nature portfolio

Corresponding author(s): Natalie W. Nairn

Last updated by author(s): Sep 24, 2024

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	
[ ]	[x]	The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
[ ]	[x]	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
[ ]	[x]	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.*
[x]	[ ]	A description of all covariates tested
[ ]	[x]	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
[ ]	[x]	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)
[ ]	[x]	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.*
[x]	[ ]	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
[x]	[ ]	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
[x]	[ ]	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	Flow cytometry took place on Becton Dickson FACSAria III or on Sony SH800S instruments with associated software, BD FACSDiva (version not noted) or Sony Cell Sorter Software v2.1.5. Plate based quantitative experiments (cell viability, serum pharmacokinetic analysis) took place on a Molecular Devices SpectraMax iD3 instrument using SoftMax Pro v7.1. Western blotting took place on a LI-COR Odyssey CLx instrument using Image Studio v5.2.
Data analysis	Data analysis involved Microsoft Excel for Mac v16.83, open-source ImageJ2 v2.14.0/1.54f with Fiji add-on, GraphPad Prism v10.2.0, BD FlowJo v10.10.0, and open-source PyMOL

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

All data associated with this study are present in the paper or the Supplementary Data. Additional information and/or materials related to this study, including

recombinant proteins and datasets, will be made available through a material transfer agreement upon request to the authors.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

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 if 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.*
Reporting on race, ethnicity, or other socially relevant groupings	*Please specify the socially constructed or socially relevant categorization variable(s) used in your manuscript and explain why they were used. Please note that such variables should not be used as proxies for other socially constructed/relevant variables (for example, race or ethnicity should not be used as a proxy for socioeconomic status). Provide clear definitions of the relevant terms used, how they were provided (by the participants/respondents, the researchers, or third parties), and the method(s) used to classify people into the different categories (e.g. self-report, census or administrative data, social media data, etc.) Please provide details about how you controlled for confounding variables in your analyses.*
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.

[x] 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	For flow cytometry experiments, analysis on a given sample was run until 5000 events were collected, corresponding to roughly 2000-4000 viable singlet cells for measurement. In vitro cell growth disruption experiments were performed in technical triplicate per drug concentration at at least 8 drug concentrations to generate the data for an accurate curve fit using at least 24 points. The pilot in vivo studies (Fig. 8) all use three animals per group. Pharmacokinetic analysis used three biological samples per timepoint, each assessed in technical triplicate. We were not designing the experiments to be powered for a given effect size, as this was a pilot study using test articles of unknown activity.
Data exclusions	No data were excluded from analysis.
Replication	All experiments were conducted once or twice. Experiments of a quantitative nature (e.g., flow cytometry, cell growth disruption) that were performed once involve interventions that were tested in other experiments or contexts, either in this dataset or unpublished, that produced consistent results.
Randomization	For the flank tumor xenograft study, tumors were implanted, and upon initiation of dosage, animals were randomized between treatment groups for equivalent average tumor mass.
Blinding	Investigators were blinded to test article identity for the PK study, while the tumor xenograft study was unblinded. Quantitative analyses (PK, Western blot quantitation, Ki67 positivity) from which we determined whether a pharmacodynamic effect is apparent all use objective measures not prone to unconscious bias.

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
[x]	Plants

Methods

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

Antibodies

Antibodies used	Primary antibodies for surface protein staining were as follows: EGFR, clone 199.12 (ThermoFisher MA5-13319); TfR, clone OKT9, APC-labeled (ThermoFisher 17-0719-42); PD-L1, clone 22C3 (Agilent M365329-1). Primary antibodies for Western blotting are as follows: rabbit anti-EGFR (Cell Signaling Technology 2646); rabbit anti-phospho-Y1068 EGFR (Cell Signaling Technology 3777); goat anti-actin (Abcam ab8229). Secondary antibodies or co-stains were as follows: Alexa Fluor 647-conjugated streptavidin (surface display staining other than TfR human/mouse cross-reactivity, ThermoFisher S21374); iFluor 647-conjugated anti-His-tag antibody (pilot CYpHER detection and TfR human/mouse cross-reactivity, Genscript A01802); Alexa Fluor 647-conjugated anti-Fc Fab (Surface protein and CYpHER quantitation, ThermoFisher Zenon Labeling Kits, mouse IgG2a for anti-EGFR 199.12 quantitation [ThermoFisher Z25108], mouse IgG1 for anti-PD-L1 22C3 quantitation [ThermoFisher Z25008], human IgG for CYpHER quantitation [ThermoFisher Z25408]); iFluor 647-conjugated sAvPhire monovalent streptavidin (catalytic soluble protein uptake, Millipore Sigma SAE178-100UG); iFluor 488-conjugated sAvPhire monovalent streptavidin (catalytic soluble protein uptake, Millipore Sigma SAE176-100UG); IRDye 680RD Donkey anti-goat (Western blotting, LI-COR 926-68074); IRDye 800CW Donkey anti-rabbit (Western Blotting, LI-COR 926-32213).
Validation	Each commercial antibody has a validation statement at the vendor's website. This includes, but is not limited to, IP-MS (ThermoFisher MA5-13319), siRNA knockdown (ThermoFisher 17-0719-42), or immunoprecipitation (Cell Signaling Technology 2646). Additional internal validation for clone 199.12 (anti-EGFR) and clone 22C3 (PD-L1) was performed to verify the absence of competition with test articles; clone OKT9 (TfR) is heavily published and known to bind distal to the transferrin binding site, where our TfR-binding domains are shown by crystallography to interact.

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	Cell lines were sourced directly from vendors ATCC or Sigma Aldrich.
Authentication	Authentication was not performed internally, instead relying on the vendor's verification of authenticity.
Mycoplasma contamination	Lines were not tested for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	None of the cell lines used in this study are among the commonly misidentified lines.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	For the pharmacokinetic experiment, 8 to 10 week old female NCr nu/nu mice were used. For the tumor growth study, 6 week old female athymic nude mice (Foxn1nu), purchased from Inotiv Laboratories (#069), were used.
Wild animals	No wild animals were used.
Reporting on sex	Females were used due to the practical utility of being able to re-house the mice without substantial stress (which can impact tumor biology), and for the athymic nude mice also because the breeding scheme requires homozygous males for propagation, so reservation of homozygous males for breeding is commonplace.
Field-collected samples	Studies used no field collected samples.
Ethics oversight	All mice were maintained in accordance with the National Institutes of Health Guide for the Care of Laboratory Animals with approval from the Seattle Children's Research Institute, Institutional Animal Care and Use Committee (protocol ACUC00682).

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

Plants

Seed stocks

Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.

Novel plant genotypes

Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.

Authentication

Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.

Flow Cytometry

Plots

Confirm that:

- [x] The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- [x] The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- [x] All plots are contour plots with outliers or pseudocolor plots.
- [x] A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

For mammalian surface display, 293F cells (ThermoFisher R79007) were grown in FreeStyle 293 expression medium (ThermoFisher 12338018) in 37°C, 8% CO2 humidified shaking incubators. Proteins were surface displayed via transient transfection (singleton testing) or lentiviral transduction (pooled screening) using vector SDGF, in which displayed proteins have a free C-terminus, or a variant thereof where the displayed protein has a free N-terminus and is connected to C-terminal GFP by a Type 1 transmembrane domain derived from human CD28. The parental C-terminal display vector was used for experiments involving CDPs (including EGF and variants thereof), while the N-terminal display variant was used for experiments involving VHH nanobodies. General growth, transfection, staining, sorting, and data interpretation methods were previously published. Staining either took place with monovalent (TfR-binding CDP or PD-L1-binding CDP work) or tetravalent (VHH nanobody or EGF variant work) protocols, with binder concentrations varying depending on the assay: 100 nM for diversity library screening (Primary EGF Rosetta variant library), 20-100 nM for maturation (EGFd1.5 affinity maturation, VHH nanobody His-doped variant library, PD-L1 binder pH maturation), and 10-50 nM for singleton validation stains. Testing for pH-dependent release involved the conventional staining protocols, but after target protein incubation, cells were pelleted and resuspended in cold pH 7.4 PBS or pH 5.5 citrate-phosphate saline buffer for 5 mins, followed by pelleting at 500xg for 5 mins (combined 10 mins incubation). Cells were then resuspended in buffer for the next step (fluorescent co-stain for monovalent staining protocols, Flow Buffer [PBS with 0.5% bovine serum albumin and 2 mM EDTA] for tetravalent staining protocols). Flow cytometry took place on Becton Dickson FACSAria III or on Sony SH800S instrumentation.

For cancer cell line and primary keratinocyte surface protein flow cytometry, cell lines were grown by conventional adherent cell culture in 37°C, 5% CO2 humidified incubators using DMEM + 10% FBS and 1x antibiotic/antimycotic (293T-EGFR-GFP, MDA-MB-231-PD-L1-GFP, A431) or RPMI + 10% FBS and 1x antibiotic/antimycotic (A549, H1975, H1650, H1650-PD-L1-GFP, H358). Cells were lifted by removing media, rinsing with room temperature PBS, and incubating with TrypLE Express (ThermoFisher 12605036) until detachment. For staining, following detachment, enzyme was inhibited with complete culture medium, and cells were pelleted at 500xg for 5 min. Cells were resuspended in cold Flow Buffer; for surface EGFR or TfR quantitation, the buffer contained either 10 nM primary antibody (either conjugated with Alexa Fluor 647 [anti-TfR] or pre-labeled with Zenon labeling kit according to manufacturer's instructions [anti-EGFR]) or an equivalent volume of a mock labeling reaction (using Zenon labeling kit reagents but with flow buffer in place of primary antibody); for surface CYpHER detection, Zenon human IgG detection reagent was added as if it were a primary antibody to 10 nM. Cells were incubated in this staining solution on ice for 30 mins, pelleted at 500xg for 5 mins, and resuspended in fresh, cold Flow Buffer containing 1 µg/mL DAPI immediately prior to flow analysis.

Instrument

Flow cytometry took place on Becton Dickson FACSAria III or on Sony SH800S instrumentation.

Software

Software for flow data collection was BD FACSDiva (version not noted) or Sony Cell Sorter Software v2.1.5. Data analysis involved Microsoft Excel for Mac v16.83, BD FlowJo v10.10.0, and GraphPad Prism v10.2.0.

Cell population abundance

All analysis and sorting used the primary cell population, gating out only cellular debris (based on FSC/SSC profile), doublet (based on FSC-H/FSC-W profile), and dead cells (DAPI+). The vast majority of samples have >70% events make it through these gates.

Gating strategy

Raw data is analyzed by FSC/SSC profile, avoiding events that are either FSC-low or have FSC or SSC levels far in excess of the primary non-debris population. Singlets are identified by events whose FSC-W/FSC-H levels are within a tight square gate that

excludes high-FSC-H/low-FSC-W or high-FSC-W/low-FSC-H events. Viable cells are then gated by excluding events with aberrantly-high DAPI stain, with DAPI+ events always falling within one of two distinct populations corresponding to 2N or 4N chromatin levels. Finally, autofluorescent outliers are eliminated in an APC vs GFP plot. Further gating for GFP or RFP positivity can take place for study of subpopulations where relevant.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.