

# A BIOINFORMATICS PIPELINE REVEALS A SHARED $I\kappa B\alpha$ INTERFACE FOR NF- $\kappa$ B AND HISTONE BINDING

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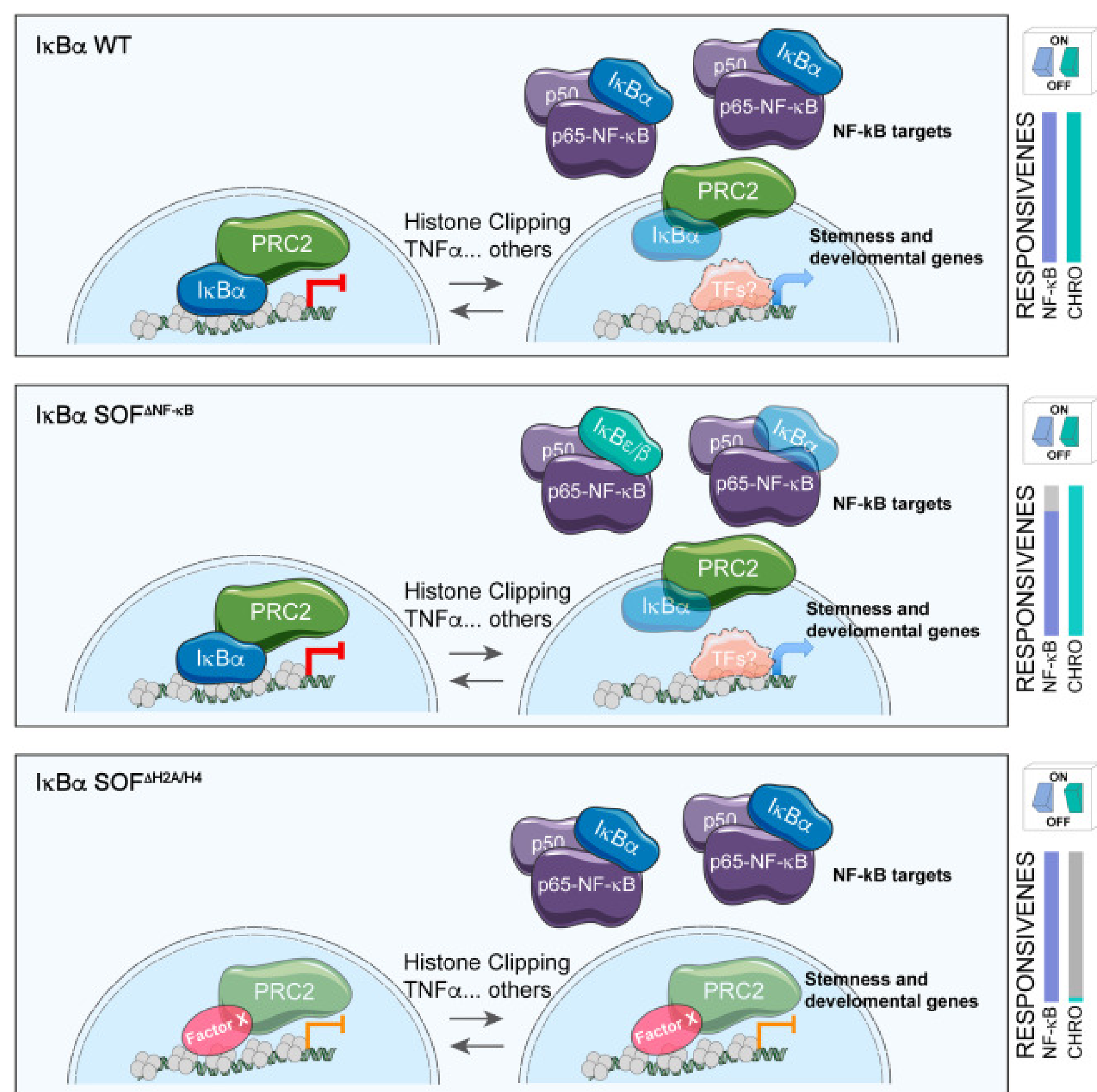
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## SUMMARY

$I\kappa B\alpha$ , traditionally a negative regulator of NF- $\kappa$ B, has recently been linked to chromatin-dependent transcriptional control (Marruecos et al., 2021). We developed a bioinformatics pipeline to identify conserved, surface-exposed residues mediating protein interactions. Our Fold-Excluded Evolutionary Conservation (FEEC) metric integrates structural packing (WCN) and evolutionary conservation (ConSurf) to prioritize  $I\kappa B\alpha$  binding residues. FEEC highlighted an ANK-repeat surface engaging both the p65 NLS and histone H4 tail. Guided by FEEC, we engineered separation-of-function (SOF) mutants disrupting either NF- $\kappa$ B binding ( $SOF^{\Delta NF-\kappa B}$ ) or histone association ( $SOF^{\Delta H2A/H4}$ ). Structural modeling, mutagenesis, and functional assays confirmed their specificity. Transcriptomic analysis of  $SOF^{\Delta NF-\kappa B}$  cells revealed repression of intestinal stem-cell genes independent of NF- $\kappa$ B, underscoring a chromatin-related role for  $I\kappa B\alpha$ . FEEC thus provides a generalizable framework to resolve multifunctional protein interfaces. (Álvarez-Villanueva et al., 2025)



Regulation of NF- $\kappa$ B and PRC2 target genes by  $I\kappa B\alpha$  WT and SOF mutants

## METHODOLOGY

The **Weighted Contact Number (WCN)** quantifies atomic contact density from interatomic distances as

$$WCN_i = \sum_{j \neq i} \frac{1}{r_{ij}^2}, \quad iWCN_i = \frac{1}{WCN_i}.$$

Sequence conservation scores ( $S_{C,i}$ ) were obtained from the CONSURF server and expressed as Z-scores. The **Fold-Excluded Evolutionary Conservation (FEEC)** score identifies residues with conservation beyond structural constraints:

$$FEEC_i = iWCN_i - S_{C,i}.$$

Interface energies were computed from Rosetta-generated conformations weighted by Boltzmann probabilities and expected binding energies:

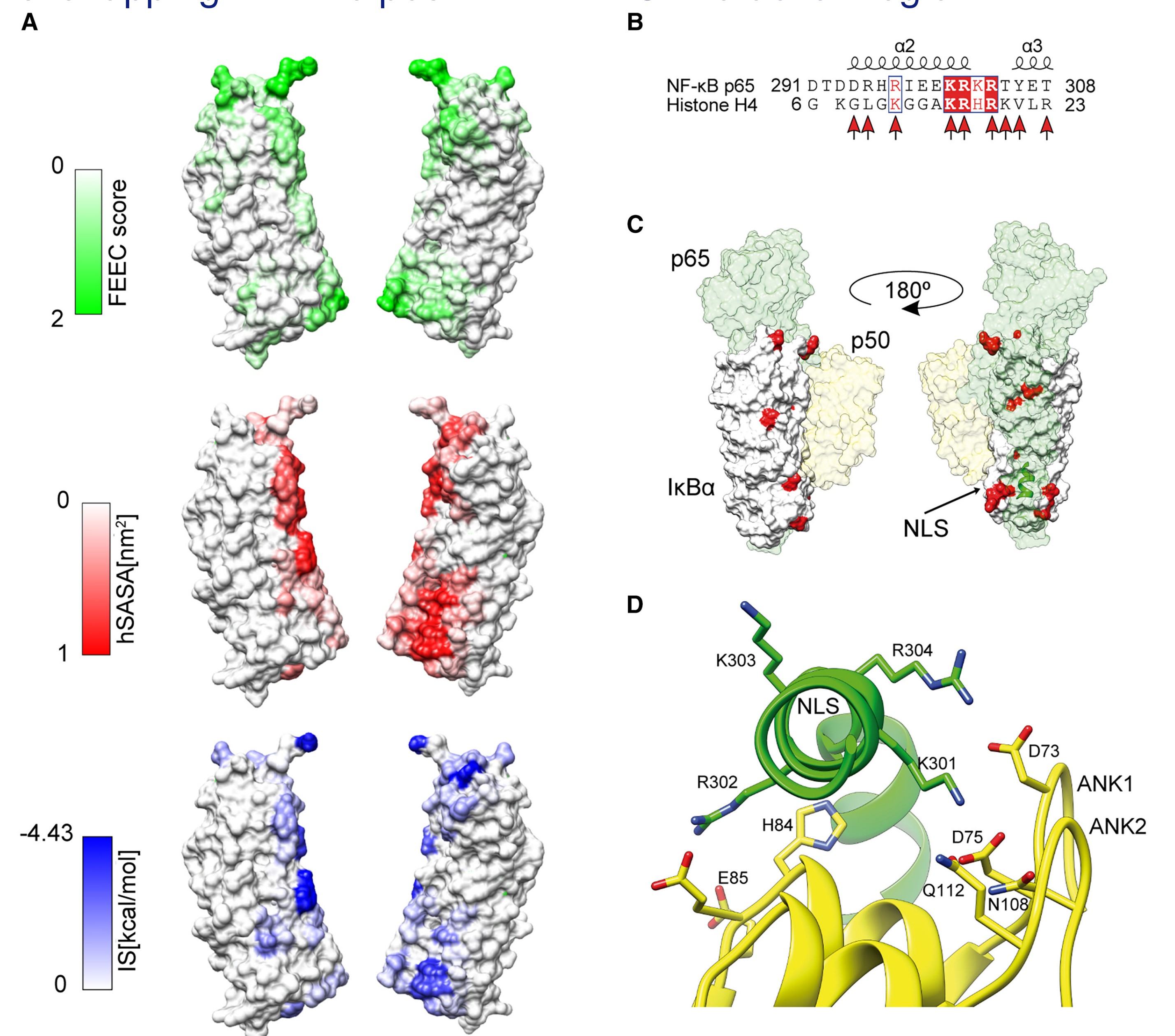
$$P_i = \frac{e^{-E_i/kT}}{\sum_{i=1}^N e^{-E_i/kT}},$$

$$\langle E_{\text{binding}} \rangle = \sum_i P_i E_{b,i}, \quad E_{b,i} = E_{\text{complex},i} - (E_{A,i} + E_{B,i}).$$

Mutational effects were estimated after repacking and minimizing residues within 8 Å of each mutation site.

## COMMON DOMAIN OF $I\kappa B\alpha$ BINDING TO P65-NF- $\kappa$ B AND H2A/H4

FEEC scores accurately predicted most  $I\kappa B\alpha$ /NF- $\kappa$ B interface residues (83% for p65, 73% for p50), validating FEEC as a reliable predictor of binding sites. Mapping positive FEEC residues revealed strong overlap with known interface regions from structural data. Negatively charged FEEC-positive residues in the ANK1-ANK2 domains (e.g., D73, D75, E85, E86) likely form the binding site for the histone H4 N-terminal tail, overlapping with the p65-NF- $\kappa$ B NLS interaction region.



(A) Surface mapping of  $I\kappa B\alpha$  residues with positive FEEC scores (capped at 2), solvent-accessible area buried upon NF- $\kappa$ B binding, and per-residue interface scores (PDB: 1NFI). (B) Alignment of the p65-NF- $\kappa$ B NLS region with the H4 N-terminal tail; conserved positions (blue), similar/identical residues (red/white), and  $I\kappa B\alpha$ -contacting residues (red arrows) are indicated (PDB: 3UW9). (C)  $I\kappa B\alpha$  (white)-NF- $\kappa$ B (p65, green; p50, yellow) complex showing negatively charged  $I\kappa B\alpha$  residues with FEEC>0 (red). (D) Interaction of the p65 NLS motif (KRKR, green) with  $I\kappa B\alpha$  ANK1-2 repeats; polar interacting residues in yellow. UniProt Q04206 (p65) and P25963 ( $I\kappa B\alpha$ ).

## REFERENCES

- Álvarez-Villanueva, Daniel et al. (2025). "Separation-of-function mutants reveal the NF- $\kappa$ B (independent) role of chromatin-bound  $I\kappa B\alpha$  in intestinal stemness and differentiation". In: *Cell Reports* 44.7, p. 115949. doi: 10.1016/j.celrep.2025.115949.
- Marruecos, L. et al. (2021). "Dynamic chromatin association of  $I\kappa B\alpha$  is regulated by acetylation and cleavage of histone H4". In: *EMBO Reports* 22.8, e52649. doi: 10.15252/embr.202152649.