as single clones and approximately equal distributions of varied amino acids were found. Pooled colonies were collected to exceed the library size 5-10-fold, to provide >95% library coverage.

**Selection of Winner Peptides** - PCA has been described in detail elsewhere\textsuperscript{13,17,22}. Briefly, target and library peptides are tagged at the genetic level to N- or C-terminal halves of the murine form of dihydrofolate reductase (mDHFR). Only two interacting helices will bring the two halves of the enzyme into close proximity, render the enzyme active, and result in colony formation on selective M9 minimal medium plates with 1 µg/mL trimethoprim to inhibit bacterial DHFR. Surviving colonies
were pooled, grown, and serially diluted in liquid cultures under selective conditions (M9 minimal medium with 1 µg/mL trimethoprim). Fastest growth, and hence the highest affinity interacting partners, were found to dominate the pool after 2 passages. Library pools as well as colonies from taken from individual colonies isolated from competition selection pools were sequenced to verify the arrival at one discreet sequence.

Peptide Synthesis and Purification – As described previously\textsuperscript{17,23}, Rink amide ChemMatrix™ resin was obtained from PCAS Biomatrix, Inc. (St.-Jean-sur-Richelieu, Canada); Fmoc L-amino acids and benzotriazol-1-yl-ox-tripyrrolidinophosphonium hexafluorophosphosphate (PyBOP) were obtained from Merck; all other reagents were of peptide synthesis grade and obtained from VWR. Peptides were synthesised on a 0.1-mmol scale on a PCAS ChemMatrix™ Rink amide resin using a Liberty Blue™ microwave peptide synthesiser (CEM; Matthews, NC) employing Fmoc solid-phase techniques\textsuperscript{24} with repeated steps of coupling, deprotection and washing (4 x 5 mL dimethylformamide). Coupling was performed as follows: Fmoc amino acid (5 eq), PyBOP (4.5 eq) and diisopropylethylamine (DIPEA) (10 eq) in dimethylformamide (5 ml) for 5 min with 35-W microwave irradiation at 90 °C. Deprotection was performed as follows: 20% piperidine in dimethylformamide for 5 min with 30-W microwave irradiation at 80 °C. Following synthesis, the peptide was acetylated using acetic anhydride (3 eq) and DIPEA (4.5 eq) in dimethylformamide(2.63 ml) for 20 min and then cleaved it from the resin with concomitant removal of side-chain-protecting groups by treatment with a cleavage mixture (10 ml) consisting of TFA (95%), triisopropylsilane (2.5%) and H₂O (2.5%) for 4 h at room temperature.

Suspended resin was removed by filtration, and the peptide was precipitated using three rounds of precipitation in ice-cold diethyl ether, vortexing and centrifuging. The pellet was then dissolved in 1:1 MeCN/H₂O and freeze-dried. Purification was performed by RP-HPLC using a Phenomenex Jupiter Proteo (C18) reverse-phase column (4 µm, 90 Å, 10 mm inner diameter x 250 mm long). Eluents used were as follows: 0.1% TFA in H₂O (a) and 0.1% TFA in ACN (b).

The peptide was eluted by applying a linear gradient (at 3.5 ml/min) of 5–95% B over 40 min. Fractions collected were examined by electrospray MS, and those found to contain exclusively the desired product were pooled and lyophilised. Analysis of the purified final product by RP-HPLC indicated a purity of >95%.

Circular Dichroism (CD) – Analysis was performed using an Applied Photophysics (Leatherhead, U.K.) Chirascan CD apparatus using a 200 µL sample in a CD cell with a 1 mm path length. Samples contained a 150 µM total peptide (Pt) concentration at an equimolar concentration for heterodimeric solutions (i.e., 75 µM per peptide) and suspended in 10 mM potassium phosphate
and 100 mM potassium fluoride (pH 7) for 30 min prior to analysis. The CD spectra of the samples were scanned between 200 and 300 nm in 1 nm steps, averaging 0.5 s at each wavelength. Three scans at 20 °C were averaged to assess overall helical content as well as the coiled coil structure. Raw data (ellipticities) were collected and averaged, and data were converted to molar residue ellipticities (MREs).

**Thermal Denaturation** – Analysis was performed using an Applied Photophysics (Leatherhead, U.K.) Chirascan CD apparatus recording the ellipticities of homotypic or heterotypic (1:1 stoichiometric mix) samples at a total peptide concentration (Pt) of 150 μM in a buffer of 10 mM potassium phosphate and 100 mM potassium fluoride (pH 7). For all thermal denaturation experiments, a stepping gradient was set from 0 to 90 °C in 1 °C increments. Each temperature point was held for 30 sec to equilibrate the sample to within 0.1°C of the target temperature before measuring ellipticity at 222 nm. The resulting sigmoidal thermal denaturation profiles were fit to a two-state model, derived via modification of the Gibbs–Helmholtz equation to yield the melting $T_m$.

**RESULTS AND DISCUSSION**

*In silico* screening both without (isPCA) and with (isCAN) off-targets has been combined with PCA to derive a 39-mer peptide that is selective for cJun in the presence of cFos. From thermal melt data coupled with dimer exchange experiments within the systems of the individual antagonists, the sequence is shown to be compatible with an existing sequence that is selective for cFos in the presence of cJun (37-mer JunWcAND1)[11]. Here we describe our approach towards co-compatible peptides that are capable of targeting specific components within the heterodimeric AP-1 complex with minimal crosstalk between partners and each other.

**Library Creation and in silico screening** – The *in silico* library used for this work was previously generated[17] to create a highly expansive set that provided options capable of balancing both library diversity and simulation feasibility. In doing so the $\Delta T_m$ parameter (difference between the $T_m$ of the desired complex and the closest non-desired complex) was set to 20°C during the initialisation stage, leading to a library of 60,466,176 peptides. Briefly, this library contained semi-randomised residues at the core and electrostatic positions, with LIVN options at $a$ positions and QEK options at $e$ and $g$ positions. As previously described, inclusion of Asn at all $a$ positions was in order to mitigate against the formation of higher order oligomeric states by driving the formation of Asn-Asn pairs with the $a3$ position on the target helix.[16,27] The peptides were next subjected to both isPCA and isCAN (Fig. 1), resulting in libraries of 73,124 and 71,667 sequences respectively. In order to create the library for PCA, the top 25 sequences (ranked according to a predicted $\Delta T_m$ of at least 20°C and the value of the desired complex $T_m$) from isPCA and isCAN libraries were combined and used to define the library.
for PCA. Both the isPCA and the isCAN library contained sequences that represented 0.12% of the original 60,466,176 member library. Within this, the top 25 sequences represent 0.03% of these secondary libraries. The combination of the top 25 sequences from both resulted in 34 unique sequences due to overlap of 16 sequences present in both. The library created from these 34 unique sequences resulted in a small high quality and PCA-accessible library of 23,328 sequences, which expanded further to 31,104 sequences with the addition of His at one core position (Figure S2). The inclusion of this residue was unavoidable when incorporating Asn (AAC) at α4 with Ile (AUC) and Leu (CUC) – requiring the use of two ambiguous nucleotides (MWC), with M = A/C and W = A/U (see SI).

**PCA Selection** - The stages of PCA demonstrate a stabilisation of the semi-randomised library through each passage, with the sequence identified from the final passage termed FosU_{PCA} (ASEIDTLEAELDQLENYALKTELANLEKEIELQGAP). The details of the sequence highlight the result of a number of conflicting selection pressures enforced by the binding and growth assay, with progression tracked in Figure S1. Prediction of the melting temperature ($T_m$) of FosU_{PCA} with its desired and off-targets (Figs 2-3) highlighted the diversity of its origins. As with previous work led by *in silico* design\textsuperscript{17}, the presence of a large difference ($\Delta T_m$) between the desired complex (FosU_{PCA}–cJun) and the non-desired complexes was enforced ($\Delta T_m = 26^\circ C$).

![Figure 2. Predicted $T_m$ values of FosU_{PCA} and related peptides.](image)

In comparison with Fos-based peptides targeting cJun with cFos as a competitor (*and cFos not duplicating values). All interactions have been predicted using the same iSCAN protocol. The $\Delta T_m$ values against the highest off-target
(predicted to be library member homodimerisation for all but cFos). FosU_{PCA} is predicted to have a T_{m} of 86°C with a ΔT_{m} of 26°C. **For both FosU_{SCAN} and FosU_{PCA}, all sequences have been extended to 39 residues with N-terminal g and C-terminal e positions filled.

This predicted value was lower relative to that of previous work with this library due to the increased predicted T_{m} of homodimeric FosU_{PCA} – FosU_{PCA} (T_{m} = 60°C) relative to that of the purely in silico derived FosU_{SCAN} (T_{m} = 41°C). Although the FosU_{SCAN} was one of the top 25 sequences used as a basis for the library, it was not selected during PCA. The contrasting selections of in silico vs in silico → in cellulo approaches is highlighted by the fact that the final selected sequence, FosU_{PCA}, is not observed in either of the top 25 sequences from isPCA or isCAN. Upon additional screening of the library however, FosU_{PCA} was identified as being within the top 20% of all library predicted members within the PCA library (predicted ΔT_{m} and desired complex T_{m}).

Figure 3. Helical wheels of FosU_{PCA} and JunW_{CANDI} interactions. The helical wheel diagram displays the residues present on the coiled coil from the position of the N-terminus to the C-terminus, looking down the axis of the alpha helices. For both FosU_{PCA} (a–c) and JunW_{CANDI} (d–f), these diagrams illustrate the hydrophobic interface at the core position (a/d) and the charged residues present at the flanking position (e/g). The helical wheel of FosU_{PCA} – cJun (c) demonstrates how FosU_{PCA} contains residues which promote favourable electrostatic and core interactions to drive coiled coil formation. JunW_{CANDI} in complex with cFos also shares this (f), with multiple attractive Glu-Arg and Glu-Lys electrostatic interactions.
**Circular Dichroism Spectroscopy** - The global secondary structures of FosU<sub>PCA</sub> in isolation and in complex with cJun and cFos were analysed to monitor for both helicity and interaction (Fig. 4a/b). CD spectra showed that at 20°C, FosU<sub>PCA</sub> in isolation displayed a low level of helical stability (fH = ~17%) and a 222nm/208nm ratio of 0.46. In combination, this data describes a peptide lacking prerequisite α-helical profile required for formation of a homodimeric coiled coil. Inspection of the helical wheel (Fig. 3a) shows the presence of six repulsive Glu-Glu interactions (60% of the possible total electrostatic interactions). Similarly, cFos (Fig. 4b) exhibited low levels of helicity (fH = ~23%) and a 222nm/208nm ratio of 0.61. However, cJun displayed increased higher levels of helicity (fH = ~31%) and a 222nm/208nm ratio of 0.73 – and is well documented to be capable of forming a coiled coil. The secondary structure of the non-desired FosU<sub>PCA</sub>–cFos complex was analysed using CD (Fig. 4b) to establish if it formed from the component peptides. Although it presented with a fH of ~28%, the 222nm/208nm ratio of 0.81 also described a structure tending towards an α-helix. Analysis of the target complex of FosU<sub>PCA</sub>–cJun provided significant evidence for the formation of a coiled coil. In particular, it displayed increased α-helicity when compared to the homodimeric and off-target heterodimeric complexes (fH = ~40%) and four net electrostatic attractions (Fig 4b).

![Figure 4. CD spectra and thermal denaturation data for FosU<sub>PCA</sub> with cJun and cFos. Shown are data for FosU<sub>PCA</sub> (red) with cJun (A and C, blue) and cFos (B and D, blue). Spectra were measured at 20 °C at a total peptide concentration of 150 μM and presented as mean residue ellipticity (MRE). The minima at 208 and 222 nm are indicative of a helical structure when coupled with fractional helicity (fH), with the 222 nm/208 nm ratio of FosU<sub>PCA</sub> showing less structure (222 nm/208 nm = 0.46 & fH =](image-url)
17.0%) than cJun (222 nm/208 nm = 0.73, fH = 30.5%) and cFos (222/208 = 0.61, fH = 22.9%). The FosU<sub>PCA</sub> - cJun complex (A and C, purple) shows increased helicity (fH = 39.8%) but similar helical structure (222 nm/208 nm = 0.78) and compared to the FosU<sub>PCA</sub> - cFos (B and D, purple) complex (222 nm/208 nm = 0.81, fH = 27.6%) Thermal denaturation profiles with cJun (C) and cFos (D) were taken using 1°C increments and tracking the 222 nm signal at 150 μM. FosU<sub>PCA</sub> - cJun shows an increase in the transition midpoint with a T<sub>m</sub> of 52.0°C compared to FosU<sub>PCA</sub> in isolation and FosU<sub>PCA</sub> - cFos, both with T<sub>m</sub> unable to be fit. This suggests that FosU<sub>PCA</sub> would preferentially bind to cJun over the potential off-target states. All experiments were performed in 10 mM potassium phosphate and 100 mM potassium fluoride (pH 7). Where possible (C), data were fitted to the two-state model.

Structural analysis of complexes containing JunW<sub>CANDI</sub> (Fig. 5a/b) showed that the homodimeric complex presented with moderate levels of α-helicity (fH = ~37%) as well as a 222nm/208nm ratio of 0.81. In complex with cJun, the α-helicity showed only a slight decrease, with the fH value dropping to ~32% and the 222nm/208nm ratio to 0.74. When in complex with the cFos target, there is a marked increase in the α-helicity measured (fH = ~46%) and the 222nm/208nm ratio (0.97).

![Figure 5](image-url)

**Figure 5.** CD spectra and thermal denaturation data for JunW<sub>CANDI</sub> with cJun and cFos. Shown are data for JunW<sub>CANDI</sub> (red) with cJun (A and C, black) and cFos (B and D, blue). Spectra were measured at 20 °C at a total peptide concentration of 150 μM and presented as mean residue ellipticity (MRE). The minima at 208 and 222 nm are indicative of a helical structure when coupled with fractional
helicity ($f_H$), with the 222 nm/208 nm ratio of JunW$_{CANDI}$ showing stability in isolation (222 nm/208 nm = 0.81 & $f_H = 37.2\%$) greater than that of cJun (222 nm/208 nm = 0.73, $f_H = 30.5\%$). The JunW$_{CANDI}$ – cJun complex (brown) shows helicity slightly greater than that of cJun ($f_H = 32.4\%$) but similar helical structure (222 nm/208 nm = 0.74). In comparison, the JunW$_{CANDI}$ – cFos complex (purple) showed an increase in helicity, with a 222 nm/208 nm ratio of 0.97 and a $f_H$ of 45.7%. Thermal denaturation profiles with cJun (C) and cFos (D) were taken using 1°C increments and tracking the 222 nm signal at 150 μM. JunW$_{CANDI}$ shows a stable complex with a $T_m$ of 32.0°C compared to JunW$_{CANDI}$- cJun, with a slightly lower $T_m$ of 27°C. In complex with cFos, there is an increase in the transition midpoint with a $T_m$ of 51°C. This suggests that although JunW$_{CANDI}$ has stability in isolation and with cJun, it would preferentially bind to cFos over the potential off-target states. All experiments were performed in 10 mM potassium phosphate and 100 mM potassium fluoride (pH 7). Where possible (C), data were fitted to the two-state model.

In context, these values describe a system in which homodimeric and off-target interactions retain relatively high levels of helical stability required to form a coiled coil (with $\alpha$-helical levels exceeded only by that of the JunW$_{CANDI}$- cFos interaction). FosU$_{PCA}$ incubated with JunW$_{CANDI}$ exhibited increased helicity compared to that of the component peptides ($f_H = ~52\%$) and a 222nm/208nm ratio of 0.89 (Fig. 6b).

Figure 6. Helical wheel and CD spectra and thermal denaturation data for FosU$_{PCA}$ and JunW$_{CANDI}$. Helical wheel (A) shows the amino acid arrangement for FosU$_{PCA}$ and JunW$_{CANDI}$ peptides. Spectra (B) were measured at 20 °C at a total peptide concentration of 150 μM and presented as mean residue ellipticity (MRE). The minima at 208 and 222 nm are indicative of various levels of helical structure, with the 222 nm/208 nm ratio of the FosU$_{PCA}$- JunW$_{CANDI}$ (purple) showing increased structure (222 nm/208 nm = 0.89) and helicity ($f_H = 51.9\%$). The lactamised form shows increased structure (222 nm/208 nm =0.74) compared to homomeric state (222 nm/208 nm =0.69). Thermal denaturation
profiles of FosU<sub>PCA</sub> – JunW<sub>CANDI</sub> (C) as well as the component peptides were taken using 1°C increments and tracking the 222 nm signal at 150 μM. The heterodimer shows an increase in the transition midpoint demonstrating a T<sub>m</sub> of 40°C compared to the only component able to have a T<sub>m</sub> fitted (JunW<sub>CANDI</sub> T<sub>m</sub> = 32°C). This suggests that the addition of JunW<sub>CANDI</sub> promotes stability and helicity to FosU<sub>PCA</sub> and that this complex is preferred over both homomeric states. This is addressed in the helical wheel, which shows a series of intermolecular attractive electrostatic interactions and repulsive intramolecular interactions. All experiments were performed in 10 mM potassium phosphate and 100 mM potassium fluoride (pH 7). Where possible (D), data were fitted to the two-state model.

**Dimer Exchange** - Dimer exchange experiments were performed for both the JunW<sub>CANDI</sub> and FosU<sub>PCA</sub> systems (Figs 7-8) in the presence of cJun and cFos cognate binding partners. Upon mixing the four component peptides, the spectra generated demonstrate the global average (hashed line) should no exchange of the component peptides take place. No exchange was deemed to have occurred in systems containing FosU<sub>PCA</sub>–cJun upon mixture with cJun–cFos (Fig. 7a) as well as JunW<sub>CANDI</sub>–cFos upon mixture with cFos–cFos (Fig. 8b). In contrast, spectra exhibiting signal exceeding the average indicated that the expected dimer exchange had indeed occurred. These changes in binding partners were observed in systems containing off-target peptides in complex with either FosU<sub>PCA</sub> (i.e. FosU<sub>PCA</sub>–cFos combined with cJun–cJun; Fig 7b) or JunW<sub>CANDI</sub> (i.e. JunW<sub>CANDI</sub>–cJun combined with cFos–cFos; Fig 8a). These data provide evidence for formation of heterospecific coiled coils with the cognate AP-1 component, in the contact of alternative cJun or cFos options available. The data also further validate the results of the in cellulo screening step through PCA.

![Figure 7](image-url)  
**Figure 7. Dimer exchange with FosU<sub>PCA</sub>, cJun, and cFos.** (A) Equimolar mixtures of cJun-cFos and FosU<sub>PCA</sub>–cJun were mixed and the observed signal resembled the average of the two constituent spectra, indicating no change has occurred. (B) Equimolar mixtures of cJun–cJun and FosU<sub>PCA</sub>–cFos were mixed and the observed spectra exceeded the average of the two constituent spectra which indicated that dimer exchange occurred to promote the system found in (A). All experiments were performed at 150 μM at 20°C in 10 mM potassium phosphate and 100 mM potassium fluoride (pH 7).
Figure 8. Dimer exchange with JunW<sub>CANDI</sub>, cJun, and cFos. (A) Equimolar mixtures of cFos-cFos and JunW<sub>CANDI</sub>-cJun were mixed and the observed spectra exceeded that of the average, indicating change. (B) Equimolar mixtures of cJun-cFos and FosU<sub>PCA</sub>-cFos were mixed and the observed spectra resembled the average of the two constituent spectra which indicated that no dimer exchange occurred. All experiments were performed at 150 μM at 20°C in 10 mM potassium phosphate and 100 mM potassium fluoride (pH 7).

Thermal Denaturation Profiles – The increased global secondary structure content for both antagonist–target complexes required further stability validation through thermal denaturation experiments (Fig. 4c/d and Fig. 5c/d). Taken in 1°C increments, the thermal melt data was generally in agreement with the spectra. In isolation, FosU<sub>PCA</sub> did not form a stable coiled coil – with only the characteristic upper baseline observed (Fig. 4c, red). Similarly, FosU<sub>PCA</sub>-cFos showed a similar upper baseline only (Fig. 4d, purple). For both of these complexes, the combination of spectra and thermal denaturation data demonstrate that weakly populated helices without the ability to associate. This scenario is beneficial for this antagonist system, as it removes two off-target states, in addition to the observation that FosU<sub>PCA</sub> incubated with cJun displayed a two-state sigmoidal thermal denaturation profile with a substantial right-hand shift (T<sub>m</sub> = 52°C). As shown from the spectra, JunW<sub>CANDI</sub> in isolation (Fig. 5c, red) formed a self-associating coiled coil with a T<sub>m</sub> of 32°C (similar to described previously<sup>11</sup>). When incubated with cJun (Fig. 5c, purple), only limited interaction was found to occur (T<sub>m</sub> = 27°C). Compared to thermal data with cJun, this represented an increase of 4°C from that previously reported (JunW<sub>CANDI</sub>-cJun<sub>37</sub> T<sub>m</sub> = 23°C). Incubated with cFos (Fig. 5d, blue), as expected, a right-hand shift was observed (T<sub>m</sub> = 51°C), representing an increase of 7°C from previous work with truncated cJun<sub>37</sub> (T<sub>m</sub> = 44°C). As observed in Fig. 3e/f, these differences in T<sub>m</sub> result from the addition of two pairs of g-e-<sup>1</sup>+1 interactions from the mismatch in length. Only in the off-target complex with cJun did this add an attractive electrostatic interaction (Glu-Arg at e-<sup>1</sup>-g<sup>0</sup>), with the presence of other residues contributing solely to increasing helicity in the complex. JunW<sub>CANDI</sub> in
complex with FosU_{PCA} showed a right-hand shift compared to the component peptides (Fig. 6c, purple). This increase in T_m to 40°C coupled with the spectra data is evidence for a stable coiled coil. In context of the helical wheel (Fig. 6a), this can be explained in part due to the fact that a net 4 of the 8 complete electrostatic interactions are attractive (Glu-Lys or Glu-Arg). In context of the other T_m values, this stability does not affect the co-compatibility as it is lower than the desired target complex T_m values.

A comparison of the predicted and experimental T_m values (Fig. 2 and Fig. 9) shows notable differences, an observation that has previously been discussed in the context of bCIPA, isCAN, and this particular library^{17,19}.

Figure 9. A comparison of measured FosU_{PCA} T_m data with previously designed peptides. The T_m of FosU_{PCA} - cJun is 52°C with a ΔT_m of 22°C. *FosU_{SCAN} and FosU_{PCA} did not form a stable homodimer or cFos heterodimer able to be fitted to get T_m. **cJun is an extended 39-mer sequence containing one extra g and one extra e position residue at the N-terminus and C-terminus respectively to add two extra interhelical electrostatic interactions with FosU_{SCAN} and FosU_{PCA}.

Primarily, the off-target FosU_{PCA}-FosU_{PCA} and FosU_{PCA}-cFos interactions were predicted to be relatively stable (60°C and 26°C respectively) whereas experimentally, this was not observed. This
highlights the role of the electrostatic interactions – as an analysis of the sequence (Fig. 3a/c) shows that the homodimer has 60% of these as repulsive Glu-Glu interactions. FosU_{PCA}-cFos exhibits a similar profile, with 40% of these interactions as Glu-Glu. In addition, the presence of Leu at g^2 and e^4 as residues incapable of forming electrostatic interactions adds to this disruption – along with the presence of two Thr and two Lys residues at the core. Although less extreme, observation of the desired complex with cJun showed that there was also a discrepancy between experimental and predicted T_m values, with bCIPA predicting a T_m value of 86°C and the experimental data displaying a T_m of 52°C. Although this was a decrease of 34°C, the system itself was not heavily altered. This is due to the fact that the ΔT_m decreased by 4°C from a predicted 26°C to an experimentally derived 22°C. In comparison to previous work, this ΔT_m value is second only to that of FosU_{isCAN} by 8°C. However, of the two, FosU_{PCA} predicted ΔT_m value sits closer to that of the experimental value (FosU_{isCAN} ΔΔT_m = -20°C). In both cases, the predicted ΔT_m was driven by the difference in T_m between the desired complex with cJun and the homodimeric library member interaction. However, with the inability of either homodimer to form a stable coiled coil, this ΔT_m relied on the difference between the desired complex and the T_m of homodimeric cJun. The thermal stability of FosU_{PCA}-cJun is comparable to previous peptides for which extensive biophysical data is available^{11,13,22,28}. In particular, the PCA derived FosW-cJun exhibited a T_m value of 63°C and a K_d value of 39 nM, whereas 4hFosW-cJun exhibited a T_m of 49°C and a K_d value of 480 nM. Since the latter exhibited thermal stability within 3°C of both FosU_{PCA}-cJun and JunW_{CANDI} – cFos, it can be estimated that the interaction K_d for both of these complexes is also within the nanomolar range.

Although the FosU_{PCA}-JunW_{CANDI} complex shows high level of stability (T_m= 40°C), it is important to note that JunW_{CANDI} was incorporated as a prototypical peptide sequence and neither peptide was explicitly screened (in-silico or in cellula) with the other as an off-target. The secondary structure and thermal data represents the formation of a stable complex, although this does not affect the co-compatibility of the two antagonists as a 11-12°C difference exists between it and the T_m values of the both FosU_{PCA}-cJun and JunW_{CANDI} – cFos. This can be credited to the fact that the increased electrostatic interactions in FosU_{PCA} and the stringency of the multi-part in silico screening of Fos-based peptides resulted in a library that exhibited higher levels of specificity to cJun itself, rather than all members of the Jun family.

**FosU_{PCA} Sequence Core Analysis** - As shown in Fig. 3, residue selection at the a positions resulted in alternations between Ile and Leu on a^1, a^2, and a^5, with a^1 selecting the former and a^2 and a^5 selecting the latter. Through all stages of PCA, the successful sequences (with the option of both Asn and Ile) selected for the Asn at this position, highlighting the importance of the interfacial Asn-Asn interaction in forming dimeric coiled coils and preventing higher order oligomeric states^{26,27}. Not
conforming to the structure or size of the otherwise ubiquitous residues found at this interface, His is thought to be ill-suited to the environment of the hydrophobic core at $\alpha$. Although not present in Jun/Fos proteins, His is present in the bHLH-ZIP cMax – along with Met – at sequential $d$ positions without disrupting the interaction required for transcriptional activity. When entering PCA, this residue was replaced at $P_1$ with Leu. One potential reason could be its position within the coiled coil – which unlike cMax – is not located in proximity of the N-terminus. As it occupies space directly between an Asn-Asn interaction as well as a position within the centre of the helix, the presence of the imidazole ring side-chain could destabilise the $\alpha$-helix and prevent successful coiled-coil formation.

**FosU_{PCA} Sequence Electrostatic Analysis** - As the electrostatic positions introduce the most diversity in options, it is interesting to first note the locations within the sequence that were not semi-randomised within the library. Importantly, at positions $g^1$, $e^2$, $g^3$, and $e^4$, Glu was selected by both isPCA/isCAN, indicating that this residue is sufficient at these positions to satisfy all conflicting design requirements. However, although this has been briefly discussed previously, it is important to interrogate this in the context of the *in cellulo* screening of a large library. At each position, Glu performs a combinatorial destabilisation and stabilisation role, depending on the complex the peptide adopts. As shown in Figure 3a-c, the selected residues at these positions may, in some cases, appear counter-intuitive. As residues at these positions interact within the coiled coil, this results in four repulsive Glu-Glu interactions in the homodimeric complex. When in complex with cJun, the presence of Glu serves to stabilise the coiled coil. $g^1$-$e^{e2}$, $e^2$-$g^{g3}$, and $g^3$-$e^{e4}$ interact beneficially in the form of 2 Glu-Lys interactions and one Glu-Arg interaction respectively ($e^4$-$g^{g3}$ forms a Glu-Thr interaction). Since isCAN additionally considers interactions with cFos, the same destabilising effect observed with the homodimer complex is selected for, with repulsive Glu-Glu interactions (and a non-optimal Glu-Leu interaction on $g^3$-$e^{e4}$). Previous work discussed the role of intramolecular interactions in the formation of the coiled-coil. Briefly, the presence of “solid-charge blocks” – a consecutive run of positively or negatively charged residues at either $e$ and/or $g$ positions within the heptad repeat – can result in intramolecular repulsion, thereby strengthening the interactions between the helices at these positions. Conversely, alternating +/- charges at these positions results in attractive intramolecular interactions which serve to lower the effect of attractive/repulsive electrostatic interactions between helices. FosU_{PCA} contains 60% Glu at electrostatic positions (i.e. 3 small charge blocks) which may serve to guide intramolecular repulsion (homomeric and in complex with off-targets) and optimise the beneficial interactions when in complex with cJun. Although it disrupts a pattern of Glu residues at this in the heptad, $g^2$ benefits from the selection of Gln due to the presence of Ala at $e^3$. Within the context of the FosU_{PCA} -cJun heterodimer, $g^{g0}$ and $g^{e4}$ on cJun
are Arg and Lys respectively, with intramolecular repulsion therefore potentially strengthening the intermolecular Arg-Glu and Lys Glu interactions with FosU_{PCA} at positions $e^4$ and $e^5$.

**Prediction vs Experimental** - As discussed previously, predictions using the bCIPA algorithm can vary in accuracy using designed peptide sequences $^{17,19}$. Elongation of designed sequences with the aim of maximising potential interactions highlights a current potential limitation in current predictive capabilities. Electrostatic charge blocks are also a sequence-specific effect which is currently not considered. bCIPA scores each residue independently of others within a helix but scores for specific interaction between helices, with only the latter taking advantage of the coupling data from the many natural peptides used to train the model. Additionally, these sequences are shorter than the sequences evaluated in this work, providing another possible explanation for discrepancy. However, the ability to impose artificial limits through an imposed $\Delta$ parameter mitigates against this – replacing the subtle differences in desired/undesired predictions with predictably larger variations. This parameter functions as a necessary safeguard for the additional layers of complexity employed. Previous work exploring this$^{10}$ has also highlighted limitations of relying solely on coupling data.

**CONCLUSIONS**

**in Silico led In Cellulo Design** – Of the 10 possible interactions within the intended system (Fig. 10), both antagonists successfully bound their respective targets while avoiding their cFos/cJun off-target complexes. Although a stable coiled-coil, the FosU_{PCA}–JunW_{CANDI} complex is not predicted to disrupt the co-compatibility of the system due to the increased thermal stability of both of the peptides with their targets.

![Figure 10](image)

**Figure 10. An overview of the FosU_{PCA} + JunW_{CANDI} system.** Analysis of $T_m$ data shows that the desired interactions (green) are preferentially formed over the off-targets (red) and the homodimers (black). Though the antagonist-antagonist dimer has a relatively high $T_m$ (40°C), this comparison highlights that the antagonist-target interactions are the preferred states.
With work into FosU_{isCAN} and now FosU_{PCA} respectively exploring purely data-driven and combinatory \textit{in silico/in cellulo} design, it has so far been shown that - aside from yielding increased stability of target homodimer complexes – the extension of the sequence length to add extra electrostatic interactions adds increased specificity but not increased stability in the heterodimeric peptide-target complex (Fig. 7) \textsuperscript{17}. Studies into the truncation of cJun antagonists have described similar issues\textsuperscript{19,22}. In these experiments, engineering stability has resulted in the antagonist peptide homodimers having near equivalent or increased thermal stability when compared to that of the desired heterodimeric complex with cJun. Due to the simplicity of coiled coil sequences and the constraint imposed by the need to specifically target certain bZIPs without disrupting the function of others, this raises the question of whether there is a limit to the ability of certain engineered peptides to be able to fulfil both the stability and specificity design requirements. PCA-derived FosW exemplifies this phenomenon, with a difference of 6°C between the T\textsubscript{m} of its homodimeric complex and heterodimeric complex, in addition to the 2°C between the latter and the T\textsubscript{m} of a potential off-target target complex with cJun (Fig. 7). As FosW–cJun, an interaction that did not consider cJun during design or selection, has a T\textsubscript{m} that is only marginally higher than that of FosU_{PCA–cJun}, this highlights the advancement of \textit{in silico} techniques in addressing the conflicting design requirements imposed by the simplicity of the coiled coil structure. Though both FosW and FosU_{PCA} were derived via PCA, there was a significant difference in the size of the two libraries generated in order to do so (with the 49,152 member FosW library being 58% larger than that of the 31,104 member isCAN/isPCA). Despite this, in the latter case the library derived a peptide that was more able to meet the criteria required by antagonist peptides – that is the maximisation of stability balanced with the ability to specifically bind its target. In the context of engineering peptides, this represents an advancement in the process and validates the combined use of combinatory \textit{in silico} and \textit{in cellulo} screening. To this end, future exploration into improving this framework would include an expansion of the library size as well as the generation of an additional library containing cJun-based peptide sequences to target cFos.
Figure 11. An overview of the proposed combined isCAN. This system incorporates the previously defined combination of predictions for off-target (1-4) and desired (6) complexes. It runs in parallel over two separate libraries (A/B) and allows for each member (L) of A to be incorporated as an additional competitor to the screening of library B and vice versa (5). This ensures that the libraries are capable of specificity relative to other designed peptides as driven by the Δ value (highlighted in red).

As shown in Fig. 11, the addition of that extra screening step (5) into this framework would allow not only for the isPCA/isCAN methods described here but also an additional isCAN constraint in which the cFos-based and cJun-based libraries are able to consider one another during the in silico screening stage. Similarly, the in cellulo screening would expand to incorporate a specific CANDI element, with both isPCA/isCAN derived peptides utilised as a competitor peptide during this process. This would allow for the library design and subsequent directed evolution of a potentially synergistic system whereby two AP-1 inhibitors designed to target different components within an oncogenic heterodimer could function specifically with minimal cross-interaction.

In summary, this work provides a step forward in the use of more stringent in cellulo screening, preceded by in silico screening, to derive peptide antagonists for coiled coil proteins in general, using AP-1 as an exemplar. The ability to utilise a combination of successful in silico and in cellulo screening methods has been validated by sequential combined screening. Within the larger context, this represents progress in the ability to derive specific peptides capable of targeting key components.
within complex bZIP systems, while increasing the experimental data required to progress data-driven design.

AUTHOR CONTRIBUTIONS

ASM and AL conducted experiments, and synthesized, purified and characterised peptides and cJun. JMM directed the research. AL and JMM participated in experimental design, analysis of the data, and writing the paper.

COMPETING FINANTIAL INTERESTS

JMM is an advisor to Sapience Therapeutics. A.L. has no financial or commercial conflict to declare.

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SUPPORTING INFORMATION

Supporting information is available in Supplementary Figures S1-S5.

REFERENCES


