Intracellular Peptide Library Screening for Covalent Transcription Factor Inhibitors

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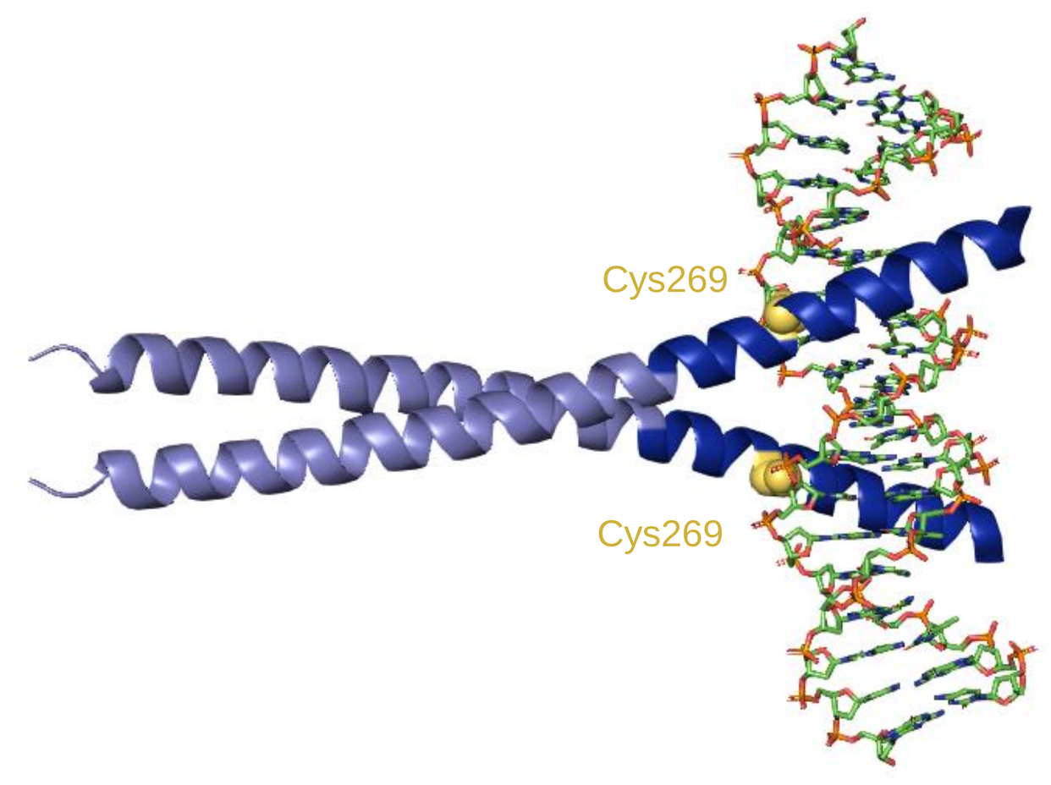
1. Introduction

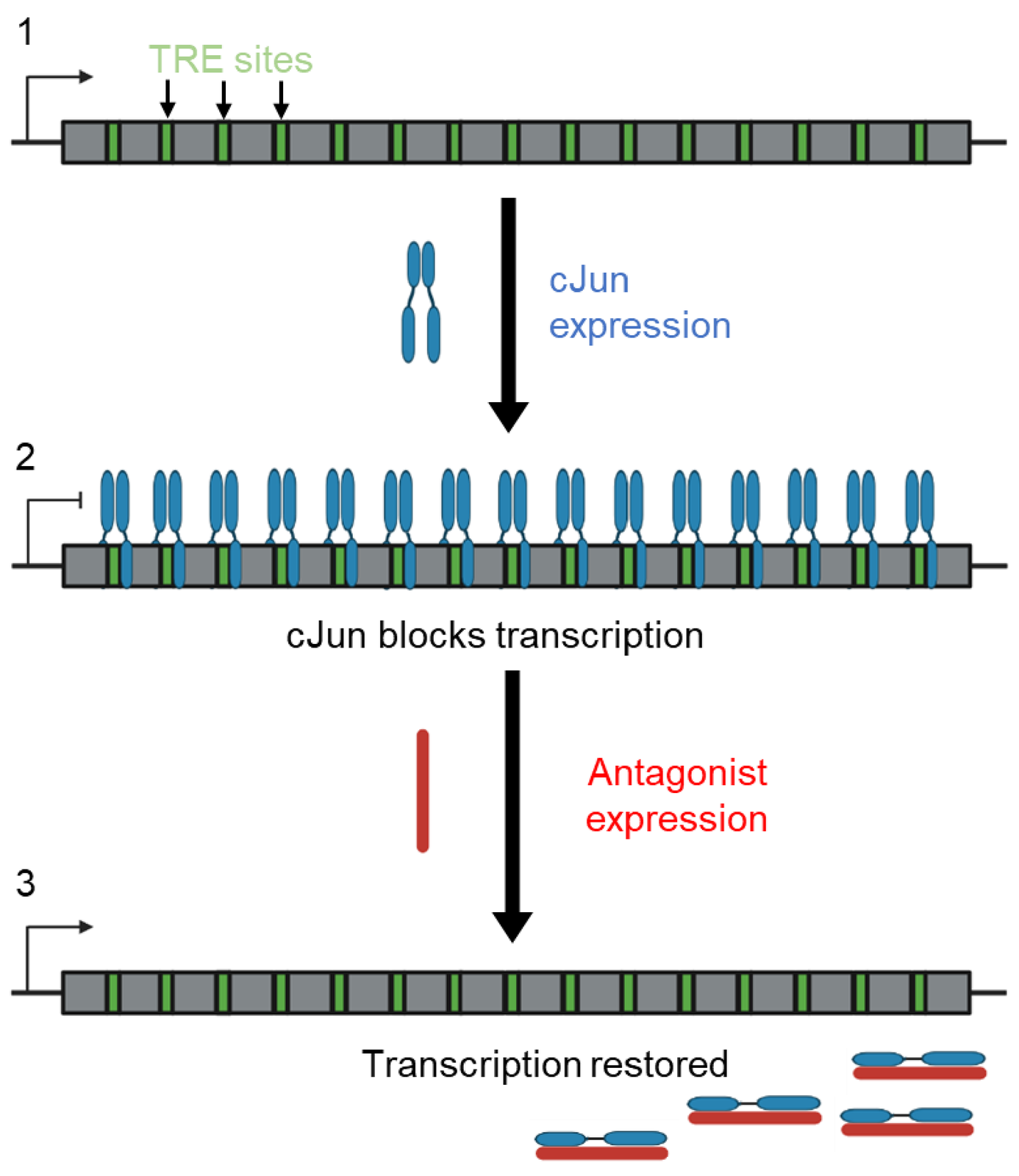
- The bZIP superfamily of transcription factors (TFs) bind DNA consensus sites as dimers, as shown for **cJun bound to TRE DNA**.[1]
- cJun is **upregulated in a range of diseases**, including cancer, making antagonism of its interaction with TRE DNA a promising therapeutic target.[2]
- The Transcription Block Survival (TBS) assay **screens peptide libraries to identify functional TF antagonists**.[3]
- **Cys269** in the cJun DNA-binding domain allows for the **possibility of selective covalent inhibition** of TF function.



Crystal structure of a cJun homodimer bound to TRE DNA.[1]

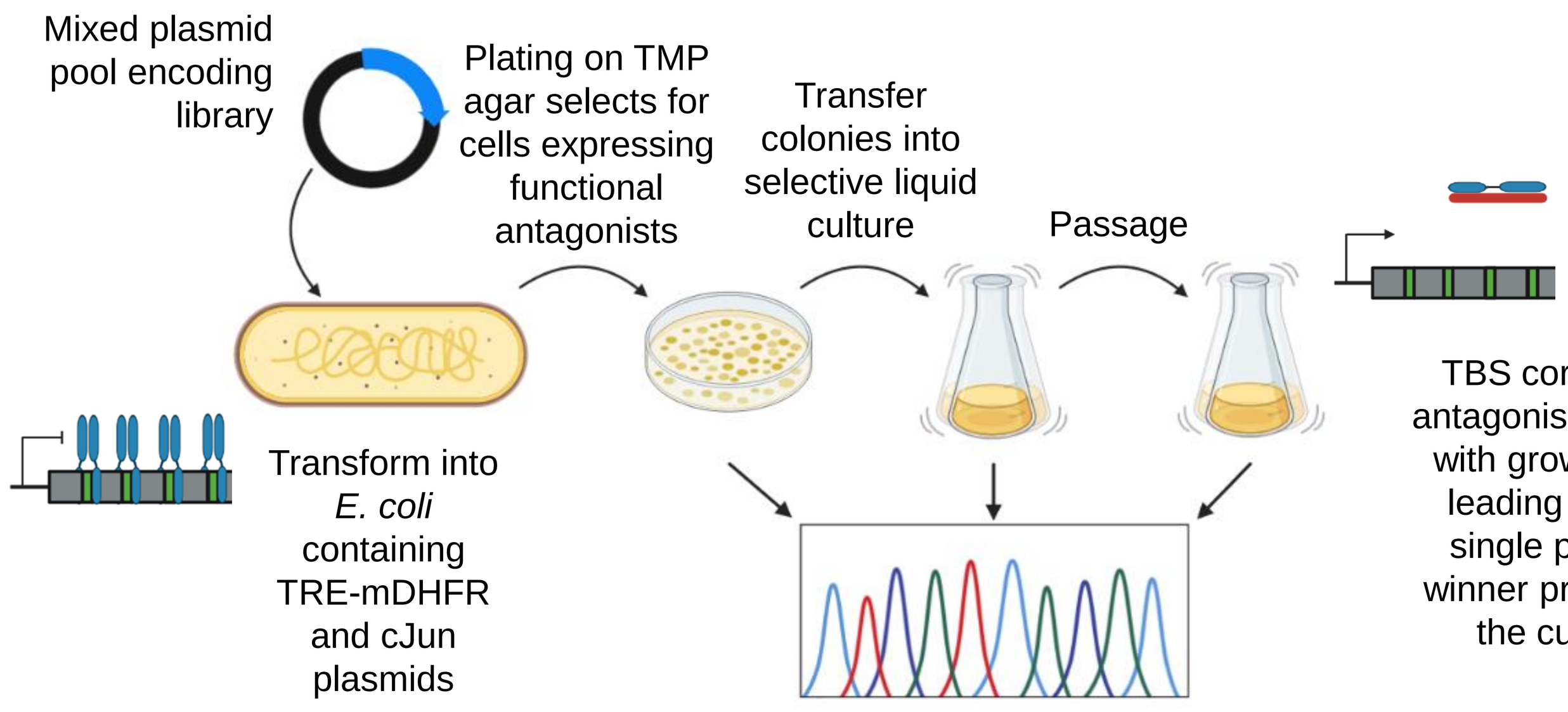
2. TBS assay design

1. Inhibition of endogenous bacterial DHFR with TMP renders bacteria dependent on an exogenous TRE-mDHFR gene for cell survival.
2. cJun binds to TRE sites within TRE-mDHFR, sterically blocking RNA polymerase from transcribing the essential gene.
3. Only functional antagonism of the cJun/TRE interaction sequesters the bZIP to restore TRE-mDHFR transcription and therefore cell growth.

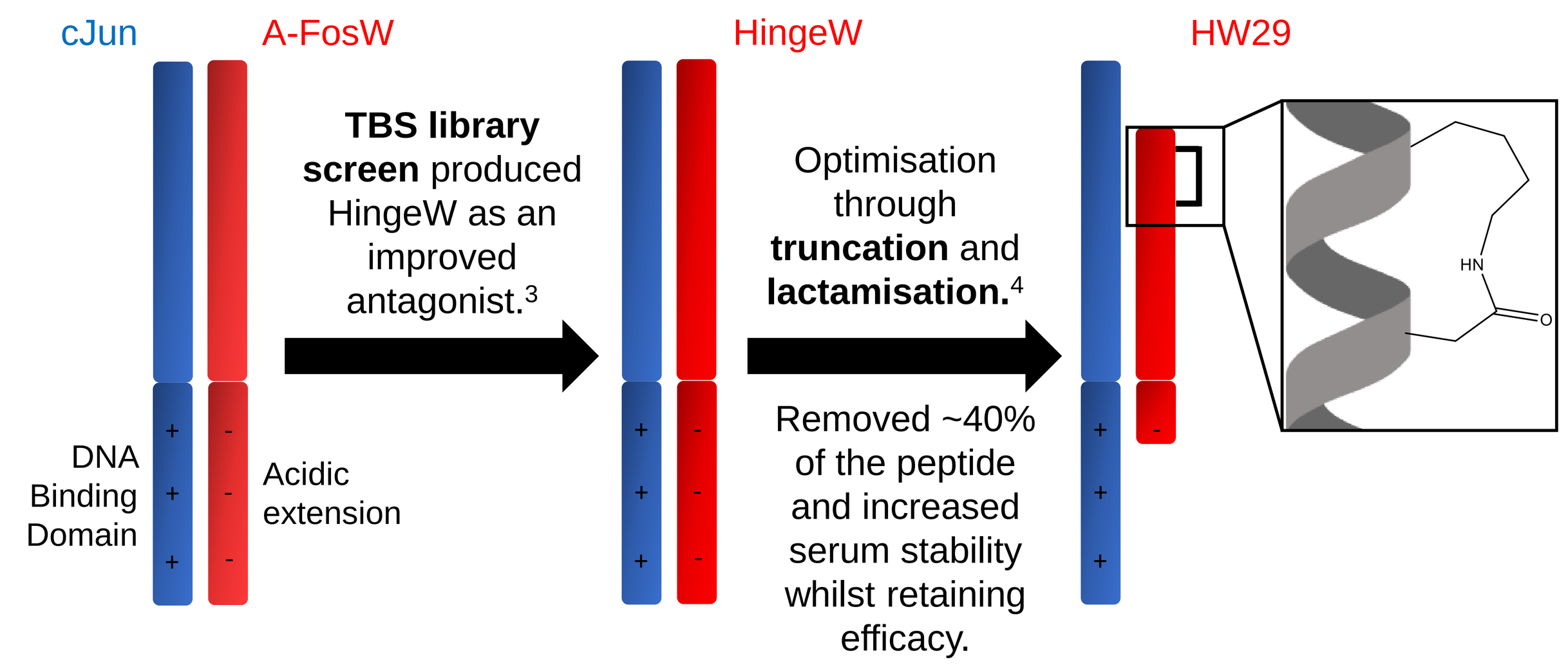


Assay benefits:

- Screen tens of millions of peptide library sequences, with growth rate as the assay readout which allows direct competition.
- Select for functional antagonism of protein-DNA interactions, not simply binding.
- Entirely tag-free system.
- *E. coli* system is robust and economical.
- Screening occurs *in vivo* which selects for favourable properties such as biostability, specificity, solubility and low toxicity.



3. Previous antagonist development using TBS and subsequent rational optimisation



4. Cells with an oxidising cytoplasm can identify a covalent antagonist

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                DBD/Acidic extension                 LZ
             1        2        3        4        5        6        7        8        9
             defgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcd
cJun         RIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNYELASTANMLREQVAQL
HW1          LEQRAEELARENEELEKEAEELVVEEDVLEEEIEQLEERNYALRKEIEDLQKQLEKL
Library                        FDRDADDFF
Options:                       CGCGSGGCV
                               LY YCYYLC
                               WC CGCCWG

OxidisedW    LEQRAEELARENEELDRDCDDLVVEEDVLEEEIEQLEERNYALRKEIEDLQKQLEKL
ReducedW     LEQRAEELARENEEFDRDSDDLVVEEDVLEEEIEQLEERNYALRKEIEDLQKQLEKL
HW29                           Ac-EAEELVVEEDVLEEEIEQLEERNYALRKEIKDLQDQ-NH2
HW31                           Ac-ECEELVVEEDVLEEEIEQLEERNYALRKEIKDLQDQ-NH2
HW33                           Ac-EMEELCVEEDVLEEEIEQLEERNYALRKEIKDLQDQ-NH2
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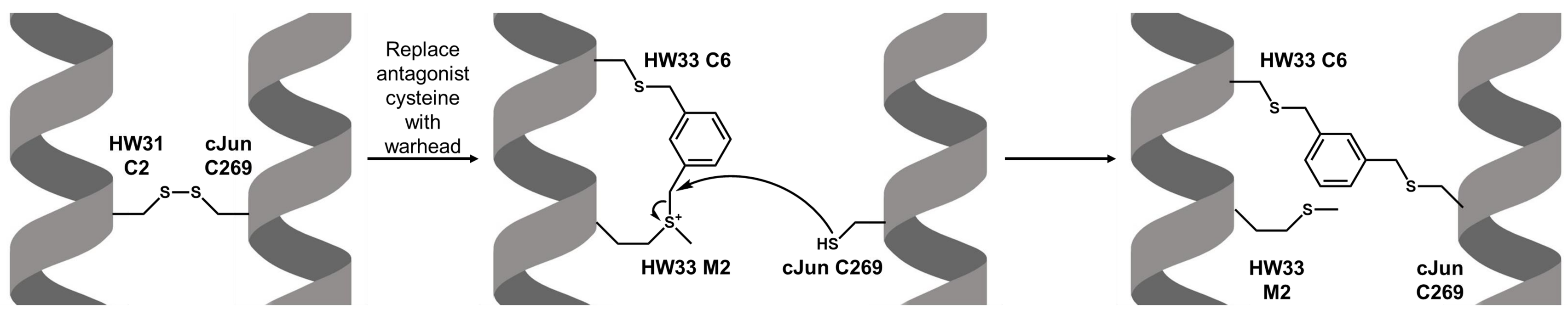
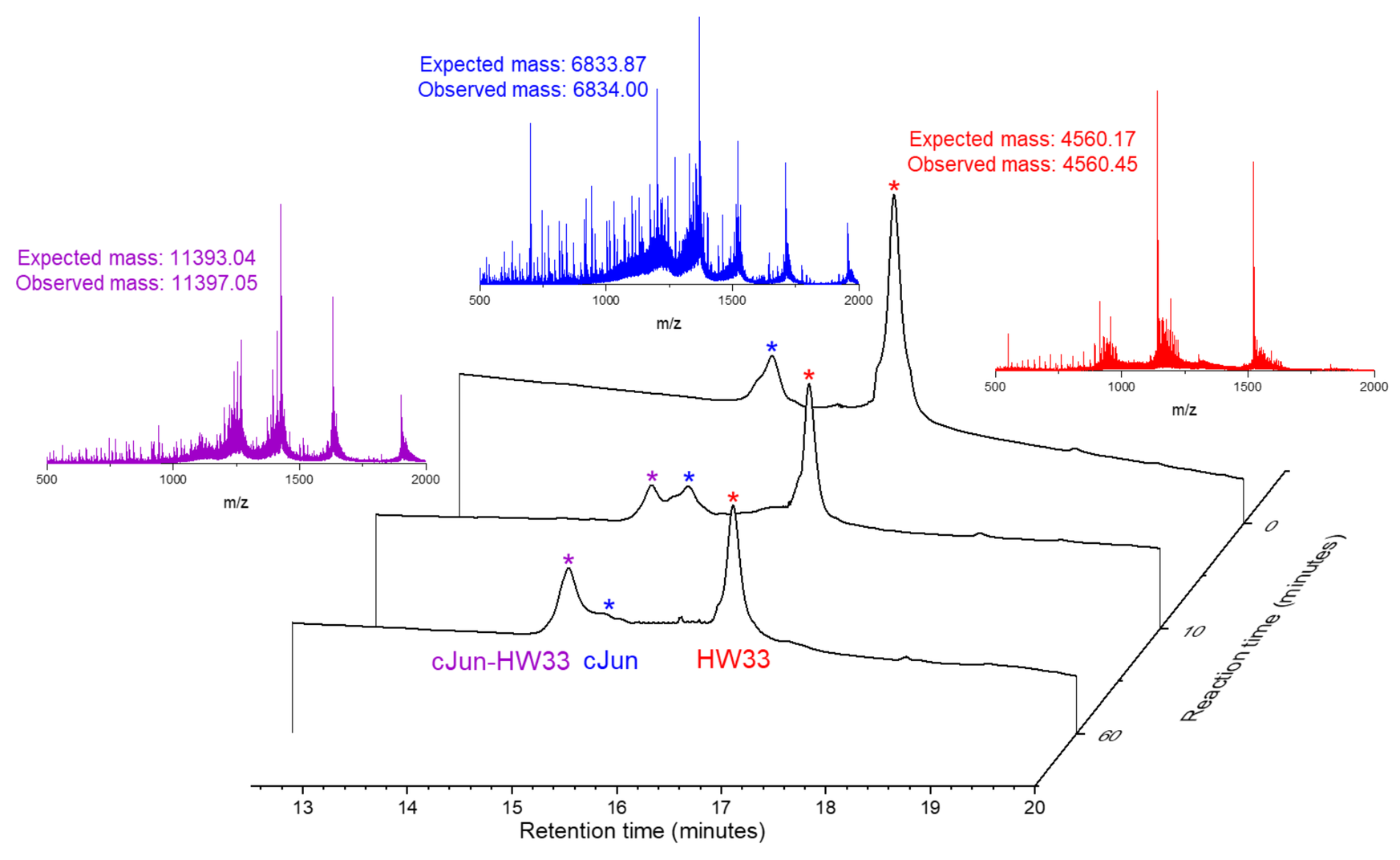
Screening a cysteine containing library in SHuffle Express cells facilitates disulphide formation between antagonist and target.

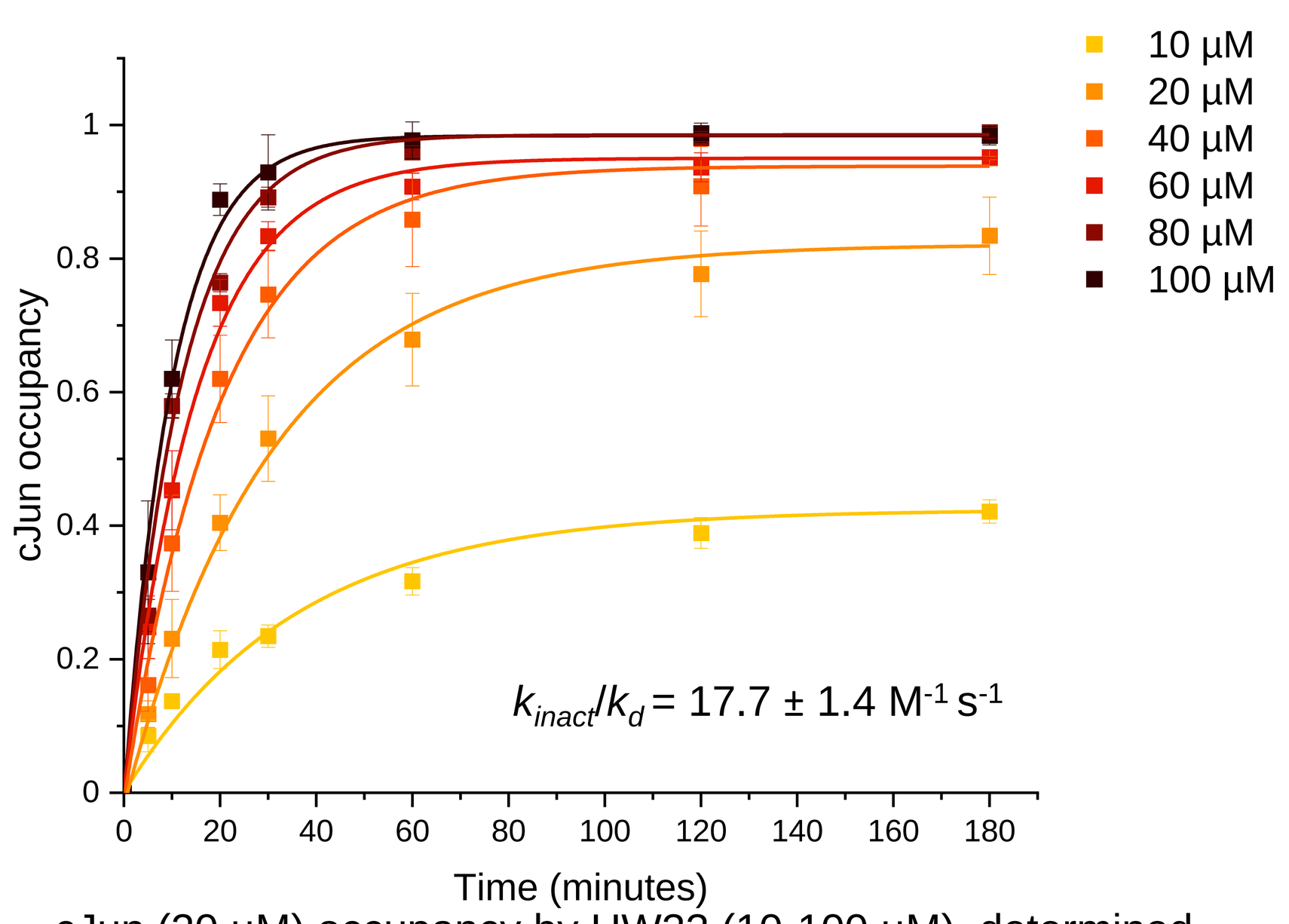
The position of the unique cysteine selected in SHuffle Express (OxidisedW) was incorporated into the optimised HW29 peptide to produce HW31 which we have shown to form an inhibitory disulphide with cJun.

5. Replacing selected cysteine with an electrophile irreversibly inhibits cJun

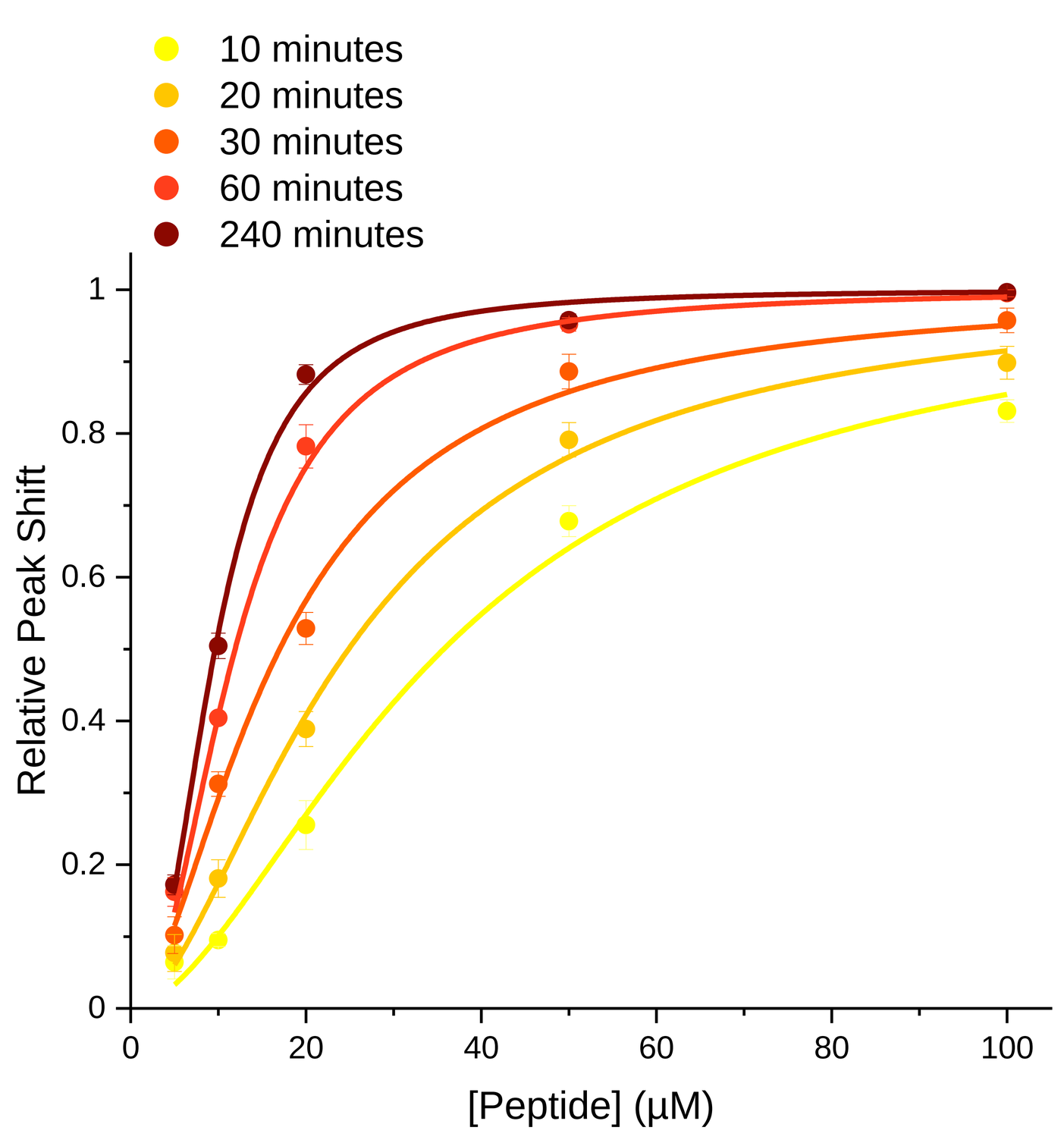




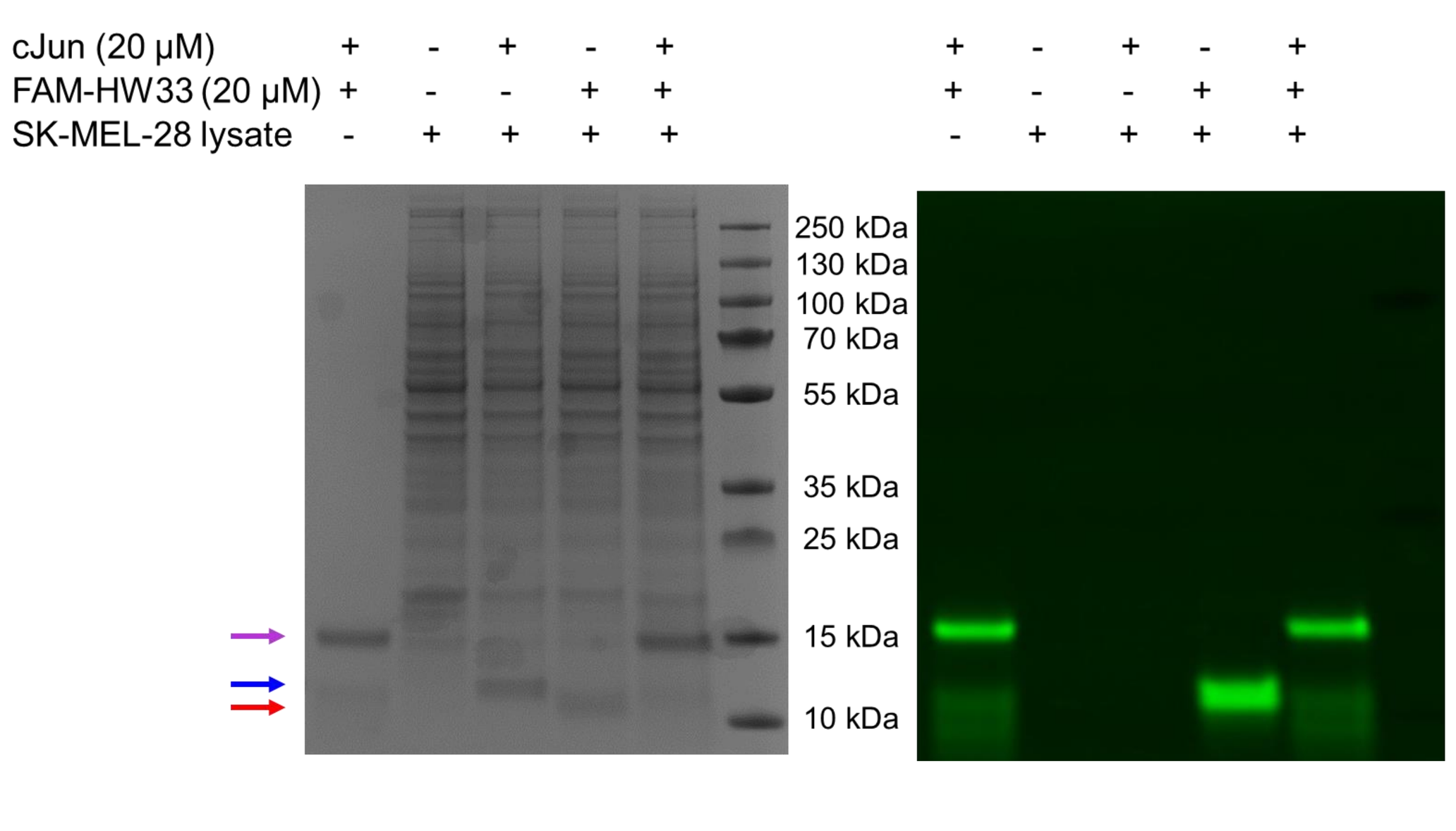
LC-MS analysis of the reaction of HW33 (40 µM) with cJun (20 µM) shows the formation of the conjugate over time.



cJun (20 µM) occupancy by HW33 (10-100 µM), determined by LC-MS, plotted over time and fit to determine the k_{inact}/k_d.

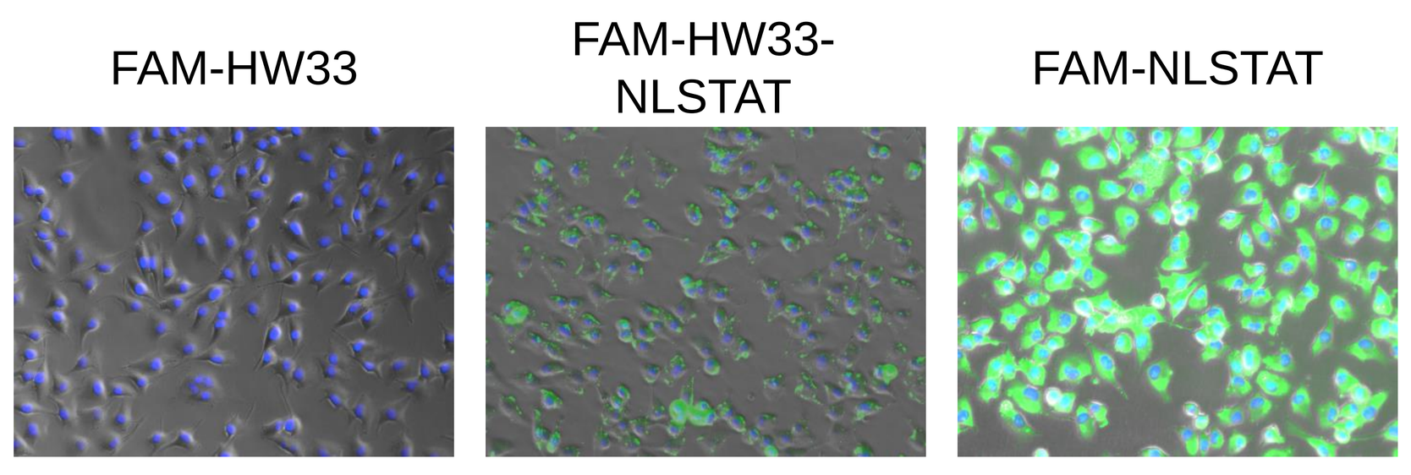


Circular dichroism cJun/TRE antagonism experiment shows an increase in IC_{50} over time as HW33 irreversibly reacts with cJun and prevents it binding to TRE DNA.



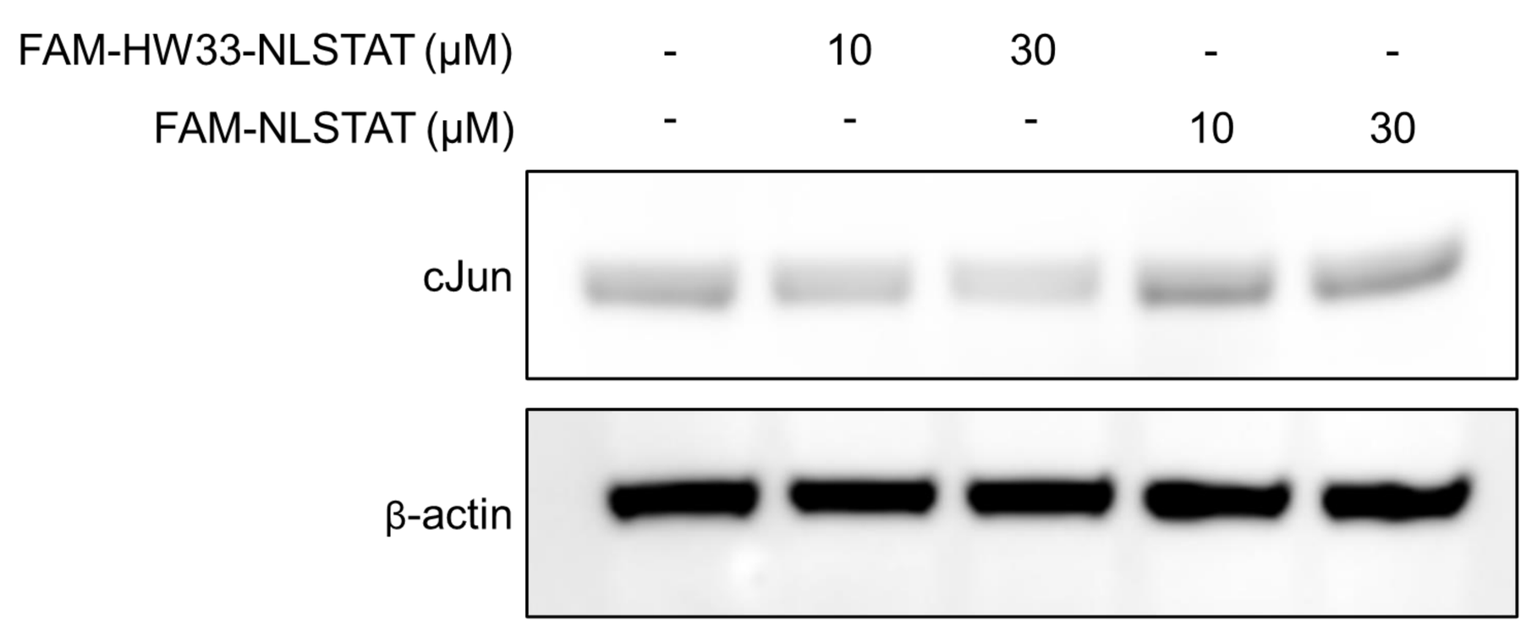
SDS-PAGE analysis of the reaction between cJun (20 µM) and FAM-HW33 (20 µM) after 24 hours incubation in SK-MEL-28 cell lysate which shows the selective reaction with the cJun bZIP.

6. HW33 shows efficacy in melanoma cells via cJun depletion

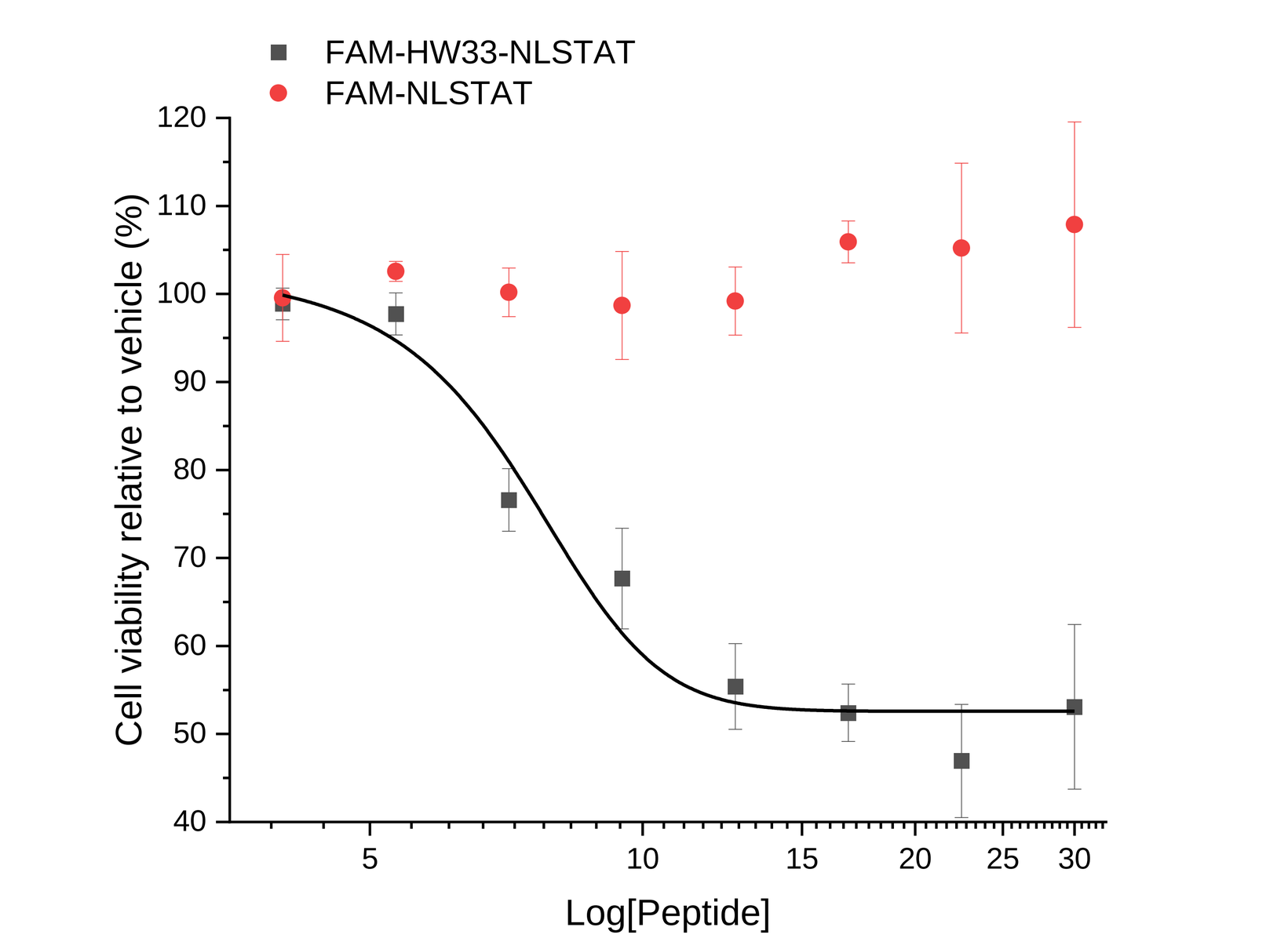


Imaging (overlay of brightfield, DAPI and FAM images) of SK-MEL-28 cells treated with 30 µM peptide for 6 hours at 37°C shows that NLSTAT is required to transport FAM-HW33-NLSTAT into cells, but the cargo reduces cell penetrance.

Western blot: 6 hours

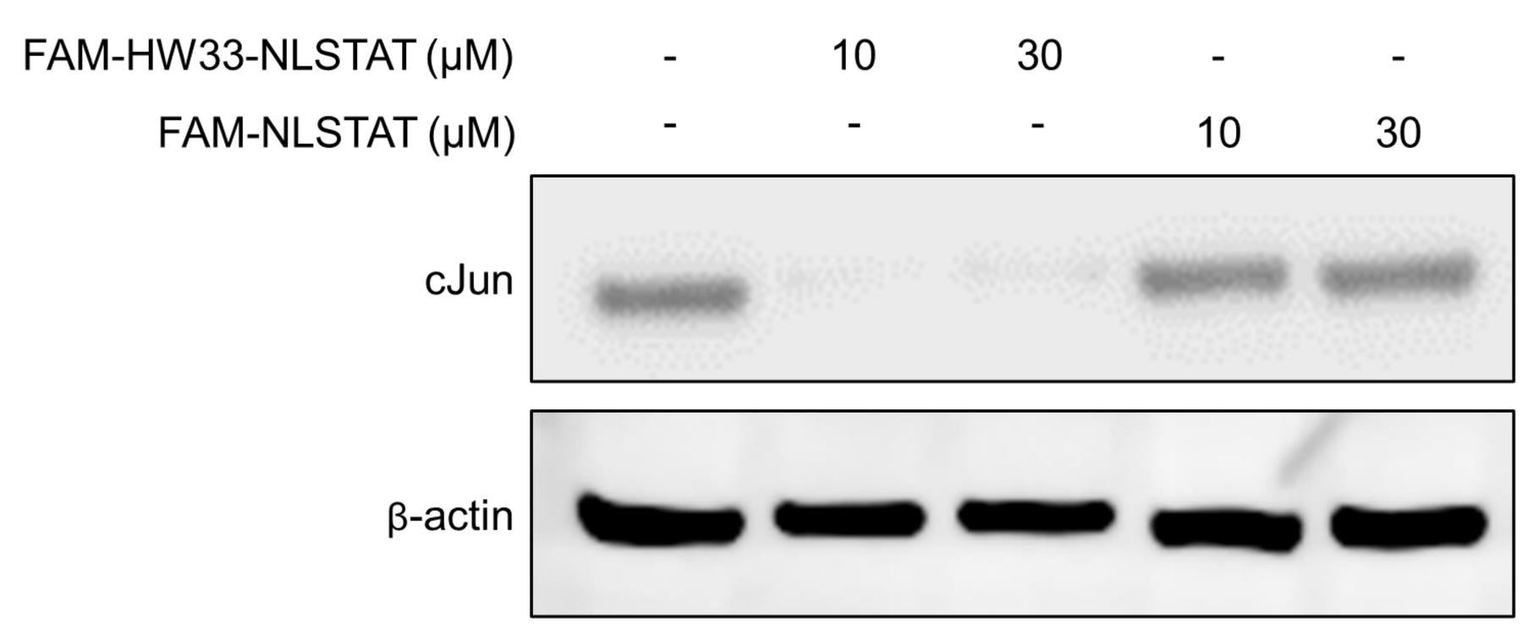


MTT: 24 hours



MTT assay on peptide-treated SK-MEL-28 cells (24 hours) shows a reduction in cell viability upon treatment with FAM-HW33-NLSTAT with an IC_{50} of 7.1 µM but the CPP alone produces minimal effect.

Western blot: 24 hours



Western blot of peptide-treated cells (6 and 24 hours) shows time- and dope-dependent depletion of cJun upon treatment with FAM-HW33-NLSTAT.

7. Conclusions

- TBS correlates growth rate and protein-DNA antagonism, directly competing peptide library members to produce a single assay winner in the complex cellular environment.
- Screening cysteine containing libraries in SHuffle Express *E. coli* allows for the selection of cysteines capable of forming a disulphide with the target protein.
- The selected cysteine position was converted into an irreversible covalent antagonist capable of reducing melanoma cell viability via cJun depletion.

References

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3. Brennan, A., *et al*., *JACS Au*., 2022. **2**(996).
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