Supplemental Material

Supplemental Methods

Mass Spectrometry
Intact mass LC-MS analysis of ΔFOSB/JUND bZIP:compound complexes was carried out as follows. Samples were injected as 1 µl aliquots of 20 ng/µl total protein onto a PepSwift Monolithic Trap (200 um x 5 mm), desalted, and then eluted (1 µl/min) onto a PepSwift Monolithic analytical column (PS-DVB monolith, 200 µm x 50 mm). Proteins were eluted using the following gradient program: isocratic at 10% B, 0–5 min; 10% to 55% B, 5–20 min; 55% to 85% B, 20-24 min; isocratic 85% B, 24-25 min; 90% to 10% B, 25-26 min; isocratic 10% B, 26-27 min; 10% to 85% B, 27-29 min; isocratic 85% B, 29-30 min; 85% to 10% B, 30-31 min; and isocratic 10% B, 31-45 min. Intact mass LC-MS data were acquired using Xcalibur version 4.3.73.11 (Thermo Fisher Scientific) in positive mode. Full scan MS1 spectra (m/z 600-2000) were acquired with an RF Lens setting of 60%, with Orbitrap detection using 120,000 resolution (at m/z = 200) and 3 microscans in profile mode, with a Normalized MS1 AGC target of 250%, and a maximum injection time of 100 ms.

Deconvolution of intact mass spectral data and analysis were carried out as follows. LC-MS spectra corresponding to the intact mass profiles of ΔFOSB and JUND bZIP domains were analyzed using BioPharma Finder. Intact mass spectra were analyzed using a time-resolved LC-MS spectral deconvolution approach based on the combined use of the Xtract and Sliding Window algorithms as we have described before (Bailey et al., 2018). Xtract deconvolution considered an Output Mass Range of 1,000 to 60,000 Da, a S/N Threshold of 3, Charge State Range of 5 to 50, and a Minimum Number of Detected Charges set to 3. Sliding Window parameters included an RT Range of 10 to 16 min, a Target Average Spectrum Width of 0.5 min, Scan Offset of 1, a Merge Tolerance of 3 ppm, and a Minimum Number of Detected Intervals of 10. Intact masses were searched against protein sequence databases of the ΔFOSB and JUND bZIP domains including variable modifications of the cysteine residues (i.e., oxidation, double oxidation), and modification of certain compounds (i.e., loss of HCl for Z2159931480; average mass of 204.18 Da). Searches were performed with a mass tolerance of 50 ppm.

To identify Z2159931480 binding sites in the ΔFOSB/JUND bZIP domains, peptide mapping of the trypsin-digested ΔFOSB/JUND bZIP:Z2159931480 complex was carried out via nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) coupled inline to a Thermo Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific) through a Nano-flex nanospray ion source (Thermo Fisher Scientific) via a FAIMS Pro (Thermo Fisher Scientific) differential ion mobility interface.
Peptide samples were directly injected into the LC path, avoiding the use of a trap column to maximize peptide recovery and MS detection. After equilibrating the column in 98% solvent A (0.1% formic acid in water) and 2% solvent B (0.1% formic acid in acetonitrile (ACN)), the samples (5 µl in solvent A) were injected onto an Aurora series C18 analytical column (1.6 µm particle size, 50 µm x 25 cm, IonOpticks, Fitzroy VIC, Australia) and subsequently eluted (300 nl/min) by a gradient program as follows: isocratic at 2% B, 0–5 min; 2% to 6% B, 5–6 min; 6% to 32% B, 6–65 min; 32% to 50% B, 49–50 min; 50% to 90% B, 71–72 min; isocratic 90% B, 72–73 min; 90% to 5% B, 73–74 min; isocratic 5% B, 74–74.5 min; 5% to 90% B, 74.5–75 min; isocratic 90% B, 75–76 min; 90% to 2% B, 76–77 min; and isocratic 2% B, 77–90 min.

All LC-MS/MS data were acquired using XCalibur version 4.4.14.14 (Thermo Fisher Scientific) in positive ion mode using a top speed data-dependent acquisition (DDA) method with a 3 s cycle time. The survey scans (m/z 300–2000) were acquired in the Orbitrap at 120,000 resolution (at m/z = 200) in profile mode, with a FAIMS Pro compensation voltage of -45 V, a maximum injection time of 50 ms, and a Normalized MS1 AGC target of 250%. The S-lens RF level was set to 30. Isolation was performed in the quadrupole with a 1.6 Da isolation window, and HCD MS/MS acquisition was performed in profile mode in the Orbitrap at 30,000 resolution (at m/z = 200) with the following settings: parent threshold = 20,000; stepped collision energy = 20%, 30%, 40%; maximum injection time = 50 ms; Normalized MS2 AGC target = 250%. Monoisotopic precursor selection (MIPS) and charge state filtering were on, with charge states 2–7 included. Dynamic exclusion was used to remove selected precursor ions, with a ±10 ppm mass tolerance, for 15 s after the acquisition of one MS/MS spectrum.

**Cell Stimulation**

The AP1 luciferase reporter human embryonic kidney 293 recombinant cell line (AP1-luc HEK293) was obtained from BPS Bioscience (USA) and cultured with growth medium 1B (BPS Bioscience) containing 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Hyclone) at 37°C in 5% CO₂. The cells were plated in octuplicate in seven 6-well microplates at a concentration of 3.5 x 10⁵ cells/well and incubated for 24 h for stabilization. Subsequently, the medium in all wells, except the control wells, was changed to assay medium 1B (BPS Bioscience) containing 0.5% FBS to starve the cells for 24 h. Then, cells were treated with 20% FBS (serum-stimulated) containing growth medium 1B for 2-24 h. Following serum stimulation, cells were lysed using RIPA buffer and sonicated (25 s on/25 s off, repeated 4 times) and centrifuged at 12,000 rpm for 10 min at 4°C to collect cell lysate. The protein concentration of cell lysates was determined with a BCA assay. Cell lysates were mixed with an appropriate volume of 4X sample buffer containing dithiothreitol (DTT), heated at 75°C for 15 min, and stored at -80°C for Western blot analysis.
**Immunoblot Analysis**

Western blotting analysis was performed on 10 µg cell lysates using 8-16% SDS-PAGE gels. Proteins were separated and transferred electrophoretically to a nitrocellulose membrane. Under agitation, membranes were blocked for 1 h in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) blocking solution with 5% milk (TBST+5% milk) and washed five times for 5 min with TBST. Membranes were incubated overnight at 4°C with appropriate primary antibodies in TBS with 5% Milk and 0.2% Tween-20 under agitation, washed five times with TBST, and incubated for 1 h at room temperature with appropriate secondary antibodies in TBST+5% milk on a shaker. Membranes were washed with TBST three times, 10 min per wash, followed by two 15 min washes with TBS. Western blots were developed using Western Lightning™ Chemiluminescence Reagent Plus (Perkin-Elmer) and the iBright imaging system (Thermo Fisher Scientific).

**Antibodies**

Rabbit anti-FOSB and mouse anti-β-actin antibodies (Cell Signaling; dilutions of 1:500 and 1:1000, respectively) were used for the immunoblot analysis. Horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse and goat anti-rabbit antibodies were obtained from SeraCare and used at a dilution of 1:10,000.

**Fluorescence Polarization Binding Assay**

We performed a fluorescence polarization (FP)-based DNA-binding assay as we have described (Jorissen et al., 2007; Wang et al., 2012). Briefly, (His)$_6$-ΔFOSB homomers or (His)$_6$-ΔFOSB Cys$^{172}$Trp homomers were dispensed in a concentration series (0-280 nM or 0-320 nM). To each well, 25 nM or 50 mM TAMRA-labeled oligonucleotide (TMR-cdk5 or TMR-SCR) was added. The FP buffer was 20 mM HEPES pH 7.5, 50 mM NaCl with 1 mM TCEP (reducing conditions). The protein:oligonucleotide samples were mixed and dispensed in quadruplicates into 384-well round bottom low-volume black microtiter plates (Corning) with 20 µl per well and then incubated at room temperature for 15 minutes. The FP signal was measured using a BioTek Synergy Neo2 plate reader (excitation 530 nm, emission 590 nm) or using a Pherastar plate reader (BMG Labs; excitation 540 nm, emission 590 nm, 100 flashes per well); the target was set to 20 mP for each individual TMR-oligonucleotide by adjusting the gain on a well with oligonucleotide in the absence of protein. As a baseline, the FP signal observed for the free oligonucleotide (i.e., no protein) was used and subtracted from the FP values measured for oligonucleotides in the presence of varying amounts of protein. Data were processed using Prism 6.0 (GraphPad). The binding curves were fitted with a sigmoidal curve (best fit obtained using a ‘Boltzmann Sigmoidal’ curve). Each data point represents the mean of four replicates, and the error bar represents the standard deviation of the mean.
| Compound       | Identified species                                      | Relative abundance (%) | Observed deconvoluted monoisotopic protein mass (Da) | Chemical formula of modification | Mass change of modification (Da) | Theoretical monoisotopic mass (Da) | Measured mass error (ppm) |
|----------------|--------------------------------------------------------|------------------------|-----------------------------------------------------|---------------------------------|---------------------------------|-----------------------------------|---------------------------|
| Z2159931480    | ΔFOSB + Z2159931480 (minus HCl)                         | 100.0                  | 8447.448                                            | C\(_{11}\)H\(_8\)O\(_4\)        | 204.042                         | 8447.387                          | 7.3                       |
|                | ΔFOSB + Z2159931480                                     | 13.0                   | 8483.417                                            | C\(_{11}\)H\(_9\)ClO\(_4\)      | 240.019                         | 8483.364                          | 6.3                       |
|                | ΔFOSB unmodified                                        | 0.0                    | N/D                                                 | none                             | 0                               | 8243.345                          | N/D                       |
|                | JUND + Z2159931480 (minus HCl)                          | 24.5                   | 8167.661                                            | C\(_{11}\)H\(_8\)O\(_4\)        | 204.042                         | 8167.631                          | 3.7                       |
|                | JUND + Z2159931480                                     | 6.9                    | 8203.632                                            | C\(_{11}\)H\(_9\)ClO\(_4\)      | 240.019                         | 8203.608                          | 3.0                       |
|                | JUND unmodified                                         | 0.0                    | N/D                                                 | none                             | 0                               | 7963.589                          | N/D                       |
| 2196-99-8      | ΔFOSB + 2196-99-8 (minus HCl)                           | 100.0                  | 8391.394                                            | C\(_9\)H\(_8\)O\(_2\)           | 148.052                         | 8391.397                          | -0.3                      |
|                | ΔFOSB minus ~16-18 Da; loss of 1x O                     | 24.9                   | 8225.617                                            | N/D                             | N/A                             | N/A                               | N/A                       |
|                | ΔFOSB plus 98 Da; unknown modification                  | 23.5                   | 8339.615                                            | N/D                             | N/A                             | N/A                               | N/A                       |
|                | JUND + 2196-99-8 (minus HCl)                           | 36.3                   | 8111.627                                            | C\(_9\)H\(_8\)O\(_2\)           | 148.052                         | 8111.641                          | -1.7                      |
| 456-04-02      | ΔFOSB + 456-04-2 (minus HCl)                            | 100.0                  | 8379.373                                            | C\(_8\)H\(_6\)ClFO               | 136.032                         | 8379.377                          | -0.4                      |
|                | JUND + 456-04-2 (minus HCl)                            | 32.6                   | 8099.609                                            | C\(_8\)H\(_6\)ClFO               | 136.032                         | 8099.621                          | -1.4                      |
| Z3247353427    | ΔFOSB unmodified                                        | 100.0                  | 8243.398                                            | none                            | 0                               | 8243.345                          | 6.5                       |
|                | JUND unmodified                                         | 30.7                   | 7963.609                                            | none                            | 0                               | 7963.589                          | 2.6                       |
| 1183031-77-7   | ΔFOSB unmodified                                        | 100.0                  | 8243.341                                            | none                            | 0                               | 8243.345                          | -0.4                      |
|                | ΔFOSB + 456-04-2 (minus HCl)                            | 1.9                    | 8458.459                                            | C\(_{14}\)H\(_{17}\)NO           | 215.131                         | 8458.476                          | -2.0                      |
|                | JUND unmodified                                         | 34.6                   | 7963.578                                            | none                            | 0                               | 7963.589                          | -1.3                      |
|                | JUND + 456-04-2 (minus HCl)                            | 1.4                    | 8178.707                                            | C\(_{14}\)H\(_{17}\)NO           | 215.131                         | 8178.720                          | -1.5                      |

**Table S1.** Intact mass analysis of ΔFOSB/JUND bZIP incubated with compounds. High-resolution LC-MS data were analyzed generating a single, averaged mass spectrum using spectra acquired during the elution time frame followed by Xtract charge-state deconvolution, which provides monoisotopic mass and relative abundance for observed isoforms as well as mass accuracy (ppm) for identified species.
Figure S1. Small molecules targeting ΔFOSB disrupt DNA binding. a) Group A compounds assessed in FP-based dose response assays (shown for ΔFOSB homomers). b) Group B compounds assessed in FP-based dose response assays (shown for ΔFOSB homomers). For a) and b), compounds were tested by incubating 25 nM TMR-cdk5 oligo with increasing amounts of compound (0-200 µM) in presence of 320 nM ΔFOSB full-length protein (●) or in absence of protein (○). The controls, '100%-inhibition' i.e., oligonucleotide alone (♦) and '0%-inhibition', i.e., ΔFOSB+cdk5 oligo (■) are indicated. The data points were collected in quadruplicate; error bars indicate the standard deviation. IC$_{50}$ values are averaged over two independent experiments and the standard deviation given. The compounds were also tested against ΔFOSB/JUND, see Figure 3.
Figure S2. Crystal packing of ΔFOSB/JUND\textsuperscript{red} and ΔFOSB/JUND\textsuperscript{cmpd}. a) Molecules contacting the ΔFOSB/JUND\textsuperscript{red} bZIP in the asymmetric unit. ΔFOSB (orange), JUND (cyan), redox-switch cysteines (magenta spheres) and DNA-binding motifs (darker shades). b) Molecules contacting the ΔFOSB/JUND\textsuperscript{cmpd} bZIP in the asymmetric unit. Colors as in a). The location of ΔFOSB Cys\textsuperscript{172} with Z2159931480 bound is encircled. In a) and b), interactions with the central molecule are shown as sticks and colored as follows: salt bridges (hot pink), hydrogen bonds (limon), and nonpolar (marine). c) Three contacts may influence the compound binding site. Contact #1 and #3 are discussed in the text. Contact #2 likely does not affect the bend of the helices or pry the
forceps open, because a symmetry-related JUND bZIP subunit interacts only with the main JUND bZIP subunit and not with the ΔFOSB bZIP subunit.
Figure S3. Quantification of FOSB and ΔFOSB in AP1-luc HEK293 cells by Western blotting.

a) Representative Western blotting analysis of FOSB and ΔFOSB from control (no treatment) and 2, 4, 6, and 24 h serum-stimulated AP1-luc HEK293 cells. b) Quantified abundance of FOSB. c) Quantified abundance of ΔFOSB. Abundances of FOSB and ΔFOSB were normalized to the abundance of β-actin. Data are means ± SEM (standard error of the mean; N=5 for FOSB and N=8 for ΔFOSB).
Figure. S4. T5224 and Z3247353427 tested in FP-based dose response curves (DRC). a) T5224, and b) Z3247353427 were assessed against full-length ΔFOSB/JUND, ΔFOSB Cys^{172}Ser/JUND, ΔFOSB/ΔFOSB, and ΔFOSB Cys^{172}Ser/ΔFOSB Cys^{172}Ser in an FP-based dose response assay. Compounds were tested by incubating 50 nM TMR-cdk5 oligo with increasing amounts of compound (0-200 µM) in presence of 280 nM ΔFOSB/JUND full-length protein or mutant (●) or in absence of protein (○). The controls, '100%-inhibition', i.e., oligonucleotide alone (♦) and '0%-inhibition', i.e., ΔFOSB/JUND+cdk5 oligo (■) are indicated. The data points were collected in quadruplicate; error bars indicate the standard deviation. The compounds were also tested against 280 nM ΔFOSB full-length protein or mutant. In the FP-DRCs for Z3247353427, lower activity was noted for the wild-type proteins compared to data shown in Figure 3, but they were carried out on the same 384-well plates as the mutants enabling side-by-side comparison.
Figure S5. DNA binding properties of ΔFOSB and mutant ΔFOSB Cys^{172}Trp homomers. 
a) purified full-length wild-type ΔFOSB WT and ΔFOSB Cys^{172}Trp homomers assessed by SDS-PAGE under standard reducing conditions. Markers (M) are shown in the first lane. b) DNA-binding properties of full-length ΔFOSB and ΔFOSB Cys^{172}Trp homomers assessed in fluorescence polarization studies using both a specific TMR-\textit{cdk5} AP1 oligonucleotide (25 nM) and a non-specific, scrambled TMR-\textit{scr} AP1 oligonucleotide (25 nM). The oligonucleotides were incubated with increasing amounts of protein (0, 5.1, 10.25, 20.5, 41.0, 82.0, 164.0, 205.0, 256.0, and 320.0 nM with respect to monomers) and the change in the fluorescence polarization signal monitored as a function of protein concentration. Data points are averages of quadruplicate measurements with the error bars indicating the associated standard error of the mean (SEM).
a) The table lists various genes and their UniProt IDs, along with their corresponding sequences.

b) The second table provides a similar alignment of sequences, focusing on different genes.

Figure S6. Multi-sequence alignment of the human bZIP sequences with a counterpart equivalent to the redox-switch residues ΔFOSB Cys\textsuperscript{172} and JUND Cys\textsuperscript{285}. a) all 29 human sequences. CREBL2 (CAMP Responsive Element Binding Protein Like 2) was considered an outlier at positions 11 and 36 and omitted from sequence-conservation assignment. b) FOS and JUN family members only.
Red: conserved; cyan: semi-conserved; not colored: not conserved; semi-conserved groups are defined as (R, K) and (Q, E) for a), and as (R, K), (Q, E), (A, S, T) and (V, L) for b).

“bZIP”: bZIP residue numbers (first-last); “Res”: number of residues in bZIP domain; “Sequence”: heptad repeats 0-5 are indicated.