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Protocol

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A nanobody-based strategy for rapid and scalable purification of human protein complexes

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Abstract

The isolation of proteins in high yield and purity is a major bottleneck for the analysis of their three-dimensional structure, function and interactome. Here, we present a streamlined workflow for the rapid production of proteins or protein complexes using lentiviral transduction of human suspension cells, combined with highly specific nanobody-mediated purification and proteolytic elution. Application of the method requires prior generation of a plasmid coding for a protein of interest (POI) fused to an N- or C-terminal GFP or ALFA peptide tag using a lentiviral plasmid toolkit we have designed. The plasmid is then used to generate human suspension cell lines stably expressing the tagged fusion protein by lentiviral transduction. By leveraging the picomolar affinity of the GFP and ALFA nanobodies for their respective tags, the POI can be specifically captured from the resulting cell lysate even when expressed at low levels and under a variety of conditions, including detergents and mild denaturants. Finally, rapid and specific elution of the POI (in its tagged or untagged form) under native conditions is achieved within minutes at 4 °C, using the engineered SUMO protease SENP^{EuB}. We demonstrate the wide applicability of the method by purifying multiple challenging soluble and membrane protein complexes to high purity from human cells. Our strategy is also directly compatible with many widely used GFP-expression plasmids, cell lines and transgenic model organisms. Finally, our method is faster than alternative approaches, requiring only 8 d from plasmid to purified protein, and results in substantially improved yields and purity.

Key points

- The protocol describes the lentivirus-based expression of high amounts of soluble and membrane-bound tagged proteins of interest (POI) in human suspension cells. This is combined with rapid nanobody-based purification of the POI and its complexes for downstream structural analysis and functional assays.
- The protocol provides guidelines for the isolation of a POI in its tagged (TagON) and scarless untagged (TagOFF) forms using an engineered SUMO protease (SENP^{EuB}).

Key references

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Introduction

A key prerequisite for many basic and pharmacological applications is the preparation of highly pure protein samples. Sample quality underlies the success of diverse experimental techniques including structural approaches (e.g., cryo-electron microscopy (cryo-EM) and X-ray crystallography), proteomics (e.g., interactome studies) and high-throughput studies of protein function (e.g., biophysical assays or in vitro drug screens).

Human proteins can be notoriously difficult to express and purify at the scale and purity needed for their structural and functional characterization. Their heterologous expression (e.g., in bacteria) frequently results in extensive degradation, formation of insoluble aggregates or lack of essential posttranslational modifications. These problems are exacerbated for multisubunit protein complexes and membrane proteins, which may rely on mammalian-specific factors for their biogenesis. Mammalian expression systems are thus, in many cases, the only alternative. However, culturing mammalian cells can be costly, making it essential to streamline both expression and protein purification workflows, to maximize yield and purity while saving valuable time and resources.

Here, we present a rapid and easily scalable workflow to purify soluble and membrane-spanning human proteins and protein complexes from human suspension cells for their structural and functional analysis. Our workflow combines advances in both expression cell line generation[1] and purification strategy[2], and employs two highly versatile and complementary affinity tags, GFP and the small ALFA peptide tag[3].

First, we describe how to rapidly generate stable polyclonal human suspension cell lines expressing a GFP- or ALFA–tagged protein of interest (POI) using lentiviral transduction. The use of lentivirus combines multiple advantages of both transient transfection and monoclonal cell line generation[1]. In fact, lentivirus allows users to quickly generate stable cell lines within only a few days, using lentivirus produced from a single well of a six-well plate. Also, it enables highly tunable expression: transduction efficiencies can be titrated to either >80–90% to maximize yield, or to <30% to ensure a single copy of cDNA per cell. When coupled with fluorescence-activated cell sorting (FACS), polyclonal cell lines can be generated that contain uniform expression across all cells. Finally, lentivirus ensures reproducible and scalable expression, because once generated, cell lines can be stored or expanded indefinitely. To streamline the cloning of suitable expression plasmids, we provide an extensive plasmid toolbox (Table 1), an accompanying cloning guide (Supplementary Data 1) and general recommendations for GFP/ALFA tag placement.

Second, we present a nanobody (Nb)-based strategy for the isolation of the tagged POI under native conditions, which ensures higher yield and purity than canonical epitope tag-based approaches. This is achieved through the combination of high-affinity binding to anti-GFP or ALFA nanobodies with selective elution by an engineered SUMO protease ($SENP^{EuB}$)[4]. $SENP^{EuB}$ allows nearly 1,000-fold faster release of resin-bound proteins than tobacco etch virus (TEV) or 3C proteases, and elution can therefore be performed quickly under gentle conditions on ice. Rapid and selective elution of Nb-captured proteins ensures high sample purity and preserves delicate protein complexes. We provide details on bacterial expression plasmids and protocols to inexpensively produce both nanobodies and protease in amounts sufficient for hundreds of purifications.

Through the combination of lentivirus-based cell line generation and Nb-mediated affinity purification, our protocol reduces the time and costs required to prepare high-quality protein samples. Together, these strategies can therefore substantially accelerate the structural and functional analysis of otherwise difficult-to-express human protein complexes.

Applications

One advantage of this protocol is that the Nb-based purification strategy is highly flexible and modular. It can be used for purification of proteins or protein complexes under a variety of conditions, and from lysates prepared from various eukaryotic or prokaryotic sources. In particular, utilizing the anti-GFP Nb for protein capture, our method can leverage the vast

Table 1 | TagON- and TagOFF-compatible lentiviral transfer plasmid toolbox and *E. coli* expression plasmids

Plasmid	Open reading frame	Addgene ID
pTS093	GFP–22xGS–MCS	199346
pTS094	GFP–22xGS–3C–MCS	199347
pTS095	GFP–22xGS–TEV–MCS	199348
pTS096	GFP–22xGS–SUMO^{Eu}–MCS	199349
pTS097	GFP–22xGS–SUMOstar–MCS	199350
pTS098	TagBFP–P2A–ALFA–22xGS–MCS	199351
pTS099	TagBFP–P2A–ALFA–22xGS–3C–MCS	199352
pTS100	TagBFP–P2A–ALFA–22xGS–TEV–MCS	199353
pTS101	TagBFP–P2A–ALFA–22xGS–SUMO^{Eu}–MCS	199354
pTS102	TagBFP–P2A–ALFA–22xGS–SUMOstar–MCS	199355
pTS103	MCS–22xGS–GFP	199356
pTS104	MCS–3C–22xGS–GFP	199357
pTS105	MCS–TEV–22xGS–GFP	199358
pTS106	MCS–3C– SUMO^{Eu}–22xGS–GFP	199359
pTS107	MCS–TEV– SUMO^{Eu}–22xGS–GFP	199360
pTS108	MCS–3C–SUMOstar–22xGS–GFP	199361
pTS109	MCS–TEV–SUMOstar–22xGS–GFP	199362
pTS110	MCS–22xGS–ALFA–P2A–TagBFP	199363
pTS111	MCS–3C–22xGS–ALFA–P2A–TagBFP	199364
pTS112	MCS–TEV–22xGS–ALFA–P2A–TagBFP	199365
pTS113	MCS–3C–SUMO^{Eu}–22xGS–ALFA–P2A–TagBFP	199366
pTS114	MCS–TEV– SUMO^{Eu}–22xGS–ALFA–P2A–TagBFP	199367
pTS115	MCS–3C–SUMOstar–22xGS–ALFA–P2A–TagBFP	199368
pTS116	MCS–TEV–SUMOstar–22xGS–ALFA–P2A–TagBFP	199369
pTP341	TagBFP–3xFLAG (constitutive)	199391
pTP924	GFP (constitutive)	199392
pTP396	His_{14}–Avi–27xGS–SUMO^{Eu}–19xGS–anti GFP Nb	149336
pTS117	His_{14}–Avi–45xGS–anti GFP Nb	199370
pTP298	His_{14}–Avi–27xGS–SUMO^{Eu}–2xGS–anti ALFA tag Nb	199390
pTS118	His_{14}–Avi–28xGS–anti ALFA tag Nb	199371
pAV0286	His_{14}–TEV–SENP^{EuB} protease	149333
pTP264	His_{14}–*bd*NEDD8–BirA	149334

All plasmids are available from Addgene for use in academic research. 3C, human rhinovirus 3C protease recognition site; GS, glycine/serine-rich spacer of indicated length in aa; MCS, multiple cloning site; TEV, tobacco etch virus protease recognition site.

number of existing sources for GFP-tagged proteins (e.g., yeast, flies, worms and mice)[5–10]. Publicly available and commercial sources are enumerated in Supplementary Table 1. Further, the Nb–affinity tag pair can easily be exchanged to other natural, laboratory-evolved or computationally designed binding proteins, as well as protein-specific binders[11,12], making the system highly adaptable. A selection of potential options are listed in Supplementary Table 2 (refs. 13–21). We therefore envision that this strategy will be suitable for any structural or functional application requiring protein purification from cells, extract or tissue.

One particularly powerful application of this protocol is generating samples for structural analysis, which requires large-scale production of highly pure samples[2,22]. For example, we have used this strategy to purify the nine-subunit human endoplasmic reticulum (ER) membrane protein complex (EMC), for structure determination using single-particle cryo-EM[2]. For the EMC, and many other protein complexes, we have found that expression of a single tagged subunit results in its incorporation into the intact complex in place of the endogenous subunit. Any excess unassembled subunits are typically degraded by the ubiquitin–proteasome

pathway, as has been previously described[23,24]. Therefore, by exploiting endogenous protein quality control machinery in human cells, it is often possible to introduce a tag into a protein complex without expressing multiple subunits or first generating knockout cell lines.

Similarly, our protocol can be used to generate highly pure samples for functional assays (e.g., to characterize a protein's enzymatic activity, stability or DNA-/RNA-/lipid-binding properties). We recently used it to isolate the 33 kDa mitochondrial outer membrane insertase MTCH2 to reconstitute its protein-insertion activity in vitro[25].

Further, because of the high specificity, efficient capture and rapid elution, this protocol is also well suited for analysis of physical interaction partners by mass spectrometry (K. Page, V.N.N. and T.P., unpublished observation).

Overview of the procedure

After cloning of the POI into a lentiviral transfer plasmid as a GFP- or ALFA-tag fusion (Supplementary Data 1), our protocol comprises three main stages (Fig. 1) that can be carried out in parallel and can be summarized as follows:

- Recombinant expression of anti-GFP or ALFA nanobodies and protease in *Escherichia coli* followed by Ni^{2+}-chelate affinity purification (Part 1, Steps 1–34)
- Generation of lentivirus and transduction of human suspension cells, to stably integrate the desired open reading frame (Part 2, Steps 35–66)
- Nb-mediated purification of the POI (in its tagged or untagged form) from the expanded suspension cell culture (Part 3, Steps 67–89)

In its fastest format, our procedure can be completed in only 8 d; however, the daily workload can be reduced by using the multiple pause points highlighted in the 'Procedure' section. In addition, some steps are marked as 'optional' and performing them will require additional time. However, researchers should consider implementing them as quality control procedure for critical reagents or to obtain higher final sample yield (sorting) and homogeneity (size-exclusion chromatography (SEC)).

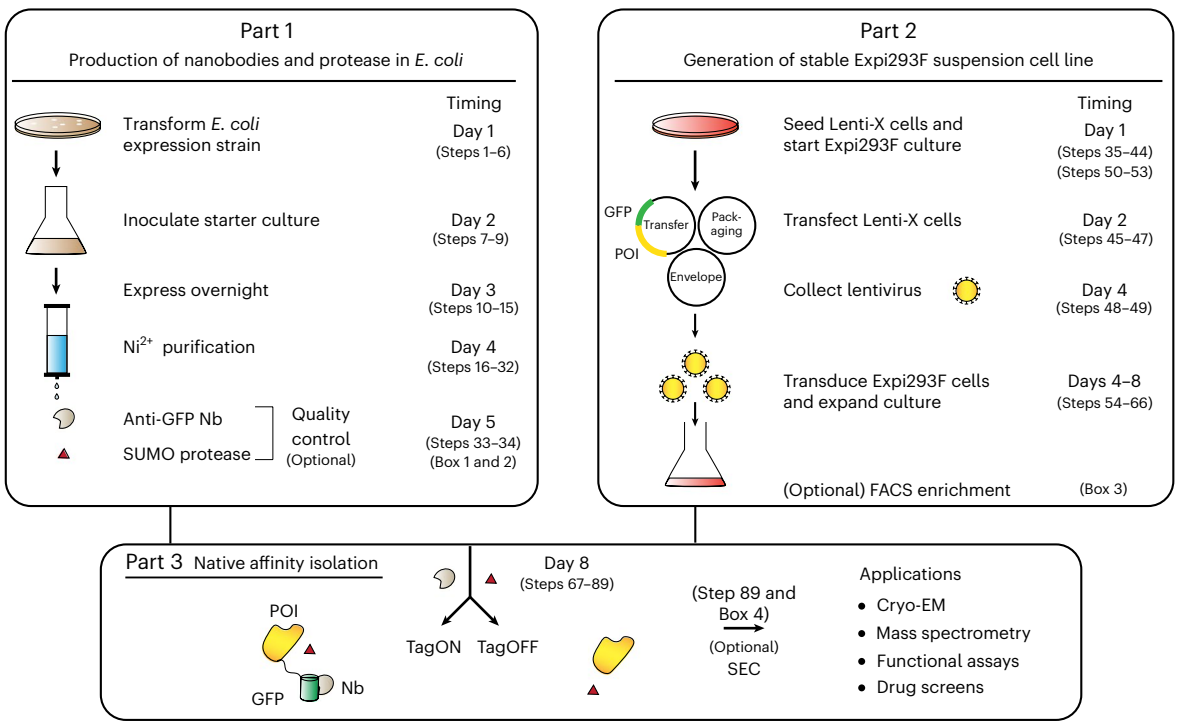

Fig. 1 | Schematic overview of the protocol. Anti-GFP or ALFA Nb-based capture agents, SUMO protease and biotin ligase BirA are separately expressed in *E. coli* and purified via Ni^{2+}–chelate affinity chromatography (left, Part 1). The lentivirus encoding a GFP- or ALFA-tagged POI is generated and used to transduce human Expi293F suspension cells (right, Part 2). The resulting stable cell line is expanded and either directly used for first small-scale purification trials (bottom, Part 3) or sorted using FACS (optional step, Box 3). In its fastest format, our protocol can be completed in only 8 d, going from plasmid to purified protein.

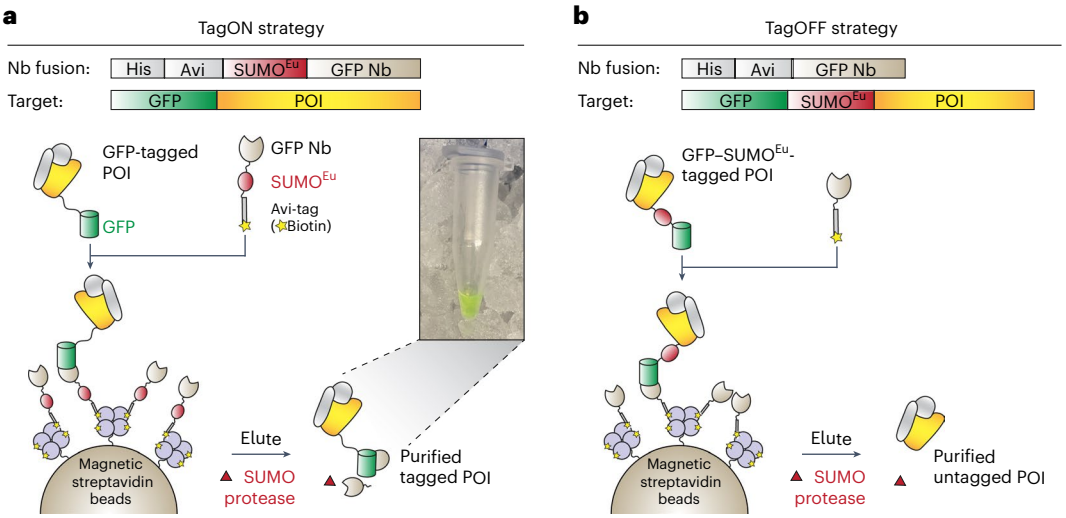

Fig. 2 | Rapid isolation of GFP- or ALFA-fused proteins in tagged (TagON) or untagged (TagOFF) form. a, Schematic of the TagON strategy. A biotinylated SUMO^{Eu}-fused anti-GFP Nb is immobilized onto magnetic streptavidin beads. Nb-decorated beads are then incubated with cell lysate containing an expressed GFP-tagged POI. Following wash steps, GFP-tagged POI and bound interaction partners (gray) are rapidly eluted by cleavage with the engineered SUMO protease SENP^{EuB}. The inset depicts the result of a high-yield TagON purification (GFP–AE2, Fig. 5c). **b**, Schematic of the TagOFF strategy. SUMO^{Eu} is placed between the GFP-tag and the POI, and the POI is captured by a noncleavable Nb. This allows scarless tag-free elution of the POI by SENP^{EuB}.

Two purification strategies: TagON and TagOFF

We present two different strategies to purify a GFP- or ALFA-tagged protein in either a tagged (TagON) or scarless untagged form (TagOFF) (Fig. 2). Both strategies employ biotinylated anti-GFP or ALFA nanobodies that are immobilized onto streptavidin beads to capture GFP- or ALFA-tagged proteins from cell lysate. In the TagON strategy (Fig. 2a), a SUMO^{Eu} module is inserted between the biotin acceptor peptide (Avi) tag and Nb, so that SENP^{EuB} cleavage releases the Nb along with its bound protein. The eluted protein therefore retains the desired tag, which may be useful for detection during downstream applications, such as fluorescence detection SEC[26]. In the TagOFF strategy (Fig. 2b), a SUMO^{Eu} module is placed in between GFP/ALFA tag and the protein so that cleavage by SENP^{EuB} only releases the protein while both Nb and tag are retained on the resin. This strategy is useful if the presence of the tag interferes with downstream applications.

Comparison with other methods

The advantages of this purification protocol over existing methods stem primarily from combining two innovations.

First, we achieve higher sample purity by using highly specific nanobodies and affinity beads. Our approach exploits the picomolar affinity between the nanobodies and their epitope tags to selectively capture tagged proteins from cell lysate. Additionally, both the Nb fusion proteins and affinity resin are fully orthogonal to eukaryotic and prokaryotic host proteins. For example, the Nb-decorated streptavidin beads are passivated by blocking any excess binding sites with free biotin or charged PEGylated biotin derivatives. Therefore, in contrast to the TwinStrep:Streptactin system[27], our strategy does not capture endogenous biotinylated proteins.

Second, rapid and selective protease elution of Nb-captured proteins additionally ensures high sample purity and quality. In contrast, commonly used epitope tag-binding monoclonal antibodies (e.g., FLAG, HA, Myc or V5), rely on native elution using excess epitope peptide. This is often highly inefficient and requires prolonged incubation at room temperature (RT; 23–25 °C), which releases background binders, causes aggregation of fragile proteins

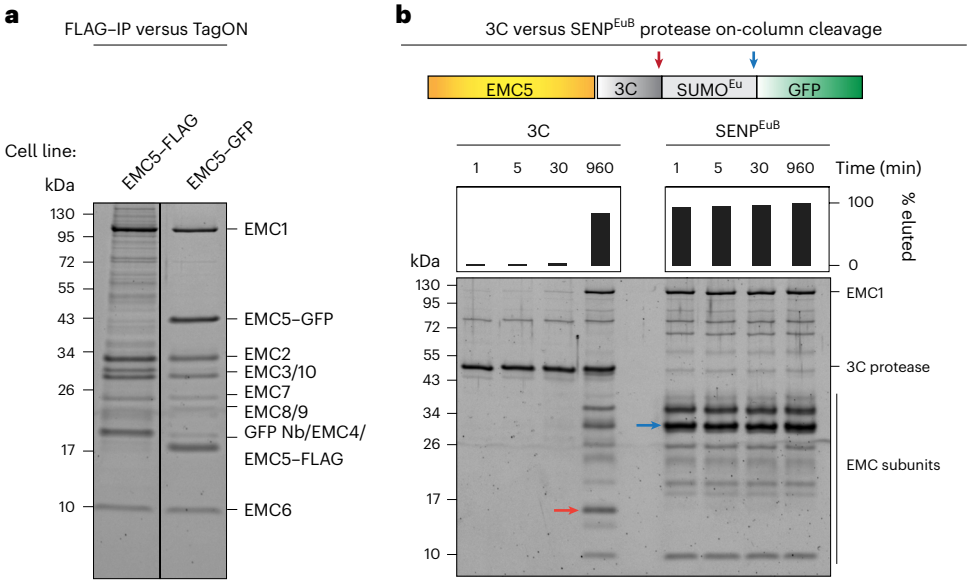

Fig. 3 | Comparison with other methods. a, Comparison of FLAG and TagON purification of the EMC. Elutions from either EMC5–FLAG (using M2 FLAG affinity resin (Millipore–Sigma)) or EMC5–GFP (using TagON) purifications performed at equivalent scale were analyzed by SDS–PAGE and Sypro Ruby staining. The results from this experiment demonstrate a strong reduction of the presence of contaminating proteins when using the TagON strategy compared with a traditional FLAG peptide elution. **b**, Comparison of 3C and SENP^{EuB} protease on-column cleavage efficiency. Purification of EMC from a EMC5–3C–SUMO–GFP expressing cell line (using the TagOFF strategy). The cell lysate was incubated with magnetic streptavidin beads, containing immobilized noncleavable biotinylated anti-GFP Nb (expressed from pTS117). After washing, beads were split in half and incubated either with 2 µM 3C or 250 nM SENP^{EuB} protease at 4 °C for the indicated time frames. Under these conditions, SENP^{EuB} cleaves nearly 1,000-fold faster, allowing for rapid elution in just 1 min as opposed to lengthy overnight incubations typically required for elution with 3C or TEV protease. The products of 3C and SENP^{EuB} cleavage are marked with red and blue arrows, respectively.

or dissociation of weaker binding partners. A side-by-side comparison shows that EMC isolated from an EMC5–GFP cell line using SENP^{EuB} cleavage is substantially purer than EMC isolated from an EMC5–3×FLAG cell line using traditional anti-FLAG purification (Fig. 3a). The combination of specific nanobodies, passivated affinity resin and protease elution therefore yields high final sample purity.

Protease cleavage is thus a powerful alternative over inefficient competitive peptide elution. However, common proteases, such as human rhinovirus 3C protease or TEV protease, inefficiently cleave resin-bound proteins, especially on ice. Often, large amounts of contaminating protease and lengthy overnight incubations (>13 h) are needed to circumvent these issues. In contrast, low nanomolar concentrations of SENP^{EuB} are sufficient to gently release resin-bound proteins on ice within a few minutes (Fig. 3b).

Through both innovations, our protocol thus improves the signal-to-noise ratio of affinity purifications and better preserves sensitive protein complexes and their transient interaction partners. This yields fewer false interactors for affinity purification mass spectrometry experiments and should also reduce contaminants that may interfere with functional experiments.

Limitations

One limitation of this approach is that the ectopic expression of a protein complex subunit can, in some cases, result in its purification in excess over other endogenous complex members. This especially affects subunits that are stable in the unassembled state. Frequently, these excess subunits can be separated by an additional SEC purification step. However, multiple preventative measures can improve protein complex yield and stoichiometry. One strategy could involve the use of Freestyle 293-F suspension cells instead of Expi293F cells. Indeed, we observed that overexpression of unassembled subunits is more limited in these cells, probably

due to tighter regulation by the ubiquitin–proteasome system (unpublished observation, T.A.S. and T.P.). However, Freestyle 293-F cells cannot be grown to as high density as Expi293F cells, decreasing yield per liter of expression. As our lentiviral vectors are regulated by doxycycline-inducible promoters, the expression level of the POI can also be fine tuned by varying induction time in Expi293F TetR+ cells. Alternatively, the expression level can be reduced by exchanging the strong cytomegalovirus (CMV) promoter for a weaker one[28] and/or by removing the expression-enhancing woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) element[29]. If the POI is membrane bound, it can be beneficial to first enrich a membrane fraction to remove excess nonincorporated and aggregated subunits in the cytosol. Purification from the solubilized membrane fraction can therefore yield higher sample quality and better complex stoichiometry.

If these approaches do not yield the expected outcome, it is possible to overexpress multiple subunits in parallel. This can be achieved via cotransduction with multiple distinct lentiviral particles, via fusion of multiple subunits into a single expression plasmid using P2A sites[30] or via a combination of the first two options. The efficiency of dual or triple transduction is typically lower than transduction with a single lentivirus. Multicolor sorting via FACS is therefore required to obtain a fully transduced cell line. Our lentiviral transfer plasmid toolbox already allows compatible GFP and blue fluorescent protein (BFP) expression plasmids to be generated. A third compatible plasmid can easily be generated by introducing mCherry.

Another limitation to consider is that the constitutive ectopic expression of a protein can be toxic or reduce cellular fitness. In a heterogeneous cell population containing untransduced cells, this can lead to a gradual loss of more slowly growing transduced cells. In such cases, the use of the inducible expression system is likely to alleviate the negative selection pressure resulting from the constitutive expression of a toxic protein. Alternatively, successfully transduced cells can be sorted via FACS to remove faster-growing untransduced cells.

The yield of lentiviral particles decreases sharply with increasing transfer plasmid size, resulting in strongly reduced titers for transfer plasmids encoding large proteins (e.g., inserts between 5′ and 3′ long-terminal repeats (LTRs) above 8 Kbp). After subtraction of essential elements, this leaves ~5 Kbp for the GFP- or ALFA-tagged POI (~180 kDa). If necessary, removal of the WPRE element can yield an extra ~600 bp.

Expertise needed to implement the protocol

Our purification protocol requires only basic skills in recombinant protein production in *E. coli* and cell culture handling, and can even be carried out by undergraduate students with prior training. The generation of stable human suspension cell lines using lentiviral transduction, however, requires special biosafety training and conditions in compliance with the relevant institutional and governmental biosafety regulations. Elegheert and colleagues expertly summarized common safety practices associated with lentiviral work[1]. However, if use of lentivirus is not feasible, multiple other strategies for plasmid delivery can be used instead (see below).

Experimental design

Preparation of Nb fusion proteins and SENP^{EuB} protease

All recombinant proteins required for this protocol can easily be purified in very high yield and purity from single 1 L *E. coli* cultures (Steps 1–34). Such preparations generate sufficient reagents for hundreds of purifications from eukaryotic cells. We provide expression plasmids and protocols for the generation of SENP^{EuB} protease, as well as biotinylated anti-GFP and anti-ALFA nanobodies with or without the SUMO^{Eu} module in between the Avi-tag and Nb (for Addgene IDs, see Table 1).

The anti-GFP Nb can easily be biotinylated during expression in the *E. coli* strain AVB101, which allows IPTG-inducible co-expression of the biotin ligase BirA. The anti-ALFA Nb, however, expresses better in the *E. coli* Rosetta-gami2 strain, which does not express BirA. Purified ALFA Nb therefore needs to be biotinylated with purified BirA in solution (Box 1). For this, we provide a BirA expression plasmid (pTP264) and purification protocol. BirA can, however, also be obtained commercially.

BOX 1

In vitro biotinylation of ALFA nanobodies with purified BirA

● **TIMING 5 h**

▲ **CRITICAL** Ni^{2+}-purified and buffer-exchanged ALFA nanobodies expressed from pTP298 and pTS118 are biotinylated with purified BirA in solution. Excess biotin then needs to be removed by an additional buffer exchange step.

Procedure

1. Set up the biotinylation reaction mix as detailed in the table below:

Component	Amount
ALFA Nb purified from pTP298 or pTS118	100 nmol
BirA enzyme	1.5 nmol
5× Biotinylation buffer	300 µL
ddH_2O	Up to 1.5 mL

▲ **CRITICAL STEP** In vitro biotinylation is most efficient when the substrate is provided at a concentration of at least 40 µM. Reaction volumes can be scaled up or down.

2. Incubate the reaction for 3 h at 25 °C in a thermomixer without mixing. Mix by inverting the tube every 30 min.

▲ **CRITICAL STEP** BirA activity is highly temperature dependent. If performed on ice the biotinylation reaction typically needs at least 24 h for completion.

3. Load the entire 1.5 mL biotinylation reaction on a PD-10 desalting column, pre-equilibrated in ALFA Nb storage buffer according to the manufacturer's instructions.
4. Wait for the entire volume of the reaction (1.5 mL) to completely sink into the column and discard the flow through.
5. Add 1 mL of ALFA Nb storage buffer and discard the flow through.
6. Elute stepwise by adding 0.5 mL of ALFA Nb storage buffer to the PD-10 desalting column and collect 6× 0.5 mL fractions, each in a clean 1.5 mL Eppendorf tube.
7. Measure the absorbance of each individual fraction at 280 nm as in Step 28, as well as the 260/280 nm absorbance ratio.
8. In a fresh 2 mL tube, combine only fractions with a 260/280 ratio close to the ratio of the purified Nb before biotinylation (measured in Step 28).

▲ **CRITICAL STEP** The expected ratio should be between 0.5 and 0.7. Including later fractions with increased 260/280 ratios (>0.7) risks contaminating the final biotinylated ALFA Nb preparations with excess free biotin that will compete with Nb binding to streptavidin beads.

9. Measure the final concentration as in Step 28 of the procedure, prepare aliquots as advised in Step 32 and flash-freeze them in liquid nitrogen.

■ **PAUSE POINT** Frozen protein aliquots are stable at −80 °C for years.

Affinity resins containing immobilized (noncleavable) anti-GFP or ALFA nanobodies are commercially available and are directly compatible with the TagOFF strategy.

Optimal introduction of a tag on a protein subunit or complex

The first critical consideration for the purification of a multisubunit protein complex is to choose a subunit to tag. We frequently found that ectopic expression of a single tagged subunit of a protein complex in human cells can result in the replacement of its endogenous counterpart[24]. This strategy yields stoichiometric complexes in human cells when efficient cellular quality control mechanisms exist that degrade excess tagged and endogenous subunits of the chosen complex. Existing functional and structural data can often be used to inform subunit choice. For example, scaffold subunits of protein complexes typically contain multiple hydrophobic interfaces and are especially short lived in the unassembled state, ensuring rapid elimination of excess subunits. Such subunits might thus represent prime candidates for first tagging trials[24] and can be identified from mass spectrometry studies that analyzed proteome-wide degradation kinetics[31]. More stable subunits can accumulate after overexpression and may need to be removed by additional purification steps.

The next important consideration is to identify the optimal location of the affinity tag to minimize its interference with protein function and stability. For protein complexes, the tag must be compatible with complex assembly. Compatibility can often be inferred from prior published studies or large-scale GFP-tagging efforts (Supplementary Table 1), provided a careful analysis of localization and function was performed. Alternatively, existing structures or structural models of the protein can be analyzed. A flexible unstructured terminus that is not part of a folded domain can usually tolerate a protein tag. Conversely, regions with high

sequence conservation might indicate a functional requirement or binding site. In general, smaller peptide tags (such as the ALFA tag) tend to be less disruptive than larger globular tags. The ALFA tag can also be placed internally into exposed loops and thus provides an alternative for proteins that cannot be tagged at either terminus.

If the presence of a tag will interfere with downstream applications, the TagOFF strategy should be used to completely remove the tag during purification. For this, the appropriate noncleavable Nb fusion protein needs to be generated and combined with a protein expression vector that contains a protease cleavage site. As described below, N-terminal tags can be removed completely (scarless), yet C-terminal tag removal will always generate a cleavage scar.

Cloning of a lentiviral transfer plasmid encoding the tagged POI

Once a tagging and purification strategy is identified, the coding sequence of the POI needs to be cloned into a lentiviral transfer plasmid. We provide an extensive toolbox of second generation lentiviral transfer plasmids to facilitate fusion of a POI to a GFP or ALFA tag. All plasmids are available via Addgene (for IDs, see Table 1). Each tag is either N-terminal (pTS93–pTS102) or C-terminal (pTS103–pTS116) and either noncleavable for TagON or cleavable for TagOFF purification (Supplementary Fig. 1). A detailed cloning guide is provided in Supplementary Data 1.

All constructs contain a CMV promoter fused to two Tet operator sequences (CMV–TetO2), which are Tet repressor (TetR) binding sites. The CMV–TetO2 promoter is thus doxycycline inducible only in a TetR+ cell line but will otherwise express constitutively.

We strongly recommend homemade SENP^{EuB} protease for tag removal, but we also provide plasmids encoding the cleavage sites of the commercially available 3C, TEV and SUMOstar proteases. As these proteases all cleave at the C-terminus of their recognition site, N-terminal tags can thus be nearly completely removed. For C-terminal tags, however, this results in a cleavage scar. The scars of 3C and TEV protease (both of six amino acids (aa) in length) are smaller than those of the SENP^{EuB} (96 aa) and SUMOstar proteases (99 aa). If desired, these larger C-terminal scars can be removed after elution by an additional 3C or TEV protease cleavage step in solution. Our SUMO protease-cleavable C-terminal GFP- or ALFA-tag encoding plasmids thus contain additional 3C or TEV cleavage sites between multiple cloning site and SUMO module.

A final consideration when selecting the right construct is that protein yield can be maximized by isolating successfully transduced cells via FACS to obtain a homogeneous population. While cells expressing GFP-tagged proteins can easily be sorted using GFP fluorescence, we additionally equipped expression vectors encoding ALFA-tagged proteins with a TagBFP expression cassette. TagBFP is separated from the ALFA-tagged protein using a porcine teschovirus P2A sequence, which mediates peptide bond skipping by the ribosome[30] and thus results in the efficient synthesis of two separate proteins from a single mRNA (i.e., TagBFP and the ALFA-tagged protein).

Generation of stable human suspension cell lines using lentiviral transduction

Lentiviral particles can be generated by cotransfecting adherent Lenti-X 293T cells with a second generation lentiviral transfer plasmid encoding a GFP- or ALFA-tagged POI and second generation packaging (e.g., psPAX2) and envelope plasmids (e.g., pMD2.G) (Steps 35–49). Transfected cells secrete lentiviral particles into the culture medium that are pseudotyped with the VSV-G envelope protein, which binds the widely expressed low-density lipoprotein receptor on the target cell surface and thus confers broad host cell tropism[32]. Lentiviral particles can then be used to transduce multiple human suspension and adherent cell lines that are compatible with our purification protocol.

We favor a very rapid, easy-to-use, lentiviral transduction approach[1] to generate stable cell lines expressing a GFP- or ALFA-tagged POI (Steps 50–61). Generally, we prefer Expi293F (TetR−) and Expi293F inducible cell lines (TetR+), because they maintain high viability even at very high cell density, and thus maximize the yield of cells per liter of culture medium. To express secreted or membrane proteins without complex *N*-glycans, the *N*-acetylglucosaminyltransferase I knockout (GnTI−) derivatives of these cell lines should be used.

During infection, lentiviral particle-containing culture medium is simply mixed with human Expi293F suspension cells. This leads to the stable integration of the open reading frame encoded on the transfer plasmid into a random genomic location. For first small-scale purification trials, we recommend expanding transduced cells to medium density in ~50 mL. This allows testing different tags, tag positions or expression conditions. For monomeric or homo-oligomeric proteins, a reduction of expression temperature from 37 °C to 30 °C or the inclusion of histone deacetylase inhibitors such as sodium butyrate or valproic acid were previously shown to lead to large increases of final protein yield[31]. For individually expressed subunits of protein complexes, strong overexpression might reduce final complex yield and expression conditions instead need to be optimized to maximize subunit incorporation. Once optimal conditions are found, purifications from 1 to 2 L high-density cultures often provide enough material for structural and functional assays.

If lentiviral work is not feasible, multiple alternative approaches can be used instead to generate human suspension cell lines expressing a GFP- or ALFA-tagged POI. These include, for example, transient transfection using polyethylenimine or Expifectamine, baculovirus transduction[33] and adaptation of stable adherent cell lines to suspension growth[34]. Such stable adherent cell lines can be generated by Flp-In recombination[35], antibiotic selection[34] or clustered regularly interspaced short palindromic repeats (CRISPR) knock-in[36–38].

Cell lysis method and purification conditions

The subcellular localization and stability of the POI dictates the appropriate lysis method to prepare cell extracts for affinity purification (Step 66). Mechanical lysis is appropriate to purify soluble cytosolic or nuclear proteins. Nuclear DNA-bound proteins additionally need to be dissociated from DNA using a high-salt lysis buffer[39]. Membrane proteins must be solubilized from whole cells or enriched membrane fractions using detergents or other membrane mimetics[40,41]. The choice of detergent for solubilization can affect membrane protein extraction efficiency and stability.

Another critical factor to consider is the protein's intrinsic stability. In general, lysis buffers with slightly elevated salt concentration (>150 mM) and detergent can strongly reduce nonspecific binding of lysate proteins to affinity resins. For example, the widely used RIPA lysis buffer contains high concentrations of ionic (deoxycholate and sodium dodecyl sulfate (SDS)) and nonionic detergents (Triton-X-100 or NP-40) and is used for both soluble and membrane protein purification. Generally, these harsher detergents, if tolerated, enable purifications with higher purity; however, some proteins and protein complexes can be very sensitive to high ionic strength or detergents, and in this case, gentle detergents such as glyco-diosgenin (GDN) are strongly preferred to maintain protein stability as well as transient protein–protein interactions. To determine the optimal conditions for a protein or complex of interest, we recommend optimizing lysis buffer composition alongside expression conditions in pilot small-scale purification trials. For GFP-tagged proteins, this can be done using fluorescence detection SEC, as described before[26,33].

Protein purification by Nb capture and protease release

The major components of our purification strategy are highly stable proteins that are compatible with the most commonly used lysis buffer compositions.

For example, we use the anti-GFP Nb Enhancer (12.8 kDa), which binds GFP with high affinity (K_D ~ 590 pM) and specificity in many different systems[42,43]. Various widespread GFP variants (including wildtype (WT) GFP, EGFP and superfolder GFP[44]), as well as closely related fluorescent proteins, are compatible (Extended Data Fig. 1). The ALFA Nb (13.4 kDa) binds a rationally designed, 15 aa α-helical peptide with very high affinity (K_D ~ 26 pM)[3]. Both nanobodies, as well as GFP and ALFA tags are stable and bind efficiently even under harsh conditions, including high concentrations of most detergents, salt and even urea (Supplementary Table 3).

For our purification strategies (Steps 67–89), both nanobodies are immobilized onto streptavidin beads using a fused biotin acceptor peptide (Avi tag), which is modified with a single biotin moiety by the biotin ligase BirA, either during expression in *E. coli* or

after purification in vitro[45–47]. As one of the strongest noncovalent interactions in nature, the biotin–streptavidin interaction withstands harsh purification conditions.

Finally, our protocol makes use of the SUMO^{Eu} protein (10.5 kDa), which was engineered by the Görlich laboratory (Max Planck Institute for Multidisciplinary Sciences, Germany) to be resistant against cleavage by endogenous eukaryotic SUMO proteases[4]. SUMO^{Eu}-tagged nanobodies or proteins are thus stable in a variety of eukaryotic cells and cell extracts. The more commonly used yeast SUMO is useful for expression in prokaryotic systems, but is very rapidly cleaved in eukaryotic cells and cell lysate[4,48]. The engineered SUMO^{Eu}•SENP^{EuB} pair therefore extends the use of the SUMO protease technology to eukaryotes. A similar but completely orthogonal SUMOstar system[49] is commercially available (LifeSensors), but is slightly less resistant against host SUMO protease cleavage[4].

Controls

If our protocol is to be used for the identification of interaction partners via mass spectrometry, it is important to include controls to account for trace contaminants that nonspecifically bind to the affinity tag, Nb or affinity resin. A good control is a matched cell lysate expressing solely the affinity tag without a fused protein (e.g., only GFP, generated using plasmid pTP924). Less optimally, the cell lysate containing the POI can be split into two even fractions and additionally incubated with beads that either do not contain any immobilized Nb or that contain a nonspecific Nb (e.g., anti-ALFA Nb for a GFP-tagged protein).

Similarly, for functional assays, it is important to control that the observed activity of the purified protein is not caused by a contaminant. If possible, a catalytically dead mutant should be purified in parallel under identical conditions.

For cell sorting enrichment, nontransduced (negative) and single-color (positive) controls are typically required to determine efficient sort gate placement. Including these controls is even more important when sorting cell lines transduced with multiple different lentiviruses, encoding compatible fluorescent proteins, to allow multicolor compensation to correct for cross-channel fluorescence bleed through. Plasmids pTP341 and pTP924, which respectively express TagBFP and GFP both from a constitutive CMV promoter, can be used for this purpose (Table 1).

Materials

Biological materials

Cell lines

- Lenti-X 293T cell line (Takara Bio, cat. no. 632180)
- Gibco Expi293F cells (TetR−; Thermo Fisher Scientific, cat. no. A14527; RRID: CVCL_D615)
- Gibco Expi293F GnTI− cells (TetR−; Thermo Fisher Scientific, cat. no. A39240; RRID: CVCL_B0J7)
- Gibco Expi293F inducible cells (TetR+; Thermo Fisher Scientific, cat. no. A39241; RRID: CVCL_B0J8)
- Gibco Expi293F inducible GnTI− cells (TetR+; Thermo Fisher Scientific, cat. no. A39242; RRID: CVCL_B0J9)
- Gibco Freestyle 293-F cell line (Thermo Fisher Scientific, cat. no. R79007; RRID: CVCL_D603)

E. coli strains

- NEBExpress I^{q} chemically competent cells (New England Biolabs, cat. no. C3037I)
- AVB101 chemically competent cells (Avidity, cat. no. CVB101)
- Stellar competent cells (Takara Bio, cat. no. 636763)
  ▲ **CRITICAL** Cloning DNA with repetitive elements, such as our lentiviral transfer plasmid toolbox, requires a recA− *E. coli* strain such as *E. coli* Stellar.
- Rosetta-gami 2 competent cells (Novagen/Millipore–Sigma, cat. no. 71350-3)

Reagents

Common reagents

- Gibco Expi293 expression (Expi) medium (Thermo Fisher Scientific, cat. no. A14351-01)
- Gibco FreeStyle 293 expression medium (Thermo Fisher Scientific, cat. no. 12338018)
- Gibco Dulbecco's modified Eagle medium (DMEM), high glucose, no glutamine (Thermo Fisher Scientific, cat. no. 11960051)
- Gibco L-glutamine 200 mM (100× Gln) (Thermo Fisher, cat. no. 25030081)
- Gibco penicillin–streptomycin 5,000 U/mL (100× pen–strep) (Thermo Fisher Scientific, cat. no. 15070063)
- HyClone FBS (Cytiva, cat. no. SH30071.03)
- Gibco Dulbecco's phosphate-buffered saline (DPBS), no calcium, no magnesium (Thermo Fisher Scientific, cat. no. 14190136)
- Gibco Trypsin–ethylenediaminetetraacetic acid (EDTA) (0.25%), phenol red (Thermo Fisher Scientific, cat. no. 25200056)
- Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, cat. no. A13280.36)
- Gibco Opti-MEM I (1×) reduced serum medium (Thermo Fisher Scientific, cat. no. 31985-062)
- TransIT-293 transfection reagent (Mirus Bio, cat. no. MIR 2704)
- LB medium, Miller (Fisher Scientific, cat. no. BP1426-2)
- LB agar, Miller (Powder) (Fisher Scientific, cat. no. BP1425-500)
- Tryptone (Fisher Scientific, cat. no. BP9726-5)
- Yeast extract granulated (Fisher Scientific, cat. no. BP9727-5)
- Super Optimal broth with Catabolite repression (SOC) recovery medium (Thermo Fisher Scientific, cat. no. 15544034)
- Carbenicillin (disodium salt) (Fisher Scientific, cat. no. BP26485)
- Kanamycin (kan) sulfate (Fisher Scientific, cat. no. BP906-5)
- Chloramphenicol (cam) (Fisher Scientific, cat. no. BP904-100)
- IPTG (Millipore–Sigma, cat. no. I6758-5G)
- Imidazole (Millipore–Sigma, cat. no. I2399-500G)
- NaCl (Fisher Scientific, cat. no. S9888-5KG)
- Tris base, Trizma (Millipore–Sigma, cat. no. T6066-5KG)
- HEPES (Millipore–Sigma, cat. no. H3375-500G)
- 1 M Magnesium acetate solution (Millipore–Sigma, cat. no. 63052-100ML)
- Magnesium chloride (VWR, cat. no. MK5958-04)
- Potassium acetate (Millipore-Sigma, cat. no. 60035-1KG)
- Hydrochloric acid concentrated 37%/12 N (Millipore–Sigma, cat. no. HX0603-3)
- Potassium hydroxide pellets (VWR, cat. no. 6984-06)
- Glycerol (Fisher Scientific, cat. no. BP229-4)
- Sucrose (Millipore–Sigma, cat. no. 50389-5KG)
- DTT (Millipore–Sigma, cat. no. D9163-25G)
- Ethanol 200 proof (VWR, cat. no. TX-89125172CAL)
- Methanol (VWR, cat. no. BDH1135-4LG)
- PMSF (Thermo Fisher Scientific, cat. no. 36978)
- Ribonuclease A from bovine pancreas (Millipore–Sigma, cat. no. R6513-50MG)
- Adenosine 5′-triphosphate dipotassium salt hydrate (Millipore–Sigma, cat. no. A8937-1G)
- D-Biotin (Millipore–Sigma, cat. no. B4501-1G)
- $dPEG_{24}$–biotin acid (Quanta Biodesign, cat. no. 10773)
- Triton-X-100 (Millipore-Sigma, cat. no. X100-500)
- Tween-20 (Millipore-Sigma, cat. no. P1379-1L)
- GDN (Anatrace, cat. no. GDN101 25 GM)
- Doxycycline hyclate (Millipore–Sigma, cat. no. D9891-1G)
- 20% (wt/vol) SDS (VWR, cat. no. 97062-442)
- Bromophenol blue (Millipore–Sigma, cat. no. B0126-25G)
- Urea (Millipore–Sigma, cat. no. U0631-500G)
- Roche cOmplete EDTA-free protease inhibitor cocktail (Millipore–Sigma, cat. no. 11873580001)

- Ni–NTA agarose resin 25 mL (Qiagen, cat. no. 30210)
- Pierce streptavidin magnetic beads (Thermo Fisher Scientific, cat. no. 88816)

Plasmids

▲ **CRITICAL** All plasmids are available through the nonprofit Addgene plasmid repository and are subject to a Uniform Biological Material Transfer Agreement. Details of the plasmids supplied with this protocol are listed in Table 1.

- *E. coli* expression plasmids: pTP264 (Addgene ID #149334)/pTP298 (Addgene ID #199390)/pTP396 (Addgene ID #149336)/pTS117 (Addgene ID #199370)/pTS118 (Addgene ID #199371)/pAV0286 (Addgene ID #149333)
- Transfer plasmids: pTS093–pTS116 (Addgene ID #199346–199369)/pTP341 (Addgene ID #199391)/pTP924 (Addgene ID #199392)
- pMD2.G lentivirus envelope plasmid (Addgene ID #12259)
- psPAX2 lentivirus packaging plasmid (Addgene ID #12260)

Sequencing primers

▲ **CRITICAL** These nonmodified DNA oligos are custom orders (Integrated DNA Technologies).

- CMV forward (5′–CGCAAATGGGCGGTAGGCGTG–3′)
- WPRE reverse (5′–GTTGCCTGACAACGGGCC–3′)
- GFP C-terminus forward (5′–GGAGACGGTCCCGTCCTC–3′)
- BFP C-terminus forward (5′–GATACTGCGACCTCCCTAGC–3′)
- GFP N-terminus reverse (5′–TGGCCATTCACGTCTCCGTC–3′)
- BFP N-terminus reverse (5′–CTTGAAGTGATGGTTGTCCACGGTGC–3′)

Equipment

- 125 mL Erlenmeyer flask, vent cap, plain bottom, polyethylene terephthalate glycol (PETG) (Celltreat, cat. no. 229801)
- 490 cm^2 tissue culture treated roller bottle, vented cap (1 L roller bottle; Celltreat, cat. no. 229383)
- 850 cm^2 tissue culture treated roller bottle, vented cap (2 L roller bottle; Celltreat, cat. no. 229385)
- Tissue culture six-well plates (Genesee Scientific, cat. no. 25-105)
- Tissue culture 150 mm plates (Fisher Scientific, cat. no. FB012925)
- 5 mL serological (Genesee Scientific, cat. no. 12-102)
- 10 mL serological (Genesee Scientific, cat. no. 12-104)
- 25 mL serological (Genesee Scientific, cat. no. 12-106)
- 50 mL serological (Genesee Scientific, cat. no. 12-107)
- Steriflip sterile disposable vacuum filter units with pore size 0.22 µm (Millipore–Sigma, cat. no. SCGP00525)
- GenClone vacuum filter system with 0.22 µm polyethersulfone (PES) membrane, 500 mL, sterile (Genesee Scientific, cat. no. 25-227)
- Corning cryogenic vials, external thread (2.0 mL; Millipore–Sigma, cat. no. CLS430661)
- 5 mL microcentrifuge tube, sterile (Eppendorf, cat. no. 0030119487)
- 15 mL disposable centrifuge tube, sterile (Fisher Scientific, cat. no. 05-539-12)
- 50 mL disposable centrifuge tube, sterile (Fisher Scientific, cat. no. 05-539-8)
- 5 mL round-bottom flow cytometry tubes with strainer cap (VWR, cat. no. 76449-658)
- Tissue culture CO_2 incubator (Thermo Fisher Scientific, model no. Heracell 240i)
- Tissue culture S41i CO_2 incubator shaker (Eppendorf, cat. no. S41I120010)
- Class II, type A2 biosafety cabinet (Baker, model: SterilGard III SG403)
- Cell sorter (Sony Biotechnology, model no. SH800S)
- Automated cell counter (Thermo Fisher Scientific, model no. Countess 3)
- Cell counting slides (Bulldog-bio, cat. no. DHC-N01)
- Tissue culture Floid cell imaging station (Thermo Fisher Scientific, cat. no. 4471136)
- Benchtop centrifuge model no. 5810 with swing bucket rotor S-4-104 (Eppendorf, cat. no. 022627110) including 4× 750 mL swing buckets + 15 mL conical tube

adapters, as well as matching aerosol-tight caps (cat. no. 022638661) for lentiviral collection
- Refrigerated floor centrifuge (Thermo Fisher Scientific, model no. Sorvall RC6+) with Fiberlite F9-4x1000y rotor
- Corning CoolCell cell freezing vial container (Fisher Scientific, cat. no. 07-210-002)
- Fast performance liquid chromatography system (Cytiva, model no. Äkta Pure 25 M)
- SEC column (Cytiva, model no. Superose 6. Increase 3.2/300)
- Hamilton gas-tight syringe 50 µL (Millipore-Sigma, cat. no. 26280-U)
- Amicon Ultra centrifugal filters for protein concentration (Millipore–Sigma, model no. Amicon Ultra 0.5 or 4 with protein-specific molecular weight cut-off (e.g., 3, 10, 30, 50 or 100 kDa))
- Disposable gravity-flow chromatography column, 20 mL (Bio-Rad, cat. no. 7321010)
- PD-10 desalting columns (Cytiva, cat. no. 17085101)
- Wheaton Dounce tissue grinder, 15 mL, tight pestle (DWK Life Sciences, cat. no. 357544)
- Branson Ultrasonics sonifier SFX250 cell disruptor (Fisher Scientific, cat. no. 15-345-138) with disruptor horn (cat. no. 22-020860)
- Invitrogen DynaMag-2 Magnet (Thermo Fisher Scientific, cat. no. 12321D)
- Invitrogen DynaMag-15 Magnet (Thermo Fisher Scientific, cat. no. 12301D) (less expensive magnetic racks are available at https://sergilabsupplies.com)

Reagent setup

LB medium

To make 1 L of LB, dissolve 25 g of LB medium in 1 L of ddH_2O. Sterilize by autoclaving for 45 min at 121 °C using a liquid cycle. Store at RT for up to 1 year.

Super broth (SB)

To prepare 5 L of SB, weigh in 175 g tryptone, 100 g yeast extract, 25 g NaCl and dissolve in 4.5 L of ddH_2O. Adjust the pH to 7–7.5 with 1 M NaOH and top up to 5 L. Aliquot 1 L into 2 L Erlenmeyer flasks and sterilize by autoclaving for 45 min at 121 °C using a liquid cycle. Store at RT for up to 1 year.

LB agar plates

Dissolve 25 g LB agar in 1 L of ddH_2O. Sterilize by autoclaving for 45 min at 121 °C using a liquid cycle. When the solution has cooled to 45–50 °C, add 1 mL of a 1,000× stock of antibiotic, mix by swirling and pour into 100× 15 mm Petri dishes. Allow to solidify and dry at RT. Store at 4 °C for up to 6 months.

50 mg/mL Kanamycin (kan, 1,000× stock)

To prepare 10 mL of a 50 mg/mL stock solution of kanamycin, dissolve 0.5 g of kanamycin sulfate in 9.5 mL of H_2O. Top up the volume to 10 mL and filter-sterilize using a disposable Steriflip 0.22 µm filter unit. Store in 1 mL aliquots at −20 °C for up to 1 year.

100 mg/mL Carbenicillin (1,000× stock)

To prepare 10 mL of a 100 mg/mL stock solution of carbenicillin, dissolve 1 g of carbenicillin (disodium salt) in 9.5 mL of H_2O. Top up the volume to 10 mL and filter-sterilize using a disposable Steriflip 0.22 µm filter unit. Store in 1 mL aliquots at −20 °C for up to 6 months.

50 mg/mL Chloramphenicol (cam, 1,000× stock)

To prepare 10 mL of a 50 mg/mL stock solution of chloramphenicol, dissolve 0.5 g of chloramphenicol in 9.5 mL 100% ethanol. Top up the volume to 10 mL and filter-sterilize using a disposable Steriflip 0.22 µm filter unit. Store in 1 mL aliquots at −20 °C for up to 1 year.

1 M IPTG

To make up 10 mL of 1 M IPTG, weigh in 2.38 g of IPTG and dissolve in 8 mL ddH_2O. Top up the volume to 10 mL and filter-sterilize using a disposable Steriflip 0.22 µm filter unit. Store in 1 mL aliquots at −20 °C for up to 1 year.

2 M Tris/HCl pH 7.5
To make 1 L of 2 M Tris/HCl at pH 7.5, weigh in 242.2 g Tris base and dissolve in 800 mL ddH2O. Add 134.3 mL concentrated 37% (12 N) HCL. Top up the volume to 1 L with ddH_2O and sterilize using a 0.22 µm GenClone vacuum filter system. Can be stored at RT for many years.
▲ **CAUTION** HCl is a strong acidic solution and should be handled with caution. Wear appropriate personal protective equipment (PPE; butyl rubber gloves, laboratory coat and goggles) and handle concentrated solutions in a chemical fume hood.

1 M HEPES/KOH pH 7.5
To make 0.5 L of 1 M HEPES/KOH at pH 7.5, weigh in 119.2 g HEPES and dissolve in 350 mL ddH_2O. Adjust pH to 7.5 with potassium hydroxide pellets. Top up the volume to 0.5 L with ddH_2O and sterilize using a 0.22 µm GenClone vacuum filter system. Store at 4 °C for up 2 years.
▲ **CAUTION** Potassium hydroxide pellets are an irritant and corrosive substance. Wear appropriate PPE (gloves, laboratory coat and goggles)

5 M NaCl
To make 1 L of 5 M NaCl, dissolve 292 g of NaCl in 700 mL ddH_2O and add ddH_2O up to 1 L. Sterilize using a 0.22 µm GenClone vacuum filter system. Can be stored at RT for many years.

5 M Potassium acetate (KAc)
To make 200 mL of 5 M KAc, dissolve 98.14 g of KAc in 180 mL ddH_2O and add 800 µL concentrated 37% (12 N) HCl. Add ddH_2O up to 200 mL and sterilize using a 0.22 µm GenClone vacuum filter system. Can be stored at RT for many years.
▲ **CAUTION** HCl is a strong acidic solution and should be handled with caution. Wear appropriate PPE (butyl rubber gloves, laboratory coat and goggles) and handle concentrated solutions in a chemical fume hood.

1 M $MgCl_2$
To make 100 mL of 1 M $MgCl_2$, dissolve 20.3 g of $MgCl_2$ in 80 mL ddH_2O and add ddH_2O up to 100 mL.
Sterilize using a 0.22 µm GenClone vacuum filter system. Can be stored at RT for many years.

2 M Imidazole pH 7.5
To make 250 mL of 2 M imidazole pH 7.5, dissolve 34.04 g imidazole in 200 mL ddH_2O. Adjust pH to 7.5 with concentrated 37% (12 N) HCl and add ddH_2O up to 250 mL. Sterilize using a 0.22 µm GenClone vacuum filter system and store at 4 °C, protected from light.
▲ **CAUTION** HCl is a strong acidic solution and should be handled with caution. Wear appropriate PPE (butyl rubber gloves, laboratory coat and goggles) and handle concentrated solutions in a chemical fume hood.

1 M DTT
To make 10 mL of 1 M DTT, dissolve 1.5 g of DTT in 8 mL ddH_2O and add ddH_2O up to 10 mL. Make 1 mL aliquots and store at −20 °C for up to 3 months.
▲ **CAUTION** DTT is toxic. Handle with gloves.

100 mM PMSF
To make 50 mL of 100 mM PMSF, weigh in 0.87 g PMSF and dissolve in 50 mL methanol. Store at −20 °C for up to 1 year.
▲ **CAUTION** Both PMSF and methanol are toxic. Handle under a fume hood with gloves.

100 mM ATP
To make 10 mL of 100 mM ATP, weigh in 551 mg of adenosine 5′-triphosphate disodium salt hydrate and dissolve in 8 mL ddH_2O. Adjust to pH 7.0 with potassium hydroxide pellets. Top up the volume to 10 mL with ddH_2O and make 0.5 mL aliquots. Store at −20 °C for up to 1 year.

10 mg/mL RNase A
To make 5 mL of a 10 mg/mL stock of RNaseA, reconstitute 50 mg lyophilized RNase A in 5 mL 1× phosphate-buffered saline (PBS) and freeze in 1 mL aliquots. Can be stored at −20 °C for many years.

50 mM Biotin stock
To make 10 mL of a 50 mM biotin stock in 50 mM HEPES/KOH pH 7.5, weigh in 122 mg biotin and add 9.95 mL 50 mM HEPES/KOH pH 7.5. Add 50 µL 5 M NaOH to dissolve biotin. Make 0.5 mL aliquots and store at −20 °C for many years.

50 mM $dPEG_{24}$–biotin acid stock
To make 1 mL of a 50 mM $dPEG_{24}$–biotin acid stock in 50 mM HEPES/KOH pH 7.5, weigh in 68.6 mg $dPEG_{24}$–biotin acid and dissolve in 1 mL 50 mM HEPES/KOH pH 7.5. Make 100 µL aliquots and store at −20 °C for many years.

8 M Urea
To prepare 2 mL of 8 M urea, weigh in 0.96 g urea and dissolve in 0.8 mL ddH_2O. Add ddH_2O up to 2 mL. Prepare freshly before each use.

1 mg/mL Doxycycline
To prepare a 50 mL of 1 mg/mL doxycycline, weigh in 50 mg doxycycline and dissolve in 50 mL ddH_2O. Sterilize using a disposable Steriflip 0.22 µm filter unit inside a tissue culture hood. Make 1 mL aliquots and store at −20 °C for up to 1 year. For the induction of larger culture volumes, it is useful to prepare a 10 mg/mL stock solution.
▲ **CAUTION** Doxycycline is harmful if swallowed, and it causes skin irritation.

25× Protease inhibitor cocktail
Dissolve 1× Roche cOmplete EDTA-free protease inhibitor cocktail tablet in 2 mL ddH_2O. Use immediately or store at −20 °C for up to 12 weeks.
▲ **CAUTION** Protease inhibitor cocktail is toxic. Handle with gloves.

10% (wt/vol) GDN
To prepare 10 mL 10% (wt/vol) GDN, weigh in 1 g GDN and add 8 mL ddH_2O. Rotate head-over-tail at RT until the GDN is fully dissolved. Top up to 10 mL with ddH_2O and make 1 mL aliquots. Store at −20 °C for up to 1 year.

20% (vol/vol) Triton-X-100
To prepare 100 mL of 20% (vol/vol) Triton-X-100, transfer 20 mL 100% (vol/vol) Triton-X-100 into a 100 mL glass bottle and add 80 mL ddH_2O. Incubate tumbling or rotating until the solution is well mixed. Store at RT for years.
▲ **CAUTION** Triton X-100 is harmful if swallowed and an irritant in direct contact with eyes. Wear protective clothing and avoid exposure.

5× SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer
250 mM Tris/HCl pH 6.8, 5% (wt/vol) SDS, 50% (vol/vol) glycerol and 500 mM DTT.
Prepare 40 mL by mixing the following: 1.211 g Tris base, 10 mL 20% (wt/vol) SDS, 20 mL 100% (vol/vol) glycerol and 3.084 g DTT. Adjust pH to 6.8 by adding 0.79 mL 37%/12 N HCl and then add ddH_2O to 40 mL. Mix by inverting gently until all DTT has dissolved and then add 40 mg bromophenol blue. Mix as above and make 1 mL aliquots. Store at −20 °C for up to 1 year.

Resuspension buffer
50 mM Tris/HCl pH 7.5, 300 mM NaCl, 20 mM imidazole, 1 mM DTT and 1 mM PMSF. Prepare 1 L by mixing 25 mL 2 M Tris/HCl pH 7.5, 60 mL 5 M NaCl, 10 mL 2 M imidazole and 905 mL ddH_2O. Store at 4 °C for up to 1 year. Add 1 mM DTT and 1 mM PMSF directly before use.

Low-salt-biotin–ATP–RNase A (LSBAR) buffer
50 mM HEPES/KOH pH 7.5, 100 mM KAc, 2 mM MgAc, 1 mM DTT, 1 mM ATP, 50 µM biotin and 100 µg/mL RNAse A. Prepare 50 mL by mixing 2.5 mL HEPES/KOH pH 7.5, 1 mL 5 M KAc, 0.1 mL 1 M MgAc, 0.05 mL 1 M DTT, 0.5 mL 100 mM ATP, 0.05 mL 50 mM biotin, 0.5 mL 10 mg/mL RNase A and 45.3 mL ddH_2O. Prepare fresh shortly before use.

Low-salt ATP buffer
50 mM HEPES/KOH pH 7.5, 100 mM KAc, 2 mM MgAc, 1 mM DTT and 1 mM ATP. Prepare 50 mL by mixing 2.5 mL HEPES/KOH pH 7.5, 1 mL 5 M KAc, 0.1 mL 1 M MgAc, 0.05 mL 1 M DTT, 0.5 mL 100 mM ATP and 45.85 mL ddH_2O. Prepare fresh shortly before use.

High-salt buffer
50 mM Tris/HCl pH 7.5, 1 M NaCl, 20 mM imidazole and 1 mM DTT. Prepare 50 mL by mixing 1.25 mL 2 M Tris/HCl pH 7.5, 10 mL 5 M NaCl, 0.5 mL 2 M imidazole and 38.2 mL ddH_2O. Store at 4 °C for up to 1 year. Add 0.05 mL 1 M DTT directly before use.

Imidazole elution buffer
50 mM Tris/HCl pH 7.5, 300 mM NaCl, 500 mM imidazole, 10% glycerol and 1 mM DTT. Prepare 50 mL by mixing 1.25 mL Tris/HCl pH 7.5, 3 mL 5 M NaCl, 12.5 mL 2 M imidazole pH 7.5, 5 mL 100% glycerol and 28.2 mL ddH_2O. Store at 4 °C. Add 0.05 mL 1 M DTT directly before use.

BirA storage buffer
50 mM Tris/HCl pH 7.5, 200 mM NaCl, 1 mM DTT and 250 mM sucrose. Prepare 50 mL by mixing 1.25 mL Tris/HCl pH 7.5, 2 mL 5 M NaCl, 6.58 mL 1.89 M/65% (wt/vol) sucrose and 40.12 mL ddH_2O. Store at 4 °C. Add 0.05 mL 1 M DTT directly before use.

ALFA Nb storage buffer
50 mM Tris/HCl pH 7.5, 300 mM NaCl and 250 mM sucrose. Prepare 50 mL by mixing 1.25 mL Tris/HCl pH 7.5, 3 mL 5 M NaCl, 6.58 mL 1.89 M/65% (wt/vol) sucrose and 39.17 mL ddH_2O. Store at 4 °C for up to 1 year.

5× Biotinylation buffer
250 mM Tris/HCl pH 7.5, 500 mM NaCl, 50 mM ATP, 62,5 mM $MgCl_2$ and 50 mM biotin. Prepare 10 mL by mixing 1.25 mL 2 M Tris/HCl pH 7.5, 1 mL 5 M NaCl, 5 mL 100 mM ATP, 0.625 mL 1 M $MgCl_2$ and 2.125 mL ddH_2O. Weigh in 122 mg biotin and dissolve in 10 mL buffer as prepared above. Make 1 mL aliquots and store at −20 °C for up to 1 year.

Streptavidin test binding (STB) buffer
50 mM Tris/HCl pH 7.5, 200 mM NaCl, 0.1% (vol/vol) Triton-X-100 and 1 mM DTT. Prepare 20 mL by mixing the following: 0.5 mL 2 M Tris/HCl pH 7.5, 0.8 mL 5 M NaCl, 0.1 mL 20% (vol/vol) Triton-X-100, 0.02 mL 1 M DTT and 18.58 mL ddH_2O. Prepare freshly before use.

Solubilization buffer
50 mM HEPES/KOH pH 7.5, 200 mM NaCl, 2 mM MgAc, 1 mM ATP, 1% (wt/vol) GDN, 1× Roche cOmplete protease-inhibitor cocktail and 1 mM DTT. Prepare 10 mL by mixing 0.5 mL 1 M HEPES/KOH pH 7.5, 0.4 mL 5 M NaCl, 0.02 mL 1 M MgAc, 0.1 mL 100 mM ATP, 1 mL 10% (wt/vol) GDN, 0.4 mL 25× Roche cOmplete protease-inhibitor cocktail, 0.01 mL 1 M DTT and 7.57 mL ddH_2O. Prepare freshly before use.

Wash buffer
50 mM HEPES/KOH pH 7.5, 200 mM NaCl, 2 mM MgAc, 0.5 mM ATP, 0.01% (wt/vol) GDN, 1× Roche cOmplete protease-inhibitor cocktail and 1 mM DTT. Prepare 10 mL by mixing 0.5 mL 1 M HEPES/KOH pH 7.5, 0.4 mL 5 M NaCl, 20 µL 1 M MgAc, 0.05 mL 100 mM ATP, 10 µL 10% (wt/vol) GDN, 0.4 mL 25× Roche cOmplete protease-inhibitor cocktail, 0.01 mL 1 M DTT and 8.61 mL ddH_2O. Prepare freshly before use.

Wash buffer (–ATP)
50 mM HEPES/KOH pH 7.5, 200 mM NaCl, 2 mM MgAc, 0.01% (wt/vol) GDN, 1× Roche cOmplete protease-inhibitor cocktail and 1 mM DTT. Prepare 1 mL by mixing 0.05 mL 1 M HEPES/KOH pH 7.5, 0.04 mL 5 M NaCl, 0.002 mL 1 M MgAc, 0.001 mL 10% (wt/vol) GDN, 0.04 mL 25× Roche cOmplete protease-inhibitor cocktail, 0.001 mL 1 M DTT and 0.866 mL ddH_2O. Prepare freshly before use.

DMEM/10% FBS/1× Gln medium (DMEM:FBS:Gln)
Add 50 mL 100% FBS and 5.5 mL 100× L-glutamine to 500 mL Gibco DMEM culture medium. (Optional) Add 5.5 mL 100× pen–strep.

Procedure

Part 1: generation of biotinylated nanobodies, $SENP^{EuB}$ protease and biotin ligase BirA

● **TIMING** 4–5 d

▲ **CRITICAL** During the procedure, take samples for SDS–PAGE to help troubleshooting protein expression and purification as described in Supplementary Data 2.

1. Thaw one vial of the desired *E. coli* strain on ice for 10 min and add 1 µL of the relevant plasmid at a concentration of 100 ng/µL, following the guidelines provided in the table below:

Protein	Plasmid	*E. coli* strain	Antibiotic selection
GFP Nb	pTP396 and pTS117	AVB101	50 µg/mL kan and 10 µg/mL cam
$SENP^{EuB}$ protease	pAV0286	NEBExpress	50 µg/mL kan
BirA	pTP264	NEBExpress	50 µg/mL kan
ALFA Nb	pTP298 and pTS118	Rosetta-gami 2	50 µg/mL kan and 34 µg/mL cam

▲ **CRITICAL STEP** The ALFA Nb does not express well in *E. coli* AVB101 and thus needs to be expressed in the alternative strain Rosetta-gami 2, which does not support biotinylation during expression. The resulting anti-ALFA tag nanobodies therefore need to be biotinylated in vitro after Ni^{2+}-chelate affinity purification and buffer exchange, following the procedure described in Box 1.

2. Mix the sample by flicking the tube and incubate for 30 min on ice.
3. Heat shock the cells at 42 °C for 30 s before quickly transferring the sample on ice and incubating for 1 min.
4. Resuspend heat-shocked cells in 300 µL SOC recovery medium that was prewarmed to 37 °C and incubate at 37 °C for 1 h in a thermomixer, while shaking at 1,300 rpm.
5. Plate 50 µL of transformed cells onto an LB agar plate containing the appropriate antibiotics (see table above) using the dilution streaking method to obtain single colonies.
   ▲ **CRITICAL STEP** The cam addition to the LB agar plate and SB medium is required to maintain the BirA expression plasmid in the *E. coli* strain AVB101, as well as the pRARE plasmid in the *E. coli* Rosetta-gami 2 strain (see table above).
6. Incubate the plate at 37 °C overnight.
7. The following morning, pick a single colony from the plate using a toothpick or pipette tip and transfer into a clean 1.5 mL Eppendorf tube containing 200 µL SB medium, supplemented with the appropriate antibiotics (see table above).
8. Incubate shaking at 37 °C for 4–5 h and then inoculate the cells into 100 mL of SB medium containing the appropriate antibiotics (see table above) in a 2–5 L baffled flask.
9. Incubate the preculture at 37 °C overnight with shaking at 220 rpm.
10. The next morning, prepare a 1:10 dilution of the preculture in SB medium and measure the optical density of the diluted sample at 600 nm (OD_{600}) using a spectrophotometer.
11. Dilute the preculture with SB medium plus antibiotics to an OD_{600} of ~1 to make up 1 L of main culture.

12. (Optional) For expression of the anti-GFP Nb in *E. coli* AVB101, weigh in 12.2 mg of biotin powder, resuspend in 1 mL of SB medium by vortexing briefly and add to the main culture.
    ▲ **CRITICAL STEP** The suggested final concentration of biotin in the expression culture is 50 µM.
    ▲ **CRITICAL STEP** Biotin supplementation is crucial for efficient biotinylation of the expressed nanobodies. Biotin supplementation is unnecessary for $SENP^{EuB}$ protease, biotin ligase BirA and ALFA nanobodies.
13. Incubate the main culture at 18 °C with shaking for at least 1 h to accommodate the expression culture to this temperature.
14. Add 0.2 mM IPTG to the main culture to induce protein expression.
15. Incubate the induced main culture overnight at 18 °C if isolating anti-GFP nanobodies, or for 6 h at 18°C if isolating $SENP^{EuB}$ protease, biotin ligase BirA and ALFA nanobodies. The culture needs to be maintained under constant agitation at ~220 rpm.
16. Collect cells by centrifugation at 9,220*g* for 10 min at 4 °C.
17. Discard the supernatant and resuspend the cell pellet in 120 mL resuspension buffer.
18. Prepare 30 mL aliquots in 50 mL tubes and freeze in liquid nitrogen for a single freeze–thaw lysis cycle.
    ▲ **CAUTION** Wear safety goggles when handling liquid nitrogen.
    ■ **PAUSE POINT** Frozen *E. coli* cells can be stored at −80 °C for many months.
19. Rapidly thaw all frozen 50 mL tubes containing resuspended cells in lukewarm water and place on ice as soon as the last frozen clumps are thawed.
20. Combine thawed contents of all tubes and place the cell suspension into a 250 mL thin-walled metal beaker and transfer into an ice-water bath.
21. Lyse the cells by sonication using a Branson sonifier equipped with a flat tip. Perform four cycles of sonication, each 1 min long, at 100% amplitude. During each sonication cycle alternate 1 s pulse, with 2 s breaks. In between cycles, mix the cell lysate by swirling and allow to cool down for a minimum of 30 s, up to 1 min.
    ▲ **CRITICAL STEP** Sonication creates heat that can activate *E. coli* proteases and denature proteins, causing degradation, aggregation or excessive chaperone binding. Always keep the cell suspension in an ice-water bath. Ideally perform sonication in a 4 °C cold room. Keep the sonication probe properly submerged in the cell lysate to prevent foam formation and therefore protein aggregation.
22. Centrifuge the lysate for 30 min at 35,000*g* and 4 °C. Take off and pool the supernatant.
    ■ **PAUSE POINT** Lysate can be supplemented with either 250 mM sucrose or 10% vol/vol glycerol, and frozen in liquid nitrogen for storage at −80 °C for many months.
23. Equilibrate 2 mL settled Ni–NTA agarose resin with 20 mL resuspension buffer in a disposable 20 mL gravity flow column. Attach a long and wide, ideally blunt-ended, needle to the column to enhance the drip speed.
    ▲ **CRITICAL STEP** For the purification of the anti-GFP Nb, use 2× separate columns with 3 mL of settled Ni–NTA agarose resin in each column.
24. Transfer the equilibrated resin to a 250 mL glass bottle by resuspending it with 120 mL of cell lysate, and incubate for 1 h at 4 °C, with constant mixing.
    ▲ **CRITICAL STEP** Store the column and re-use in step 25 below.
25. Transfer the suspension back to the column (Step 23) and discard the flow through.
26. Wash the resin four times as detailed in the table below:

Wash cycle	Buffer	Volume (mL)
1	Resuspension buffer	20
2	LSBAR buffer	20
3	High-salt buffer	20
4	Resuspension buffer	20

▲ **CRITICAL STEP** If purifying the $SENP^{EuB}$ protease and the biotin ligase BirA, replace the LSABR buffer with low-salt ATP buffer (without biotin and RNase A).

Table 2 | Properties of all purified proteins

Construct	Protein	M_w (kDa)	ε_{280} (M^{-1} cm^{-1})
pTP396	His_{14}–Avi–27xGS–$SUMO^{Eu}$–19xGS–anti GFP Nb	31.8	33,920
pTS117	His_{14}–Avi–45xGS–anti GFP Nb	21.2	32,430
pTP298	His_{14}–Avi–27xGS–$SUMO^{Eu}$–2xGS–anti ALFA Nb	31.2	25,440
pTS118	His_{14}–Avi–45xGS–anti ALFA Nb	20.6	23,950
pAV0286	His_{14}–TEV–$SENP^{EuB}$ protease	32.4	45,380
pTP264	His_{14}–*bd*NEDD8–BirA	48.9	50,420

ε_{280}, extinction coefficient at 280 nm; M_w, molecular weight.

27. If using a needle to increase the flow, detach the needle, apply 1 mL imidazole elution buffer and discard the flow through. Elute stepwise by adding 0.5 mL imidazole elution buffer to the resin. Collect a total of 6× 0.5 mL fractions in separate 1.5 mL tubes.
    ▲ **CRITICAL STEP** If purifying the anti-GFP nanobody, apply 1.5 mL imidazole elution buffer to 3 mL resin and discard flow-through. Then collect 8× 0.5 mL fractions in separate 1.5 mL tubes.
28. Measure the UV absorbance of each fraction at 280 nm using a nanodrop and pool together fractions with the highest protein content. Make sure to use imidazole elution buffer as a blank control.
29. Estimate the concentration of the pooled purified protein using its specific extinction coefficient at 280 nm (ε_{280}) (Table 2).
30. Analyze protein expression and purification samples, collected as outlined in Supplementary Data 2, using SDS–PAGE and Coomassie staining. Compare the purified protein with Fig. 4a.
    ◆ **TROUBLESHOOTING**
31. (Optional) If purifying biotin ligase BirA or ALFA nanobodies, exchange the imidazole elution buffer to BirA storage buffer or ALFA Nb storage buffer, respectively, using a PD-10 desalting column. Purified ALFA nanobodies need to be further biotinylated in vitro with purified biotin ligase BirA, following the procedure outlined in Box 1.
    ▲ **CRITICAL STEP** BirA activity is sensitive to ionic strength and glycerol concentration.
32. Aliquot the purified proteins into multiple 10 and 50 µL aliquots in thin-walled 200 µL PCR tubes and flash freeze in liquid nitrogen. Store aliquots in small boxes or 50 mL tubes at −80 °C.
    ▲ **CRITICAL STEP** Although aliquots typically tolerate multiple freeze–thaw cycles, repeated freeze–thawing or prolonged incubation at elevated temperatures will result in protein aggregation. Mark freeze–thaw cycles on the tubes and keep aliquots on ice immediately after thawing.
    ■ **PAUSE POINT** Frozen protein aliquots are stable at −80 °C for years.
33. (Optional) Assess the biotinylation efficiency of purified nanobodies by following the procedure in Box 2 (Fig. 4b).
    ▲ **CRITICAL STEP** This is an optional quality control or troubleshooting step for the purified nanobodies. The percentage of nonbiotinylated nanobodies in the purified protein sample is ideally close to 100%. Lower biotinylation efficiencies can be accounted for in Part 3 by immobilizing more micrograms of of total Nb.
34. (Optional) Assess the activity of purified $SENP^{EuB}$ protease following this procedure:
    - Prepare 10 µM (0.32 µg/µL) of purified pTP396_His–Avi–$SUMO^{Eu}$-anti-GFP Nb in 10 µL of resuspension buffer, with and without 250 nM purified $SENP^{EuB}$
    - Incubate both reactions on ice for 20 min
    - Add 10 µL of 2× SDS–PAGE sample buffer to each sample and load half the volume (10 µL, ~1.6 µg of pTP396) in each lane
    - Analyze the test cleavage by SDS-PAGE and Coomassie staining. The expected outcome is shown in Fig. 4a
    - ▲ **CRITICAL STEP** This is an optional quality control or troubleshooting step for the purified $SENP^{EuB}$ protease. Nonfunctional $SENP^{EuB}$ protease preparations will preclude successful elution of the affinity isolated POI in Part 3.
      ◆ **TROUBLESHOOTING**

Part 2: generation of a stable human suspension cell line

● **TIMING 8 d**

Production of high-titer lentiviral supernatants

● **TIMING 5 d**

▲ **CRITICAL** Preparation of a stable human suspension cell line (Step 35) can be started in parallel to Step 1.

35. Thaw a single frozen aliquot of Lenti-X 293T cell, containing ~8–10 × 10^6 cells, in a water bath at 37 °C and add thawed cells to 10 mL of DMEM:FBS:Gln medium in a 15 mL tube.
36. Spin the culture for 3 min at 300*g* to pellet cells and remove DMSO.
37. Carefully aspirate the supernatant and resuspend the cell pellet in 1 mL of DMEM:FBS:Gln medium.
38. Add the cell suspension drop wise into a 15 cm dish containing 19 mL of DMEM:FBS:Gln medium. Distribute cells evenly using a careful 'figure eight' motion and incubate at 37 °C, in a 5% CO_2 cell culture incubator for 16–28 h.

    ▲ **CAUTION** Lentivirus and cell cultures are a potential biological hazard. Make sure to work in a certified biosafety cabinet and use proper sterile techniques. Adhere to the relevant institutional and governmental guidelines for recommended PPE and proper disposal of waste.

    ▲ **CRITICAL STEP** Prevent Lenti-X 293T cells from reaching confluency. We typically split cells 1:10 every 2 d. Treat these cells very gently to preserve high transfection efficiency, which is essential to reach a high lentiviral titer. Freeze aliquots of very early passages and avoid prolonged culture (<1 month).
39. Once the cells in the 15 cm dish have reached ~70% confluency, aspirate all culture medium, and wash gently with 20 mL of DPBS.
40. Detach the cells by addition of 8 mL of Trypsin–EDTA (0.25%) and incubate for 2 min at RT.
41. Add 12 mL of DMEM:FBS:Gln medium and resuspend the cells by pipetting.
42. Count resuspended cells using trypan blue staining on an automated cell counter and seed 1 × 10^6 cells per each well of a six-well plate in 2.5 mL DMEM:FBS:Gln medium.

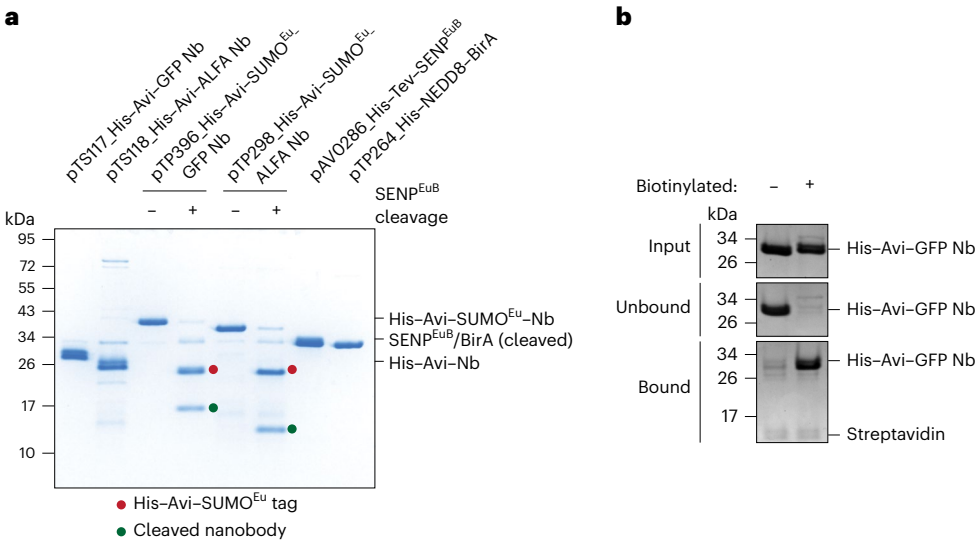

Fig. 4 | Quality control of purified proteins. a, 1 µg of each of the protein reagents required for the protocol was analyzed by SDS–PAGE and Coomassie staining to demonstrate their expected size and purity. pTP396 and pTP298 without cleavage (–) by $SENP^{EuB}$ represent the size expected for these proteins after they are initially purified from *E. coli*, while the lanes with cleavage (+) by $SENP^{EuB}$ represent the size expected for these proteins after they are used in a TagON purification. His–NEDD8-tagged BirA was purified by Ni^{2+}–chelate affinity purification and eluted by cleavage with NEDP1 protease[51,52], resulting in untagged BirA. The removal of the His-NEDD8 tag is not required. **b**, Coomassie-stained SDS–PAGE gel showing the quantitative binding of purified, biotinylated His–Avi–anti–GFP Nb (pTS117) to streptavidin beads (Box 2). The protein was expressed and purified from *E. coli* NEBExpress (–biotinylation) or *E. coli* AVB101 (+biotinylation).

BOX 2

Assessing the biotinylation efficiency of the purified Nbs

● **TIMING 4 h**

▲ **CRITICAL** The biotinylation efficiency of the purified Nbs can be assessed by testing their binding to streptavidin beads. Use prechilled buffer and handle tubes in an aluminum tube rack within an ice bath. Keep the magnetic rack on ice as well.

Procedure

1. Carefully resuspend magnetic streptavidin beads until the slurry is homogeneous and no clumps are left on the bottom or side of the bottle.
2. In a 1.5 mL tube, add 25 µL of slurry into 1 mL of STB buffer and mix well by pipetting.
3. Retrieve beads by placing the tube into a magnetic rack, wait 30–60 s to collect beads on the magnet, and then aspirate all buffer.
4. Dilute 12 µg of biotinylated Nb with STB buffer in 60 µL final volume. Take an input sample by mixing 10 µL of the dilution with 2.5 µL of 5× SDS–PAGE sample buffer in a clean 1.5 mL tube.
   ▲ **CRITICAL STEP** To assess biotinylation efficiency, it is essential to avoid oversaturating the beads. We found that the capacity of Pierce Streptavidin Magnetic Beads is ~0.5 µg of biotinylated Nb fusion protein per 1 µL slurry of beads.
5. Remove the tubes from step 3 from the magnetic rack and resuspend the beads with the remaining 50 µL of diluted Nb.
6. Incubate the samples for 30 min on ice with occasional gentle flicking of the tube to prevent beads from settling.
7. Retrieve the beads by placing the tubes into a magnetic rack. Pipette out the unbound fraction and take a sample by mixing 10 µL of the unbound fraction with 2.5 µL of 5× SDS–PAGE sample buffer in a clean 1.5 mL tube.
8. Wash the beads quickly by resuspending them in 1 mL of STB buffer.
9. Retrieve beads immediately by placing the tube into a magnetic rack and aspirate the buffer.
10. Repeat steps 8–9 above for a total of two wash steps.
11. For the last wash step, resuspend the beads in 100 µL of STB buffer to concentrate them in a smaller volume. Retrieve beads and remove the buffer completely.
12. Resuspend the beads in 60 µL of 2× SDS–PAGE sample buffer containing 0.5 M urea and boil for 10 min at 97 °C in a heat block. Retrieve the beads and transfer the elution sample into a new 1.5 mL Eppendorf tube. Boil samples of input and unbound fraction (Steps 4 and 7) under identical conditions.
13. Analyze the test binding by SDS–PAGE and Coomassie staining. Load 12.5 µL of input and unbound samples, as well as 10 µL of the elution per lane. The expected outcome is shown in Fig. 4b.
    ◆ **TROUBLESHOOTING**

43. Incubate the six-well plate for ~24 h at 37 °C in a humidified 5% CO_2 cell culture incubator.
    ▲ **CRITICAL STEP** 70–80% confluency produces the best lentiviral titers
44. (Optional) Freeze the excess of resuspended Lenti-X 293T cells from Step 42, following these steps:
    - Pellet the cell suspension for 3 min at 300*g* at RT
    - Aspirate the supernatant and resuspend the pellet in DMEM:FBS:Gln containing 10% (vol/vol) DMSO
    - Make 1 mL aliquots (each containing ~8–10 × 10^6 cells) and store them at −80 °C for 24 h in a cell freezing container
    - Transfer frozen cell aliquots into a cryo-tank for long-term storage
45. Once the cells have reached 70–80% confluency, transfect using the following procedure:
    - For each well of the six-well plate, mix the reagents in the amounts suggested in the table below:

Reagent	Amount
Opti-MEM I reduced-serum medium	250 µL
Transfer plasmid	1.25 µg
psPAX2	0.94 µg
pMD2.G	0.32 µg

    - Mix well by pipetting and add 7.5 µL TransIT 293 transfection reagent
    - Mix again by pipetting and incubate the reaction for 15 min at RT
    - Carefully dispense the newly formed transfection complexes to each well of the six-well plate in drops and mix the plate by swirling. Incubate the plate for 24–36 h, as in Step 43

▲ **CAUTION** Enhanced biosafety level measures are required for all the following steps.

▲ **CRITICAL STEP** Transfer plasmids contain repetitive sequences and should be prepared from recombination-deficient (recA$^-$) *E. coli* strains such as Stellar.

▲ **CRITICAL STEP** If multiple six wells need to be transfected, prepare a master mix without transfer plasmid. Aliquot the master mix and add transfer plasmid, followed by TransIT 293 transfection reagent.

46. After 24–36 h, verify the transfection efficiency of each well by visually assessing the percentage of fluorescent cells on a tissue-culture microscope. Nonfluorescent wells usually indicate problems with transfection and will yield low lentiviral titers.

◆ **TROUBLESHOOTING**

47. Place the plate back into the 37 °C, 5% CO_2 cell culture incubator, for an extra 24 h.
48. 48 h after the transfection, individually collect the cell culture medium from each well (~2.5 mL) in sterile 5 mL tubes and spin for 5 min at 500*g* to remove cell debris.

▲ **CRITICAL STEP** Use rotor buckets with aerosol-tight caps and assemble and disassemble them in the biosafety cabinet.

49. Collect the lentivirus-enriched supernatant in a clean 5 mL tube and use immediately to transduce Expi293F suspension cells (Step 54).

■ **PAUSE POINT** Single-use aliquots of lentiviral supernatants can be snap-frozen in liquid nitrogen for storage at −80 °C for multiple months with only slight loss of viral titer.

▲ **CRITICAL** Repeated freeze–thaw cycles result in substantial loss of lentiviral titer. It is best to not re-use once thawed aliquots.

Transduce and grow Expi293F suspension cells

● **TIMING 8 d**

▲ **CRITICAL** Step 50 can be started in parallel to Steps 1 and 35 to allow the Expi293F cells to recover for a few days before transduction.

50. Thaw a 1 mL aliquot containing ~20 × 10^6 Expi293F cells in a water bath at 37 °C and add to 10 mL of Expi medium contained in a 15 mL tube.
51. Spin the cell suspension for 3 min at 300*g* to remove the DMSO.
52. Resuspend the cell pellet in 1 mL of Expi medium and transfer to 49 mL of the same medium in a 125 mL Erlenmeyer flask with a vented cap.
53. Grow Expi293F cells in a 37 °C orbital shaker with 8% CO_2, >80% relative humidity and shaking at ~125 rpm for 72 h.

▲ **CRITICAL STEP** While in culture, maintain Expi293F stock between 0.5 and 2 × 10^6 cells/mL by diluting them 1:4 with fresh medium every 2 d. Freeze excess cells in Expi medium supplemented with 10% (vol/vol) DMSO to replenish cell aliquots.

▲ **CAUTION** Enhanced biosafety level measures are required for all the following steps.

54. In a clean 125 mL Erlenmeyer flask with a vented cap, mix 20 mL of Expi293F cells at 1 × 10^6 cells/mL density with 2.5 mL freshly collected lentiviral supernatant (Step 49).

▲ **CRITICAL STEP** Transduction efficiency depends strongly on the lentiviral titer. This can be optimized by mixing a constant amount of suspension cells with increasing volume of lentiviral supernatant. If needed, this step can easily be scaled up or down.

55. Transfer the flask to a shaking incubator and grow the cells overnight using the same conditions as described in Step 53.
56. The next day, transfer the culture into a 50 mL tube, place into rotor buckets with aerosol-tight caps in the biosafety cabinet and spin at 300*g* for 3 min to pellet transduced cells for a medium exchange.
57. Take off supernatant by pipetting and mix it with 10% bleach to inactivate the lentivirus.
58. Resuspend the cell pellet (Step 56) in 50 mL of Expi medium and transfer to a new 125 mL Erlenmeyer flask with a vented cap.
59. Grow cells for 72 h as described in step 53.
60. Evaluate transduction efficiency by analyzing the ratio of fluorescent cells 48 h after transduction. This can be done visually, using a tissue culture microscope, more quantitatively via flow cytometry (Box 3) or by western blotting. If using

BOX 3

Sorting of transduced Expi293F cells

● **TIMING 7–9 d**

Procedure

1. Collect 45 × 10^6 transduced and nontransduced Expi293F cells as described in Steps 63–65.
2. Resuspend the washed cell pellets in 3 mL of DPBS supplemented with 20% (vol/vol) FBS to a concentration of ~15 × 10^6 cells/mL.
3. Filter the resuspended cells through strainer caps into 5 mL round-bottom flow cytometry tubes and place the tubes on ice.
4. Analyze the nontransduced Expi293F cells and other relevant controls on a SONY SH800S cell sorter or equivalent, and adjust gates to select alive cells (using a FSC-Area versus SSC-A gate) and single cells (using a FSC-H versus FSC-A gate), as well as to determine background fluorescence level. Set gate for transduced fluorescent cells accordingly (e.g., using a GFP-A versus FSC-A gate to sort GFP-positive cells).
5. Collect at least 2–4 × 10^6 cells in a 15 mL collection tube prefilled with 3 mL Expi medium containing 0.5× pen–strep.
   ▲ **CRITICAL STEP** Since most cell sorters are not operated under perfectly sterile conditions, it is essential to add pen–strep to prevent contamination. However, only 0.5× pen–strep is used since Expi293F cells are more sensitive to antibiotics than regular HEK 293T cells.
6. Pellet collected cells to remove sheath fluid by centrifuging at 300*g* for 3 min at RT.
7. Resuspend the cell pellet in 10 mL Expi medium containing 0.5× pen–strep.
8. Transfer all of the cell suspension to a new empty 125 mL Erlenmeyer flask with a vented cap and grow overnight in a 37 °C orbital shaker with 8% CO_2, >80% relative humidity and shaking at ~125 rpm.
9. 24 h after sorting, pellet cells as in Step 6 and aspirate the supernatant.
10. Resuspend the cell pellet in 10 mL of Expi medium without pen–strep and transfer the culture back to the same flask.
    ▲ **CRITICAL STEP** Prolonged exposure to pen–strep reduces cell viability.
11. Let cells recover by growing them for 5–7 d as described in Step 8. Assess cell density every day, replenish medium and dilute cells to grow up a new stock of sorted cells. Freeze aliquots of early passages.

inducible cells, take off 2 mL of culture to a six-well plate and induce for 24 h with 1 µg/mL doxycycline (DOX) before analysis of transduction efficiency, as above.
◆ **TROUBLESHOOTING**

61. For small-scale expression trials, grow 50 mL of culture volume to ~8 × 10^6 cells/mL. If the expressed protein is not toxic, there should not be a notable drop in cell viability.
    ▲ **CRITICAL STEP** If using inducible cells, ensure to add 1 µg/mL DOX to the culture medium. Induction time can be varied, but 24–48 h before collection is a good starting point. Before induction, take off an aliquot of noninduced cells as control, at a density of 0.5 × 10^6 cells/mL in 50 mL and transfer them into a new flask to keep them in culture.
62. To collect cells, remove flasks from shaking incubator. If using noninducible cells, count cells and make up a separate stock at ~0.5 × 10^6 cells/mL in 50 mL in a new flask to keep them in culture in case sorting is necessary or the culture needs to be expanded for large-scale preparations.
63. Transfer the remaining cells into a 50 mL tube and pellet cells as described in Step 56.
64. Wash the pellet by resuspending it in 50 mL of DPBS and repeat the centrifugation as in Step 56.
65. Pour off the supernatant, weigh the dry cell pellet and proceed directly to cell lysis.
    ■ **PAUSE POINT** Alternatively, cell pellets can be frozen in liquid nitrogen and stored at −80 °C for multiple months.

Cell lysis

● **TIMING 2 h**

66. Lyse cells by performing mechanical lysis without detergent (Option A) or lysis in detergent (Option B). Refer to the 'Experimental design' section for guidance on choosing a lysis method.
    (A) **Mechanical lysis without detergent**
        (i) Resuspend the cell pellet (Step 65) in pre-chilled lysis buffer using ~7 mL per 1 g of cell pellet as a starting point.
        ▲ **CRITICAL STEP** Lysis buffer composition and volume is protein specific and should be optimized in small-scale experiments.
        ▲ **CRITICAL STEP** If frozen cell pellets are used, add lysis buffer to the frozen pellet and then thaw quickly in a lukewarm water bath at RT. Remove to ice immediately once thawing nears completion.
        (ii) Lyse the cells in dounce homogenizer with ~30–50 vertical strokes of a tight fit pestle. Alternatively, use a motor-driven Potter–Elvehjem homogenizer.
        (iii) Monitor the progression of cell lysis on a cell counter or tissue-culture microscope using trypan blue exclusion and continue lysing the cells until no more intact cells remain.
        (iv) Remove cell debris by centrifuging the lysate for 30 min at 35,000*g* and 4 °C.
        (v) Collect the supernatant, take a sample for SDS–PAGE and proceed to affinity purification (Step 74).
    (B) **Lysis in detergent**
        ▲ **CRITICAL** Optimal detergent type and salt concentration, as well as the ratio of solubilization buffer to cell pellet weight is membrane protein as well as application specific, and should be optimized in small-scale experiments. We routinely solubilize whole cell pellets, but in certain cases it is advantageous to prepare a membrane fraction before solubilization. The steps below offer a good starting point.
        (i) Solubilize the cell pellet by adding 7 mL of prechilled solubilization buffer containing 1% (wt/vol) of a detergent of choice per each gram of cell pellet and resuspend by pipetting.
        ▲ **CRITICAL STEP** If using frozen cell pellets, add solubilization buffer directly to frozen cells and then thaw quickly in a lukewarm water bath at RT. Remove to ice immediately once thawing nears completion.
        (ii) Incubate the suspension by mixing head-over-tail for 30 min at 4 °C.
        (iii) Discard cellular debris by centrifuging the lysate for 30 min at 35,000*g* and 4 °C.
        (iv) Collect the supernatant, take a sample for SDS–PAGE and proceed to affinity purification (Step 74).

Part 3: affinity purification

● **TIMING 3 h**

▲ **CRITICAL** The amount of beads and Nb needed depend on the expression level of the POI and should be optimized in small-scale experiments. The steps below offer a good starting point. Use prechilled buffer and handle tubes in an aluminum tube rack within an ice bath. Keep the magnetic rack on ice as well.

▲ **CRITICAL** Solubilization and wash buffers contain 1 mM DTT, which might reduce disulfide bonds in proteins that contain them. If your POI contains disulfide bonds, leave out DTT.

67. For every gram of cell pellet, equilibrate 60 µL of magnetic streptavidin beads slurry. Carefully resuspend the beads until the slurry is homogeneous and no clumps are left on the bottom or side of the bottle. Aliquot 60 µL of slurry into a 1.5 mL tube.
68. Retrieve beads by placing the tubes into a magnetic rack, wait for 30 s to 1 min to collect all beads and aspirate all liquid.
69. Wash the beads by resuspending them in 1 mL of wash buffer, retrieve beads as in Step 68 and aspirate the wash buffer.
    ▲ **CRITICAL STEP** For membrane proteins, the wash buffer should be supplemented with a detergent at a concentration above its critical micelle concentration to keep the protein solubilized. We recommend using a concentration that corresponds to 2.5× critical micelle concentration.

70. For every 60 µL of magnetic streptavidin beads slurry, immobilize 20 µg of biotinylated GFP or ALFA Nb. Resuspend the beads in 500 µL of wash buffer containing prediluted biotinylated Nb and incubate the slurry for 15 min, mixing head-over-tail at 4 °C.
71. Retrieve the beads using a magnet as in Step 68 and aspirate the wash buffer. Remove the tubes from the magnetic rack and resuspend the beads in 500 µL of wash buffer containing 100 µM biotin or $dPEG_{24}$–biotin acid.
72. Incubate the suspension for 5 min, leaving the tube resting on ice to block unoccupied biotin binding sites on streptavidin.
    ▲ **CRITICAL STEP** Blocking with biotin strongly reduces background binding of endogenous biotinylated proteins. Blocking with $dPEG_{24}$–biotin acid adds additional negative charge and further reduces nonspecific background binding to the beads.
73. Retrieve the beads using a magnetic rack (Step 68) and aspirate the blocking buffer.
74. Resuspend the beads with 1 mL of cleared cell lysate and add to the remainder of cell lysate in a clean 5, 15 or 50 mL tube, depending on scale. Mix the tube rapidly by inversion.
75. Incubate the mixture for 1 h at 4 °C, rotating.
76. Retrieve the beads using a magnetic rack (Step 68). Depending on the final scale, a magnetic rack that can hold 15 mL or even 50 mL tubes should be used at this step. Take a sample of the cleared cell lysate (unbound fraction) for SDS–PAGE.
77. Aspirate the cell lysate, resuspend the beads in 1 mL of wash buffer and transfer them into a clean 1.5 mL tube.
78. Retrieve beads immediately using a magnetic rack (Step 68).
79. Wash beads three times with 1 mL wash buffer within the same tube, performing Steps 77–78.
80. For the fourth wash, resuspend beads with 100 µL wash buffer (−ATP) and transfer to a new tube. Use slightly more volume if more beads were used.
81. Resuspend the beads in wash buffer containing 250 nM purified $SENP^{EuB}$ for the isolation of target protein in its tagged (TagON) or untagged (TagOFF) form. Use a volume corresponding to the original volume of bead slurry used. To keep the eluate more concentrated, up to one-third of the original volume can be used.
    ▲ **CRITICAL STEP** If a soluble protein is stable in the presence of detergents (such as 0.05% (vol/vol) Tween-20 or Triton-X-100), these can be included during elution to improve recovery by preventing nonspecific binding of the cleaved protein to the beads.
82. Incubate the samples for 20 min on ice for protease elution to complete.
83. Retrieve beads using a magnetic rack (Step 68) and collect the supernatant by pipetting and transfer into a clean 1.5 mL tube. Keep tube with beads for postelution as described in Steps 86–87.
84. Spin eluate for 5 min at 15,000*g* at 4 °C to pellet magnetic beads that sometimes get carried over.
85. Collect the supernatant again by pipetting and transfer into a clean 1.5 mL tube. This is your final native elution sample containing your purified POI and can be used directly for structural and functional assays. Take a sample for SDS–PAGE. Alternatively, the purified protein can be flash-frozen in multiple aliquots in liquid nitrogen for storage at −80 °C.
    ▲ **CRITICAL STEP** Freeze–thawing purified proteins might substantially decrease sample quality. Always perform a test freeze–thaw cycle with a small aliquot and verify protein quality using your assay of choice.
86. To analyze what remained on the beads, resuspend the beads from Step 83 in 2× SDS–PAGE sample buffer containing 0.5 M urea and boil for 10 min at 97 °C.
87. Retrieve beads using a magnetic rack and collect postelution sample.
88. Analyze samples of the eluate and postelution by SDS–PAGE. It is recommended loading 1, 2 and 4 µL per lane and stain the gel with either Sypro Ruby or Coomassie. Samples of the cell lysate before (Steps 66a(v) and 66b(iv)) and after (Step 76) affinity purification can be analyzed by western blotting with a POI- or tag-specific antibody to assess the degree of depletion of the tagged POI from the lysate.
    ◆ **TROUBLESHOOTING**
89. (Optional) Perform a SEC run of the native elution sample from Step 85 to remove excess tagged subunit and Nb, and to improve sample homogeneity (Box 4).

BOX 4

(Optional) Size-exclusion chromatography

● **TIMING 1 d**

▲ **CRITICAL** We prefer using the Superose 6 Increase 3.2/300 column due its small column volume of only 2.4 mL, which limits sample dilution during the run.

Procedure

1. Equilibrate HPLC, Superose 6 Increase 3.2/300 column and 50 µL sample loop in filtered and degassed wash buffer without protease inhibitor cocktail according to the manufacturer's instructions.
2. Load the sample from Step 85 into a 50 µL hamilton syringe and inject into sample loop.
   ▲ **CRITICAL STEP** If eluate volume is above 50 µL, concentrate using Amicon Ultra 0.5 mL centrifugal filters with the appropriate molecular weight cut-off.
3. Inject sample loop content onto the equilibrated column from Step 1 and elute in 100 µL fractions over 1.2× column volume.
4. Measure absorbance at 280 nm, and optionally at 490 nm to detect GFP fluorescence or 260 nm to detect nonprotein contaminants.
5. Choose fractions spanning sample peak and prepare SDS–PAGE sample by mixing 10 µL of each fraction with 2.5 µL 5× SDS–PAGE sample buffer.
   ▲ **CRITICAL STEP** If this is a high yield purification you may instead prefer to dilute a smaller volume of each fraction to 10 µL with wash buffer to avoid overloading the gel.
6. Boil samples for 10 min at 97 °C, analyze by SDS–PAGE and stain the gel with either Sypro Ruby or Coomassie.
7. Pool protein-containing fractions together in a clean 1.5 mL Eppendorf tube.
8. Wash HPLC, Superose 6 Increase 3.2/300 column and 50 µL sample loop used in Steps 1–3 with degassed ddH_2O according to the manufacturer's instructions.
9. (Optional) If necessary, concentrate protein using Amicon Ultra 0.5 mL centrifugal filters with the appropriate molecular weight cut-off.
10. Use the purified protein sample directly for structural or functional assays or flash freeze multiple aliquots in liquid nitrogen for storage at −80 °C for many years.
    ▲ **CRITICAL STEP** Freeze–thawing purified proteins might substantially decrease sample quality. Always perform a test freeze–thaw cycle with a small aliquot and verify protein quality using your assay of choice.

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
30	No protein after Ni^{2+} purification	Forgot to add IPTG to induce protein expression	Add IPTG to 0.2 mM
		Forgot to add imidazole to elution buffer	Use elution buffer containing imidazole at 500 mM
	Low protein yield after Ni^{2+} purification	Protein might have aggregated during expression due to elevated expression temperature	Make sure to adapt diluted main culture to 18 °C for 1 h before induction and maintain induced main culture at 18 °C
		Protein might have aggregated during purification	Keep *E. coli* cells, lysate and purified protein on ice at all times and prevent foaming during sonication
	E. coli purified protein is partially degraded	Activation of *E. coli* proteases during cell lysis	Make sure to add 1 mM PMSF to resuspension buffer and keep *E. coli* cells, lysate and purified protein on ice at all times
34	$SENP^{EuB}$ does not cleave nanobodies in solution	Poor-quality $SENP^{EuB}$ preparation	Check $SENP^{EuB}$ by SDS–PAGE. Repeat $SENP^{EuB}$ preparation

Table 3 (continued) | Troubleshooting table

Step	Problem	Possible reason	Solution
46	Transfected Lenti-X 293T cells nonfluorescent	Poor DNA quality	Check plasmid DNA integrity and prepare new stock. Perform additional wash steps during DNA Mini or Maxi prep as outlined in your kit's manual
60	Low transduction efficiency	Bad cell health: Lenti-X 293T cells passaged for prolonged time and/or overgrown before seeding	Thaw a new Lenti-X 293T cell aliquot and avoid overgrowing and passaging them >1 month
		Transfected Lenti-X 293T cells at too high or too low confluency	Make sure to transfect at ~70–80% confluency
		Forgot or added wrong amounts or bad quality of packaging and envelope plasmids	Make sure to add correct amounts of packaging and envelope plasmids and consider preparing new stocks
		DNA insert size comes close to or exceeds packaging limit (~8 kbp between 5′ and 3′ LTRs)	Sort transduced cells and grow up a fully transduced population
		POI is toxic to cells	Try using inducible Expi293F TetR+ cell line, expressing an inactive mutant, or transient expression via chemical transfection
88	Low protein yield from Expi293F cells	Poor lentiviral titer or transduction efficiency	Repeat lentiviral preparation or sort transduced cells and grow up a fully transduced population
		Inefficient protease elution	Analyze postelution sample by SDS–PAGE and check for uncleaved bait (TagON) or bait–prey complex (TagOFF). Check protease activity in solution (Step 34) and repeat protease preparation if inactive. In some cases, the SUMO tag may be less accessible, requiring increased protease concentration and incubation time to achieve efficient elution
		Eluted protein non-specifically sticks to beads (sometimes for soluble proteins in buffer without detergent)	Include low concentration of detergent (e.g., 0.05% (vol/vol) Tween-20 or Triton-X-100) in elution buffer to reduce sticking. Block streptavidin beads with biotin-PEG-COOH instead of biotin to increase charge repulsion between beads and protein. The cleaved protein may be released in additional wash steps
		Protein aggregated due to suboptimal lysis buffer composition	Optimize lysis buffer composition e.g., salt, or detergent concentration in small-scale pretests
	Low recovery of the prey's endogenous protein complex subunits	Tagged protein is stable on its own after overexpression	Consider tagging a different, less stable subunit of the complex. Alternatively: (1) use Freestyle 293-F cells instead; (2) use inducible Expi293F cell line and vary DOX induction time; (3) exchange CMV to weaker PGK or UbC promoter or remove WPRE element; (4) purify membrane proteins from the membrane fraction rather than whole cells
	Insufficient depletion of the tagged POI from input cell lysate detected by western blotting	Not enough Nb or beads were used	Immobilize more Nb on more beads per gram of cell pellet to increase depletion of tagged POI and therefore final yield
Box 2, step 13	GFP Nanobodies produced in *E. coli* AVB101 do not bind to streptavidin beads	Forgot to supplement AVB101 main culture with biotin or forgot to use cam to maintain BirA plasmid	Make sure to propagate AVB101 with 10 µg/mL cam and add 50 µM biotin to main culture
	In vitro biotinylated ALFA nanobodies do not bind to streptavidin beads	Poor quality BirA preparation or wrong biotinylation buffer composition	Check BirA by SDS–PAGE and check 5× biotinylation buffer composition. Repeat BirA preparation

Timing

Part 1: Steps 1–34, generation of biotinylated nanobodies, $SENP^{EuB}$, and biotin ligase BirA: 4–5 d
Steps 1–6, transformation *E. coli* cells via heat shock: 1 d
Steps 7–9, starting a preculture: 1 d
Steps 10–15, starting and inducing the main culture: 1 d
Steps 16–18, collect the main culture and freeze resuspended cell pellet: 1 h
Steps 19–22, thaw frozen cells for cell lysis by sonication: 1 h
Steps 23–28, binding to Ni^{2+}-chelate affinity resin and elution: 3 h
Steps 29–32, concentration assessment and storage: 1 h

Step 33, (Optional) assessment of biotinylation efficiency: 4 h
Step 34, (Optional) assessment of SENP^{EuB} protease activity: 3 h

Part 2: Steps 35–66, generation of a stable human suspension cell line: 8 d (unsorted), 15 d (sorted)
Steps 35–49, production of high-titer lentiviral supernatants: 4 d
Steps 35–38, culture Lenti-X 293T cells: 1 d
Steps 39–44, seed Lenti-X 293T cells into six-well plates: 1 d
Step 45–47, transfection and lenti production: 2 d
Steps 48–49, collect lentiviral supernatant: 0.5 h
Steps 50–61, transduce and grow Expi293F suspension cells: 8 d
Steps 50–53, culture Expi293F cells: 3 d
Steps 54–55, transduce with collected lentiviral supernatant: 1 d
Steps 56–59, exchange medium: 0.5 h
Step 60, evaluation of transduction efficiency: 0.5 h
Step 61, growth phase: 4 d
Steps 62–65, cell collection: 1 h
Step 66, cell lysis: 2 h

Part 3: Steps 67–89, affinity purification: 3 h

Anticipated results

This protocol has been successfully used to isolate numerous soluble and membrane-bound proteins for structural, functional and mass spectrometry analysis (Fig. 5). In particular, to demonstrate the utility of both (TagON and TagOFF) purification strategies, we used the nine-subunit EMC as a model substrate. GFP- or ALFA-tagged EMC2 or EMC5 are incorporated into the EMC in place of their respective endogenous subunits, allowing the intact complex to be isolated in high purity (Fig. 5a,b). Using the TagOFF strategy, capture of GFP–SUMO^{Eu}–EMC2 with a noncleavable anti-GFP Nb allowed isolation of completely untagged EMC under native conditions following SENP^{EuB} cleavage, while the affinity tag and Nb were retained on the beads. As a proof-of-concept, we successfully isolated other challenging soluble and membrane protein complexes by tagging a single subunit (Fig. 5c). For example, GFP–SRP72 efficiently incorporated into the ribonucleoprotein signal recognition particle (SRP), which could be isolated under native conditions. Purified SRP was fully functional and bound to stalled ribosome nascent chains exposing a transmembrane domain in a hydrophobicity sensitive manner as observed before for endogenous SRP[50] (Fig. 5d). Similarly, we used this strategy to isolate the entire 26S proteasome from cells expressing GFP-tagged RPN11, the ER-resident membrane protein complex NOMO–NCLN–TMEM147 via TMEM147–GFP, and the plasma membrane localized anion exchanger SLC4A2/AE2. Our strategy also guarantees improved yields for challenging multisubunit membrane protein complexes. For example, we could isolate 0.45 mg of EMC or ~0.8 mg of MTCH2 per 1 L of suspension cell culture. Much greater yields can be achieved for soluble protein complexes and, in particular, for soluble monomeric proteins.

Another critical consideration for the success of the protocol is to take great care to achieve high-titer lentiviral production as outlined above. Only with good lentiviral preparations can high transduction efficiencies of suspension cells be reached. In many cases, transduction efficiencies are readily between 80% and 90%, as can be seen for the transduction of Expi293F cells with an EMC3–GFP-encoding lentivirus (Fig. 6a,b). The high percentage of GFP-positive cells allowed direct purification of EMC3–GFP-containing EMC complexes without prior sorting of the cell line (Fig. 6c). In certain cases, (e.g., for much larger proteins) lower transduction efficiencies necessitate either larger-scale cultures to achieve a comparable yield or an additional sorting step (Box 3) to obtain a fully transduced

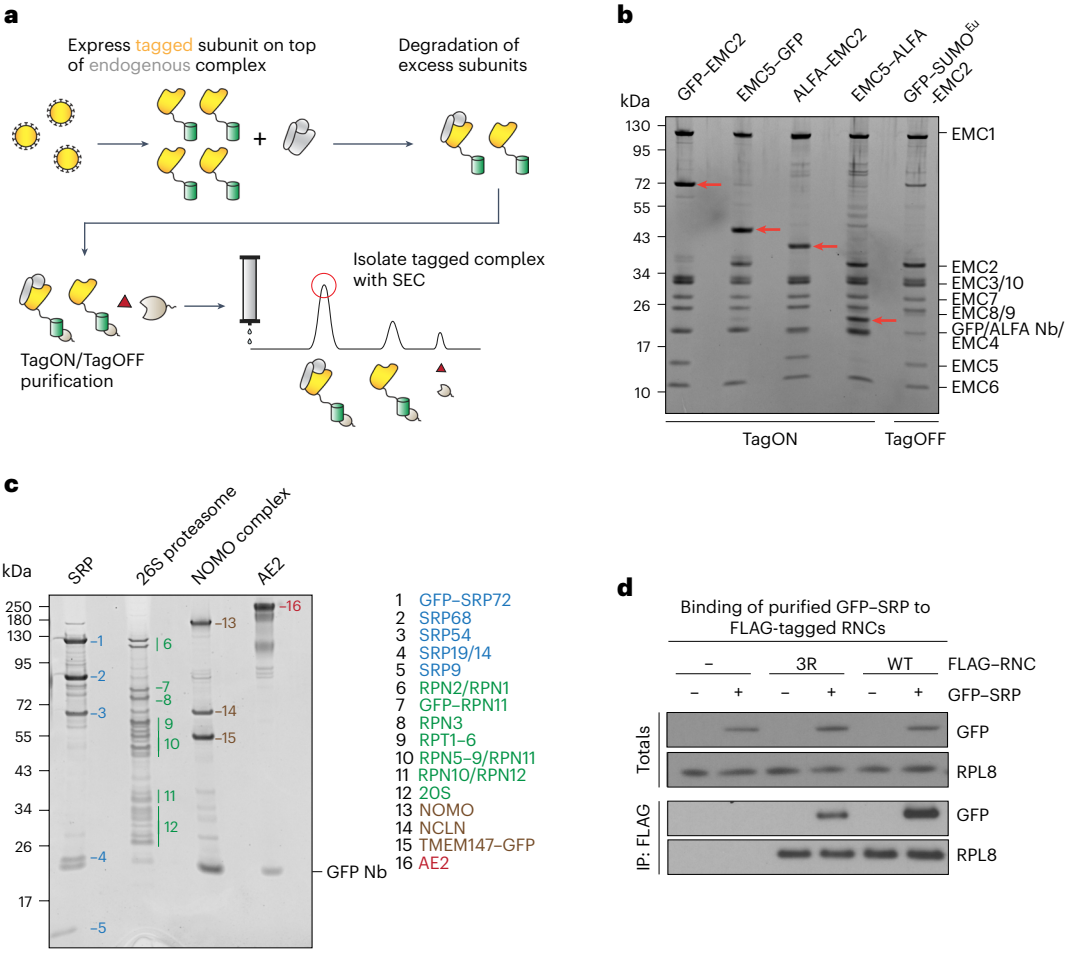

Fig. 5 | Purification of soluble and membrane protein complexes from human suspension cells. a, Ectopically expressed tagged subunits of a protein complex replace their endogenous untagged counterparts (gray) through proteasomal degradation of excess subunits. After purification, any remaining excess subunit and Nb can be removed via SEC. **b**, Peak fractions of SEC runs of the EMC purified via GFP- or ALFA-tags fused to either EMC2 or EMC5 subunits using the TagON strategy. Following the TagOFF strategy, the GFP–SUMO^{Eu}–EMC2 cell line allowed purification of completely untagged EMC. Tagged subunits are marked with red arrows. **c**, SEC peaks of various samples purified via GFP-tags, including the SRP, 26S proteasome, NOMO–NCLN–TMEM147 complex, as well as SLC4A2/AE2. **d**, Purified GFP–SRP is functional. Stalled ribosome nascent chain complexes (RNC) exposing either a WT or triple arginine mutant (3R) transferrin transmembrane domain (TMD) with 3× FLAG tag were produced by in vitro translation in rabbit reticulocyte extract supplemented with purified GFP–SRP complex where indicated. Total and FLAG–IP samples were analyzed by SDS–PAGE and western blotting. GFP–SRP co-purified strongest with WT TMD RNCs, as shown before for endogenous SRP[50].

polyclonal cell line. Sorting typically postpones first purification trials by one extra week, which is required for recovery and expansion of the sorted cells. For example, if a maximum transduction efficiency of only 10% is achieved, one could isolate 4.5 million GFP-expressing cells from an initial culture containing 45 million cells in ~2 h of sorting on a SONY SH800S cell sorter or comparable instrument (Box 3). This culture would then take ~5–7 d to recover and grow back to the original amount of 45 million cells, all of which will now be expressing the POI.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

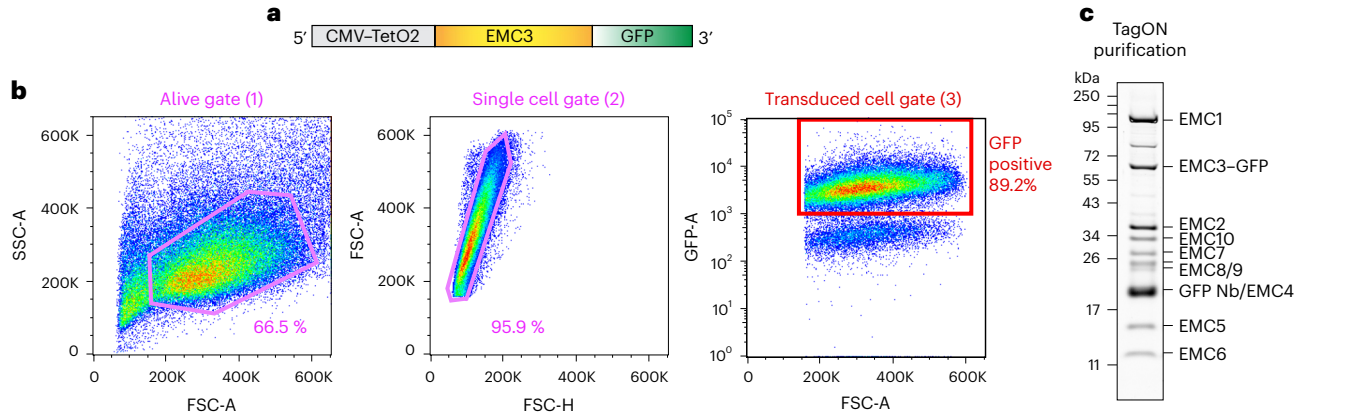

Fig. 6 | TagON purification of the EMC from a stable Expi293F EMC3–GFP suspension cell line. a, Schematic overview of the EMC3–GFP expression cassette encoded on the lentiviral transfer plasmid. **b**, Flow cytometry dot plots of Expi293F cells 48 h after transduction, as described in Box 3. Cells were gated first for alive cells (side scatter-area (SSC-A) versus forward scatter-area (FSC-A)), second for single cells (FSC-A versus forward scatter-height (FSC-H)) and third for GFP-fluorescent cells (GFP-A versus FSC-A). Gate 3 highlights a clearly separated population, which contains nearly 90% of alive, single GFP-positive cells. The placement of the GFP-positive gate is chosen based on the analysis of a nontransduced WT cell population in parallel under identical conditions. **c**, TagON purification of the EMC via EMC3–GFP. An aliquot of the elution was analyzed by SDS–PAGE and Sypro Ruby staining.

Data availability

The lentiviral transfer plasmids and bacterial expression plasmids described in this study are available from Addgene. Addgene IDs of all plasmids are listed in Table 1. Source data are provided with this paper.

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Author contributions

T.A.S., R.M.V. and T.P. conceived and designed this study. T.A.S., G.P.T., M.H., S.W., V.N.N., C.D. and T.P. carried out the experiments and interpreted data. T.A.S., R.M.V. and T.P. wrote the protocol, and all authors provided feedback on its final version.

Competing interests

R.M.V. and G.P.T. are consultants for Gates Biosciences, and R.M.V. is an equity holder.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41596-023-00904-w.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-023-00904-w.

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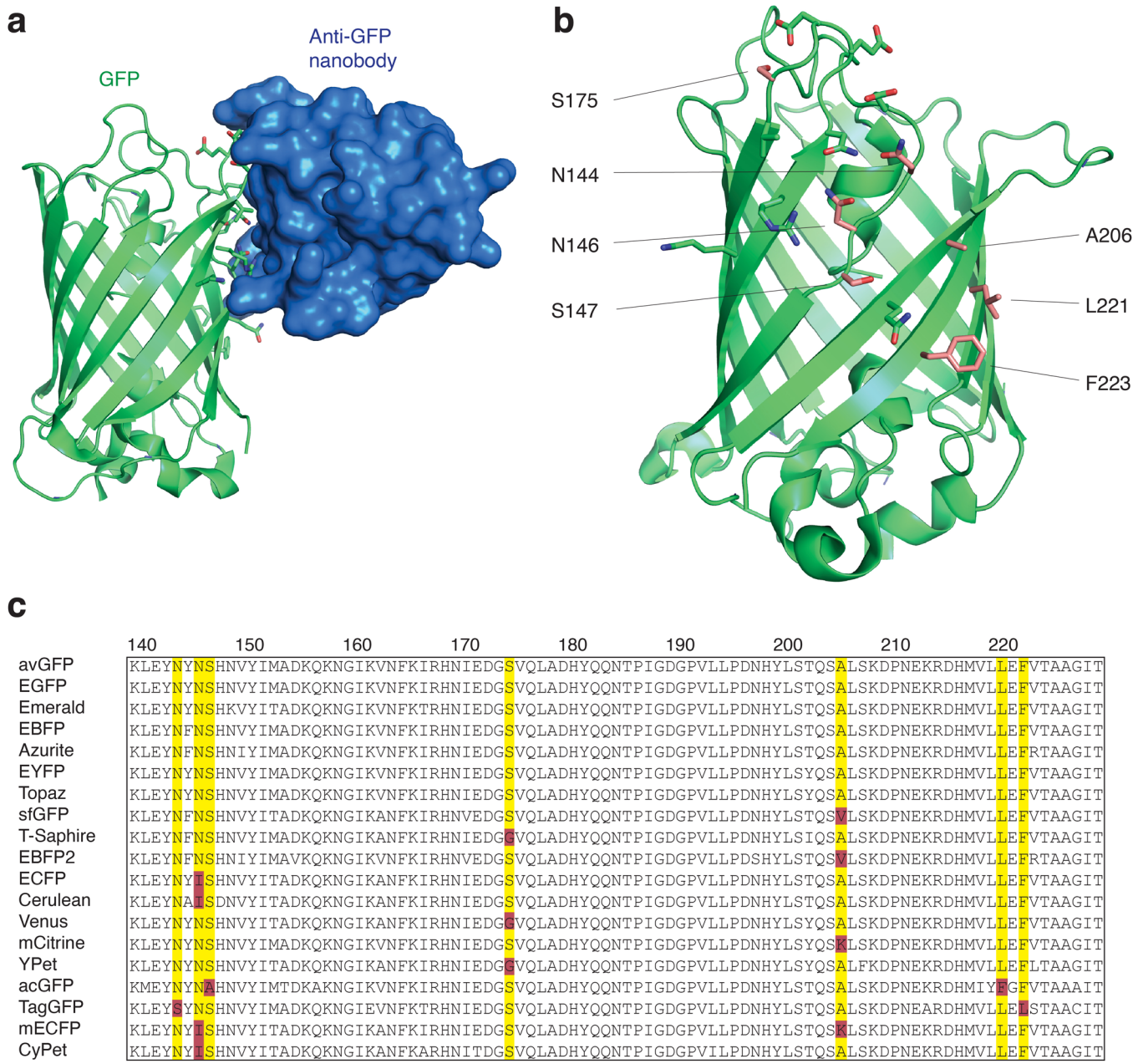
Peer review information *Nature Protocols* thanks Eric Gouaux and Jan Steyaert for their contribution to the peer review of this work.

Related links

Key references using this protocol

Pleiner, T. et al. *eLife* **4**, e11349 (2015): https://doi.org/10.7554/eLife.11349
Pleiner, T. et al. *Science* **369**, 433–436 (2020): https://doi.org/10.1126/science.abb5008
Guna, A. et al. *Science* **378**, 317–322 (2022): https://doi.org/10.1126/science.add1856



Extended Data Fig. 1 | Overview of anti-GFP nanobody compatible fluorescent protein variants. (**a**) Crystal structure of GFP bound to anti-GFP nanobody (Nb) (PDB ID: 3K1K)[43] with GFP shown in green with cartoon rendering, anti-GFP Nb shown in blue with surface rendering, and specific residues on GFP that make contact with the anti-GFP Nb shown in stick rendering. (**b**) Front-view of the anti-GFP nanobody binding surface of GFP with participating residues shown in stick rendering. Residues mutated in other fluorescent protein variants colored in salmon. (**c**) Multiple sequence alignment of various fluorescent protein variants. Columns corresponding to residues contacted by the anti-GFP Nb are highlighted in yellow, and any mutations to these positions are highlighted in red. Mutation of I146N was previously shown to restore anti-GFP Nb binding in CFP variants[53].

nature portfolio

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Policy information about availability of computer code

Data collection	Unicorn for AKTA FPLC operation, SONY SH800 software for cell sorter operation
Data analysis	Fiji image processing package (quantification of fig. 3B), FlowJo for flow cytometry data analysis

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The lentiviral transfer plasmids and bacterial expression plasmids described in this study are available from Addgene. Addgene IDs of all plasmids are listed in Table 1. Source data showing uncropped gels and western blots can be found in the supplements.

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Timing	
Data exclusions	
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Randomization	

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Timing and spatial scale	
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○	● Antibodies
○	● Eukaryotic cell lines
●	○ Palaeontology and archaeology
●	○ Animals and other organisms
●	○ Clinical data
●	○ Dual use research of concern
●	○ Plants

Methods

n/a	Involved in the study
●	○ ChIP-seq
○	● Flow cytometry
●	○ MRI-based neuroimaging

Antibodies

Antibodies used: Anti-RPL8 (Abcam #ab169538), Anti-GFP (homemade)

Validation: Anti-RPL8: https://www.abcam.com/products/primary-antibodies/rpl8-antibody-epr11630-ab169538.html

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s): Expi 293F: Thermo fisher catalog #A14527

Authentication: Cells were analyzed to confirm size and growth characteristics which matched the manufacturers description.

Mycoplasma contamination: Cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register)

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Specimen deposition

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Data collection	
Outcomes	

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○	○	Increase transmissibility of a pathogen
○	○	Alter the host range of a pathogen
○	○	Enable evasion of diagnostic/detection modalities
○	○	Enable the weaponization of a biological agent or toxin
○	○	Any other potentially harmful combination of experiments and agents

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Seed stocks	
Novel plant genotypes	
Authentication	

ChIP-seq

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Data access links
May remain private before publication

Files in database submission

Genome browser session
(e.g. UCSC)

Methodology

Replicates

Sequencing depth

Antibodies

Peak calling parameters

Data quality

Software

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☑ 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).

☑ All plots are contour plots with outliers or pseudocolor plots.

☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation: Expi293F tissue culture cells grown in suspension were used. No sample preparation was performed.

Instrument: Sony SH800S

Software: The manufacturer provided software was used to collect the data. The Flowio software population package was used to analyze

Cell population abundance: GFP-expressing cells were readily separated from non-expressing cells in the analysis. These cells were then isolated and used

Gating strategy: Live cells were gated by plotting SSC-H vs. FSC-H, single cells were gated by plotting FSC-A vs. FSC-H, and GFP-expressing cells

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

Magnetic resonance imaging

Experimental design

Design type

Design specifications

Behavioral performance measures

Acquisition

Imaging type(s)

Field strength

Sequence & imaging parameters

Area of acquisition

Diffusion MRI ○ Used ○ Not used

Preprocessing

Preprocessing software

Normalization

Normalization template

Noise and artifact removal

Volume censoring

Statistical modeling & inference

Model type and settings

Effect(s) tested

Specify type of analysis: Whole brain ROI-based Both

Statistic type for inference

(See Eklund et al. 2016)

Correction

Models & analysis

n/a | Involved in the study

- Functional and/or effective connectivity
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- Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Graph analysis

Multivariate modeling and predictive analysis