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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	The single-molecule fluorescence imaging data were collected using custom software written in LabVIEW (2015). The predicted structural data were collected using Alphafold2 software provided by https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb.
Data analysis	The custom graphical user interface (GUI) software written in MATLAB (2020a) was used for post-image analysis of the single-molecule fluorescence images. OriginPro (2022) was used for fitting curves and statistics. Visualization of structural information was performed using PyMolwin (4.6.0) software. The protein band intensity from western blot were analyzed with ImageJ (1.53a).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	*Use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design whether sex and/or gender was determined based on self-reporting or assigned and methods used. Provide in the source data disaggregated sex and gender data where this information has been collected, and consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected. Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.*
Population characteristics	*Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*
Recruitment	*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*
Ethics oversight	*Identify the organization(s) that approved the study protocol.*

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The number of single-molecule fluorescence signals from surface-immobilized bait proteins or protein-protein interaction (PPI) complexes were counted from ~12 different locations within each chamber of the imaging chip (Supplementary Fig. 1). The intra-assay coefficient of variance (CV) of single-molecule co-IP were calculated from the three independent dissociation constants (Kd) between BCL2 and BIM splice variants (Supplementary Table 1 and 3).
Data exclusions	To avoid any biased analysis, the images containing either photobleached region or aggregated large dye cluster were excluded after the post-image analysis.
Replication	The PPI reaction for determining the Kd values were repeated independently in three times (Fig. 3 and 4). The in vitro competition for determining the inhibitory constant (Ki) values were performed in two independent replicates (Fig. 4). Replications were successful for all attempts.
Randomization	Randomization was not performed because of the unbiased nature of the in vitro experiments performed in this study.
Blinding	Blinding was not performed because of the unbiased nature of the in vitro experiments performed in this study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
[ ]	[x] Antibodies
[ ]	[x] Eukaryotic cell lines
[x]	[ ] Palaeontology and archaeology
[x]	[ ] Animals and other organisms
[x]	[ ] Clinical data
[x]	[ ] Dual use research of concern

Methods

n/a	Involved in the study
[x]	[ ] ChIP-seq
[x]	[ ] Flow cytometry
[x]	[ ] MRI-based neuroimaging

Antibodies

Antibodies used

In the Methods section, all experimental conditions including the concentrations of materials used in this study are mentioned. Biotin anti-RFP antibody (ab34771; Abcam) was used to immobilize the mCherry-labeled proteins and to detect the aforelisted protein by western blot. Biotin anti-GFP antibody (ab6658; Abcam), MCL1 (94296S; Cell Signaling Technology), BCLxL (sc-56021; Santa Cruz Biotechnology), BCL2 (15071; Cell Signaling Technology), BIM (sc-374358; Santa Cruz Biotechnology), BAD (sc-8044; Santa Cruz Biotechnology), β-Tubulin (2128; Cell Signaling Technology), HRP-linked anti-rabbit IgG (7074S; Cell Signaling Technology), and HRP-linked anti-mouse IgG (7076S; Cell Signaling Technology) antibodies were used to detect the aforelisted proteins by western blot.

Validation

All antibodies are commercially validated and published previously. All informations about antibodies could be found on the manufacturer's guidelines.

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)

HEK293T cells were purchased from the Korean Cell Line Bank.

Authentication

The cell line was authenticated at source and regularly validate by molphological analysis.

Mycoplasma contamination

The cell line was confirmed to be mycoplasma negative.

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified cell lines were used.