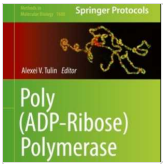

Sep 02, 2021

Validating TBMs by Fluorescence Polarization (FP)

Book Chapter

In 1 collection

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ABSTRACT

This protocol is part of a collection: Identifying and Validating Tankyrase Binders and Substrates: A Candidate Approach

The poly(ADP-ribose)polymerase (PARP) enzyme tankyrase (TNKS/ARTD5, TNKS2/ARTD6) uses its ankyrin repeat clusters (ARCs) to recognize degenerate peptide motifs in a wide range of proteins, thereby recruiting such proteins and their complexes for scaffolding and/or poly(ADP-ribosyl)ation. Here, we provide guidance for predicting putative tankyrase-binding motifs, based on the previously delineated peptide sequence rules and existing structural information. We present a general method for the expression and purification of tankyrase ARCs from *Escherichia coli* and outline a fluorescence polarization assay to quantitatively assess direct ARC–TBM peptide interactions. We provide a basic protocol for evaluating binding and poly(ADP-ribosyl)ation of full-length candidate interacting proteins by full-length tankyrase in mammalian cells.

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COLLECTIONS

Identifying and Validating Tankyrase Binders and Substrates: A Candidate Approach

KEYWORDS

Tankyrase, PARP, Poly(ADP-ribosyl)ation, Tankyrase-binding peptide motif, Enzyme–substrate relationships, Protein-protein interactions, Protein expression, Protein purification, Fluorescence polarization, FP, Structural biology

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PARENT PROTOCOLS

Part of collection

Identifying and Validating Tankyrase Binders and Substrates: A Candidate Approach

GUIDELINES

The details of many standard experimental methods not addressed here can be found in [21].

Notes

1. We recommend using vectors with a kanamycin selection marker. Ampicillin hydrolysis by secreted β-lactamase and under low pH increases the proportion of cells lacking the plasmid, which decreases protein yield [33].

2. RIL cells contain additional tRNAs for codons of Arg, Ile, and Leu that are otherwise rare in *E. coli*. The plasmid bearing these genes contains a chloramphenicol selection marker.

3. We recommend using one 5 mL column per 4 L expression culture. If using larger volumes, connect additional column(s) in series. Ni^{2+}affinity columns can easily be reconstituted by stripping from and re-charging with Ni^{2+}as per the manufacturer's instructions.

4. Use a Superdex 200 column for ARC1-5 constructs. All other ARC constructs are sufficiently small for the Superdex 75 column.

5. TCEP is more stable than DTT and not volatile, unlike β-mercaptoethanol or DTT. However, TCEP is acidic and will affect the final pH of the buffer unless the 0.5 mM stock solution is pH-adjusted with NaOH. To keep costs down, TCEP is only used in the final purification step.

6. HEPES is preferred for the final protein and in experiments due to its lower temperature dependency compared with Tris [34]. Tris was chosen for the affinity purification step due to its weak interaction with Ni^{2+}, which would help decrease background (contaminant) binding [35]. If HEPES buffer is used in the affinity purification step, the imidazole concentration may need to be increased to achieve comparably low background binding.

7. Detergent is used to reduce nonspecific binding and surface tension that may interfere with fluorescence intensity readings in the plate format. The choice of detergent and its concentration is empirical. We have also had good experience with using 100 µg/mL bovine serum albumin (BSA), but use CHAPS here since it is more effective at reducing nonspecific binding.

8. Keeping the fluorescently labeled peptides as short as possible increases the ΔFP signal window between the bound and unbound states. Ideally, peptides should be HPLC-purified; however, this is not always realistically

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achievable, especially if large numbers of peptides are to be compared and no access to in-house solid-state peptide synthesis is available. To save costs, peptides can be used at non-HPLC-purified grade, in which case a capping step is strongly recommended after each amino acid coupling reaction to prevent peptide synthesis intermediates from being linked to the fluorophore in the final coupling step and affecting the assay [7].

9. Alternatively, fluorescently labeled antibodies can be used for detection with appropriate fluorescence imaging systems. Instead of the anti-PAR antibody, an anti-pan-ADP-ribose or anti-poly(ADP-ribose) binding reagents (MABE1016 and MABE1032, respectively, Millipore) may be explored.

10. Compared to LB, TB is richer and enables higher cell densities in the log phase of growth. Cooling the cultures before IPTG induction slows down expression, thereby facilitating correct protein folding and increasing protein solubility.

11. For ARC2-3 and ARC1-5 constructs described here, a minimal NaCl concentration of 300 mM needs to be maintained, compared to 100 mM for all single-ARC constructs, and glycerol may further help stabilize the protein [7].

12. For large volumes, it may be easier to lyse cells in two batches. Alternative disruption techniques can be used, such as homogenization by an Avestin EmulsiFlex homogenizer.

13. If available with the FPLC setup, a superloop or, preferably, a sample pump can be used to load the column.

14. Depending on the ARC construct and the final concentration of imidazole in the dialysis buffer, ARCs can bind weakly to the Ni^{2+}column even after tag cleavage . They can be eluted with a further imidazole gradient. While this adds one more step to the purification protocol, it enables even higher purities to be achieved.

15. The Q column step can also be performed before concentration, directly using the flow-through from the second Ni^{2+} affinity column; however, prior concentration saves time in loading the column.

16. Confirm the pH of the peptide stock solution before measuring the concentration. Acidity, for example due to residual trifluoroacetic acid from the peptide synthesis, will strongly affect fluorophore absorption.

17. We recommend leaving wells in the outermost rows and columns empty to reduce the microplate “edge effect,” a discrepancy in readings between the central and peripheral wells [36].

18. It is important to add the different transfection reagents in the specified order to ensure proper calcium phosphate–DNA particle formation.

19. Avoid using micropipette tips to transfer the cell suspension after scraping. The small opening of the tips can cause cells to break due to shearing. You can cut off the tips to avoid this risk.

20. For directly HRP-coupled antibodies, azide as a preservative should be avoided as it inhibits HRP activity .

21. The Western blot protocol can be adapted for film-free chemiluminescence detection or fluorescence-based detection.

22. Attributing a PAR signal to a particular protein by molecular weight may be challenging, in part due to possible PAR-induced mobility shifts in SDS-PAGE. Since the tankyrase substrate candidates are immunoprecipitated from cell lysates, it is possible that the observed PAR signal corresponds to other PARylated proteins in a protein complex. Ultimate confirmation of substrates can be obtained from experiments with purified proteins or PAR site mapping by mass spectrometry, for example.

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8. Keeping the fluorescently labeled peptides as short as possible increases the ΔFP signal window between the bound and unbound states. Ideally, peptides should be HPLC-purified; however, this is not always realistically achievable, especially if large numbers of peptides are to be compared and no access to in-house solid-state peptide synthesis is available. To save costs, peptides can be used at non-HPLC-purified grade, in which case a capping step is strongly recommended after each amino acid coupling reaction to prevent peptide synthesis intermediates from being linked to the fluorophore in the final coupling step and affecting the assay [7].

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MATERIALS TEXT

Unless the supplier is explicitly mentioned, chemicals are typically obtained from Sigma-Aldrich.

2.1: Protein Expression

- Inducible bacterial expression constructs for affinity-tagged tankyrase ARCs (TNKS: NM_003747.2; TNKS2: NM_025235.2); see Table 1 for construct details:

A	B	C
ARC construct	**Construct boundaries**	**Molecular weight (kDa)**
TNKS ARC1–5	178–958	85.0
TNKS ARC1	178–336	17.2
TNKS ARC2–3	331–645	34.7
TNKS ARC4	646–807	18.0
TNKS ARC5	799–958	17.5
TNKS2 ARC1–5	20–800	85.3
TNKS2 ARC1	20–178	17.7
TNKS2 ARC2–3	173–487	35.3
TNKS2 ARC4	488–649	17.9
TNKS2 ARC5	641–800	17.3

Table 1
Human tankyrase (TNKS/TNKS2) ARC constructs for biophysical assays. The proteins include a non-native, vector-derived GAMGS sequence at the N-terminus that is retained upon cleavage of the affinity tag [7]

We recommend using vectors with a kanamycin selection marker. Ampicillin hydrolysis by secreted β-lactamase and under low pH increases the proportion of cells lacking the plasmid, which decreases protein yield [33].

- BL21-CodonPlus (DE3)-RIL *E. coli* chemically competent cells (Agilent Technologies)

RIL cells contain additional tRNAs for codons of Arg, Ile, and Leu that are otherwise rare in *E. coli*. The plasmid bearing these genes contains a chloramphenicol selection marker.

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- “Lysogeny Broth” (LB) agar plates , supplemented with kanamycin (50 µg/mL) and chloramphenicol (34 µg/mL).
- LB medium (100 mL for overnight starter culture).
- “Terrific Broth” (TB) medium (4–8 L for large-scale expression).
- 1000× stock solutions of antibiotics: 50 mg/mL kanamycin (in H_2O) and 34 mg/mL chloramphenicol (in isopropanol).
- Shaking incubators capable of a temperature range of at least 18–37 °C.
- Erlenmeyer flasks (250 mL) for pre-cultures, baffled Erlenmeyer flasks (2 L) for large-scale expression.
- Isopropyl β-d-1-thiogalactopyranoside (IPTG , 1 M stock solution)
- Refrigerated centrifuge for harvesting large volumes of bacterial cultures (4000 ×*g*, e.g., Beckman Coulter Avanti J-26XP with JLA 8.1000 rotor).
- Liquid nitrogen bath.
- 50 mL Falcon tubes or plastic film with thermal sealer for storage of bacterial pellets .

2.2: Protein Purification

- Protease inhibitors , such as Pierce protease inhibitor tablets, EDTA-free (Thermo Fisher Scientific).
- Lysozyme, 40 mg/mL stock.
- Sonicator fitted with a large probe or homogenizer capable of breaking bacterial cells .
- Ultra-filtered H_2O.
- Cell lysis buffer: 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 5 mM β-mercaptoethanol . Add protease inhibitor tablets and lysozyme (100 µg/mL final concentration) immediately before use.
- Refrigerated centrifuge for removing insoluble lysate fraction (30,000 ×*g*, e.g., Beckman Coulter Allegra 64R with F0650 rotor).
- 5.0 µm syringe filter units.
- 5 mL HisTrap HP Ni^{2+} affinity column (GE Healthcare).

We recommend using one 5 mL column per 4 L expression culture. If using larger volumes, connect additional column(s) in series. Ni^{2+} affinity columns can easily be reconstituted by stripping from and re-charging with Ni^{2+} as per the manufacturer’s instructions.

- Peristaltic pump .
- Vacuum pump and bottle filters (0.22 µm) for filtering and degassing buffers.
- Buffer A for Ni^{2+} affinity column: 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 5 mM β-mercaptoethanol , 10 mM imidazole pH 7.5—filtered and degassed.
- Buffer B for Ni^{2+} affinity column: 50 mM Tris pH 7.5, 500 mM NaCl, 5 mM β-mercaptoethanol, 250 mM imidazole pH 7.5—filtered and degassed.
- FPLC system with buffer gradient capabilities , UV absorbance detector and fraction collector (e.g., ÄKTA Purifier, GE Healthcare).
- 5 mL HiTrap Q HP column (GE Healthcare).
- Buffer A for Q column: 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol—filtered and degassed.
- Buffer B for Q column: 50 mM Tris–HCl pH 7.5, 1.5 M NaCl, 5 mM β-mercaptoethanol—filtered and degassed.
- Dialysis buffer: 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol.

For ARC2-3 and ARC1-5 constructs described here, a minimal NaCl concentration of 300 mM needs to be maintained, compared to 100 mM for all single-ARC constructs, and glycerol may further help stabilize the protein [7].

- Dialysis tubing, 3500 Da molecular weight cutoff (MWCO).
- Dialysis tubing clips.
- 2 L beaker, magnetic stirrer plate, stirrer bar.
- TEV protease , 5 mg/mL stock.
- 15 mL spin protein concentrator, 3000 Da MWCO for single-ARC constructs, 10,000 Da MWCO for double-ARC constructs, 30,000 Da MWCO for ARC1-5 constructs .
- Refrigerated centrifuge for concentrating protein (3200 ×*g*, e.g., Beckmann Coulter Allegra X12-R centrifuge with SX4750

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swinging bucket rotor).
- Refrigerated centrifuge for removing precipitate prior to size exclusion chromatography (18,000 ×*g*, e.g., Eppendorf 5417R with F45-30-11 rotor).
- HiLoad 16/600 Superdex 75 or 200 pg size exclusion column (GE Healthcare).

Use a Superdex 200 column for ARC1-5 constructs. All other ARC constructs are sufficiently small for the Superdex 75 column.

- Tris(2-carboxyethyl)phosphine (TCEP), 0.5 M stock.

TCEP is more stable than DTT and not volatile, unlike β-mercaptoethanol or DTT. However, TCEP is acidic and will affect the final pH of the buffer unless the 0.5 mM stock solution is pH-adjusted with NaOH. To keep costs down, TCEP is only used in the final purification step.

- Size exclusion buffer: 25 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 2 mM TCEP—filtered and degassed.

HEPES is preferred for the final protein and in experiments due to its lower temperature dependency compared with Tris [34]. Tris was chosen for the affinity purification step due to its weak interaction with Ni^{2+}, which would help decrease background (contaminant) binding [35]. If HEPES buffer is used in the affinity purification step, the imidazole concentration may need to be increased to achieve comparably low background binding.

For ARC2-3 and ARC1-5 constructs described here, a minimal NaCl concentration of 300 mM needs to be maintained, compared to 100 mM for all single-ARC constructs, and glycerol may further help stabilize the protein [7].

- 96-deep-well blocks for fraction collection, or fraction collector tubes (depending on the format of the fraction collector).
- UV spectrophotometer.
- 4× SDS sample buffer.
- 15% polyacrylamide gels for SDS-PAGE.
- Protein standard for SDS-PAGE.
- Coomassie stain for SDS-PAGE gels.
- 15 mL and 50 mL Falcon tubes .
- Thin-walled individual 0.2 mL PCR tubes for flash-freezing protein aliquots.
- Liquid nitrogen bath.

2.3: Fluorescence Polarization (FP) Assay

- Plate reader capable of taking FP measurements (e.g., BMG Labtech POLARstar Omega).
- Appropriate wavelength filters for chosen fluorophore, one corresponding to excitation wavelength, and two (ideally a matched pair with identical optical properties), corresponding to the emission wavelength. Here, we use a 485 nm excitation filter and two matched 520 nm emission filters for fluorescein.
- Opaque, black, 384-well, non-binding, flat-bottom plates, either in standard format (e.g., 781,900, Greiner Bio-One) or in small-volume format (e.g., 784,900, Greiner Bio-One). The latter are particularly useful if limited protein is available.
- Microplate centrifuge (1000 ×*g*, e.g., Beckman Coulter Allegra X-12R with SX4750 swinging bucket rotor, fitted with microplate inserts)
- FP assay buffer: 25 mM HEPES–NaOH pH 7.5, 100 mM NaCl, 1 mM TCEP , 0.05% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS).

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Detergent is used to reduce nonspecific binding and surface tension that may interfere with fluorescence intensity readings in the plate format. The choice of detergent and its concentration is empirical. We have also had good experience with using 100 µg/mL bovine serum albumin (BSA), but use CHAPS here since it is more effective at reducing nonspecific binding.

For ARC2-3 and ARC1-5 constructs described here, a minimal NaCl concentration of 300 mM needs to be maintained, compared to 100 mM for all single-ARC constructs, and glycerol may further help stabilize the protein [7].

- Fluorescently tagged peptide corresponding to TBM of potential tankyrase binder/substrate, 2× stock (50 nM for a final assay concentration of 25 nM) in FP assay buffer. In the present examples (3BP2 , MERIT40 , TRF1), fluorescein and 5(6)-carboxyfluorescein are used as fluorophores. The peptides have been described previously [7]. We recommend peptides of minimally the TBM octapeptide with one flanking amino acid on either side. The peptides used here are longer (*see* Fig.3b for peptide sequences). The fluorescein fluorophore is linked via β-Ala, which also provides an additional linker to minimize potential steric interference of the fluorophore with the peptide-ARC interaction.

Keeping the fluorescently labeled peptides as short as possible increases the ΔFP signal window between the bound and unbound states. Ideally, peptides should be HPLC-purified; however, this is not always realistically achievable, especially if large numbers of peptides are to be compared and no access to in-house solid-state peptide synthesis is available. To save costs, peptides can be used at non-HPLC-purified grade, in which case a capping step is strongly recommended after each amino acid coupling reaction to prevent peptide synthesis intermediates from being linked to the fluorophore in the final coupling step and affecting the assay [7].

- Tankyrase ARC protein: 2× stock of twofold dilution series, 0–400 µM, (final assay concentrations of 0–200 µM protein) in FP assay buffer .
- Software for curve fitting and analysis (e.g., GraphPad Prism 6).

2.4: Binding and PARylation of Full-Length Candidate Proteins by Tankyrase in Cells

- Mammalian expression constructs for epitope-tagged (e.g., MYC_2) tankyrases (TNKS: NM_003747.2; TNKS2: NM_025235.2) and epitope-tagged (e.g., FLAG) tankyrase-binding protein candidates (here for TRF1 /TERF1: NM_017489.2 and MERIT40/BABAM1: NM_001033549.2), corresponding empty vectors as controls .
- QuikChange Lightning mutagenesis kit (Agilent Technologies) or individual components from other sources for site-directed mutagenesis.
- Mutagenesis primers to mutate putative TBM (recommended mutation: G6R).
- PCR thermal cycler, standard setup and reagents for recombinant DNA techniques .
- Human Embryonic Kidney (HEK) 293T cells (ATCC).
- 10 cm and 15 cm cell culture dishes.
- Hemocytometer or automated cell counter.
- Dulbecco's Modified Eagle's Medium (DMEM).
- Fetal bovine serum (FBS).
- Humidified cell culture incubators at 37 °C, 5% CO_2.
- 0.2% Versene in PBS (137 mM NaCl, 2.7 mM KCl , 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 537 µM EDTA, 80 µM phenol red , final pH adjusted to 7.2, sterilization-autoclaved; all reagents cell-culture grade).
- 0.05% trypsin in Versene (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 5.5 mM D-glucose , 1.5 mM KH_2PO_4, 25 mM Tris, 1% phenol red, 0.5 g trypsin (1:250) per 1 L, 137 µM streptomycin sulfate, 168 µM benzyl penicillin, final pH adjusted to 7.5, filter-sterilized using a 0.22 µm filter).
- Ultra-filtered sterile H_2O .
- Calcium phosphate transfection reagents (all cell culture grade):

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(a)2× HEPES-buffered saline (HBS): 50 mM HEPES , 10 mM KCl, 280 mM NaCl, 1.5 mM Na_2HPO_4, 12 mM glucose, final pH adjusted to 7.05 with KOH , filter-sterilized and stored at 4 °C.
(b)25 mM chloroquine diphosphate, filter-sterilized and stored at −20 °C (1000× stock).
(c)2 M $CaCl_2$, filter-sterilized and stored at 4 °C.

- Phosphate-buffered saline (PBS).
- Cell scraper .
- Refrigerated centrifuge for collecting mammalian cells (300 ×*g*, e.g., Beckmann Coulter Allegra X-12R with SX4750 swinging bucket rotor).
- Radioimmunoprecipitation assay (RIPA) buffer: 50 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS , 1 mM DTT, 2 µM ADP-HPD PARG inhibitor and protease inhibitors (e.g., Pierce protease inhibitor tablets, EDTA-free, Thermo Fisher Scientific). Add DTT, ADP-HPD, and protease inhibitors immediately before use.
- Sonicator fitted with small probe.
- Refrigerated centrifuge for clearing lysates and settling affinity resin (800–18,000 ×*g*, e.g., Eppendorf 5417R with F45-30-11 rotor).
- Anti-FLAG M2 Agarose resin .
- Vacuum pump with inlet trap and collection flask for aspirating buffer.
- SDS-PAGE gels (e.g., 4–15% Tris–glycine polyacrylamide gradient gels for excellent resolution).
- Electrophoresis apparatus for the above and appropriate power supply.
- 4× SDS sample buffer.
- Protein standard for SDS-PAGE .
- Nitrocellulose transfer membrane.
- Wet transfer Western blotting apparatus and appropriate power supply.
- Ponceau S solution.
- Nonfat dry milk powder.
- Mouse monoclonal anti-FLAG M2 HRP-conjugated antibody (here 1 mg/mL), mouse monoclonal anti-MYC (9E10) HRP-conjugated antibody (here 1 mg/mL), rabbit polyclonal anti-PAR (4336-BPC-100, Trevigen, concentration not specified by supplier), goat anti-rabbit IgG (H+L) secondary antibody, HRP-conjugate (here 0.8 mg/mL).

Alternatively, fluorescently labeled antibodies can be used for detection with appropriate fluorescence imaging systems. Instead of the anti-PAR antibody, an anti-pan-ADP-ribose or anti-poly(ADP-ribose) binding reagents (MABE1016 and MABE1032, respectively, Millipore) may be explored.

- ECL Western blotting substrate .
- X-ray film for Western blot detection or alternative ECL detection setup.

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

Expression of Tankyrase ARCs

1

cDNA sequences encoding TNKS or TNKS2 ARC constructs are cloned into a pETM-30-2 expression vector (or similar vector) to generate fusion proteins with an N-terminal His_6-GST tag followed by a TEV protease cleavage site (ENLYFQG) [7]. *See* Table 1 for suggested ARC boundaries [7]. Chemically competent BL21-CodonPlus (DE3)-RIL *E. coli* cells for protein expression are transformed with sequence-validated plasmids. A single colony is selected to inoculate an overnight pre-culture, which is then used to inoculate the larger expression cultures (typically 4–8 L). The cultures are grown shaking at 37 °C to exponential phase, then cooled at 4 °C for 30 min before induction with IPTG overnight at 18 °C. The cells are then collected by centrifugation. Purification can either follow immediately, or cell pellets can be stored at −80 °C until processed for purification.

2

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Transform chemically competent BL21-CodonPlus (DE3)-RIL *E. coli* cells with tankyrase ARC expression constructs by heat shock. Plate cells on LB agar plates containing suitable antibiotics, here
50 microgram per milliliter (µg/mL) kanamycin and
34 microgram per milliliter (µg/mL) chloramphenicol . Incubate **Overnight** at **37 °C** .

We recommend using vectors with a kanamycin selection marker. Ampicillin hydrolysis by secreted β-lactamase and under low pH increases the proportion of cells lacking the plasmid, which decreases protein yield [33].

RIL cells contain additional tRNAs for codons of Arg, Ile, and Leu that are otherwise rare in *E. coli*. The plasmid bearing these genes contains a chloramphenicol selection marker.

3

Pick a single colony from the plate and inoculate an overnight pre-culture of **100 mL LB medium** supplemented with **50 microgram per milliliter (µg/mL) kanamycin** and
34 microgram per milliliter (µg/mL) chloramphenicol . Incubate **Overnight** in a shaking incubator at **37 °C** .

4

Add **5 mL pre-culture** to each liter of TB medium supplemented with
50 microgram per milliliter (µg/mL) kanamycin and
34 microgram per milliliter (µg/mL) chloramphenicol . Generally, the yield for individual TNKS and TNKS2 ARC constructs is 6–10 mg/L of culture.

5 30m

Incubate cultures at **37 °C** with shaking at **180 rpm** until an optical density at 600 nm (OD_{600}) of approximately 2.0 is reached. Remove cultures from incubator and cool them at **4 °C** for **00:30:00** .

Compared to LB, TB is richer and enables higher cell densities in the log phase of growth. Cooling the cultures before IPTG induction slows down expression, thereby facilitating correct protein folding and increasing protein solubility.

6 15h

Add IPTG to a final concentration of **0.5 Milimolar (mM)** (0.5 mL of 1 M stock per liter of culture) to induce

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protein production. Incubate cultures at **18 °C** with shaking **Overnight** (≈15 h).

7 30m

Collect cells by centrifugation in a precooled centrifuge at **4000 x g, 4°C, 00:30:00** .

8

Discard supernatant. The cells can be lysed for immediate purification. Alternatively, pellets can be flash-frozen in a liquid nitrogen bath for storage at **-80 °C** until required .

3.2.2 Purification of Tankyrase ARCs

9

A similar procedure is followed for the purification of all tankyrase ARCs. All buffers are ice-cold, and work is performed at 4 °C or on ice throughout. In the first step, the protein is affinity-purified by immobilized metal affinity chromatography (IMAC) using a Ni^{2+} affinity column. The affinity tag is then cleaved overnight using TEV protease, under simultaneous dialysis to remove imidazole from the elution step. A second Ni^{2+} affinity chromatography step separates the cleaved tag from the protein. An optional anion exchange chromatography step removes further impurities. Finally, size exclusion chromatography yields high-quality purified protein for downstream applications. The general procedure is applicable to all TNKS and TNKS2 ARCs with small changes (Fig.2a).

For ARC2-3 and ARC1-5 constructs described here, a minimal NaCl concentration of 300 mM needs to be maintained, compared to 100 mM for all single-ARC constructs, and glycerol may further help stabilize the protein [7].

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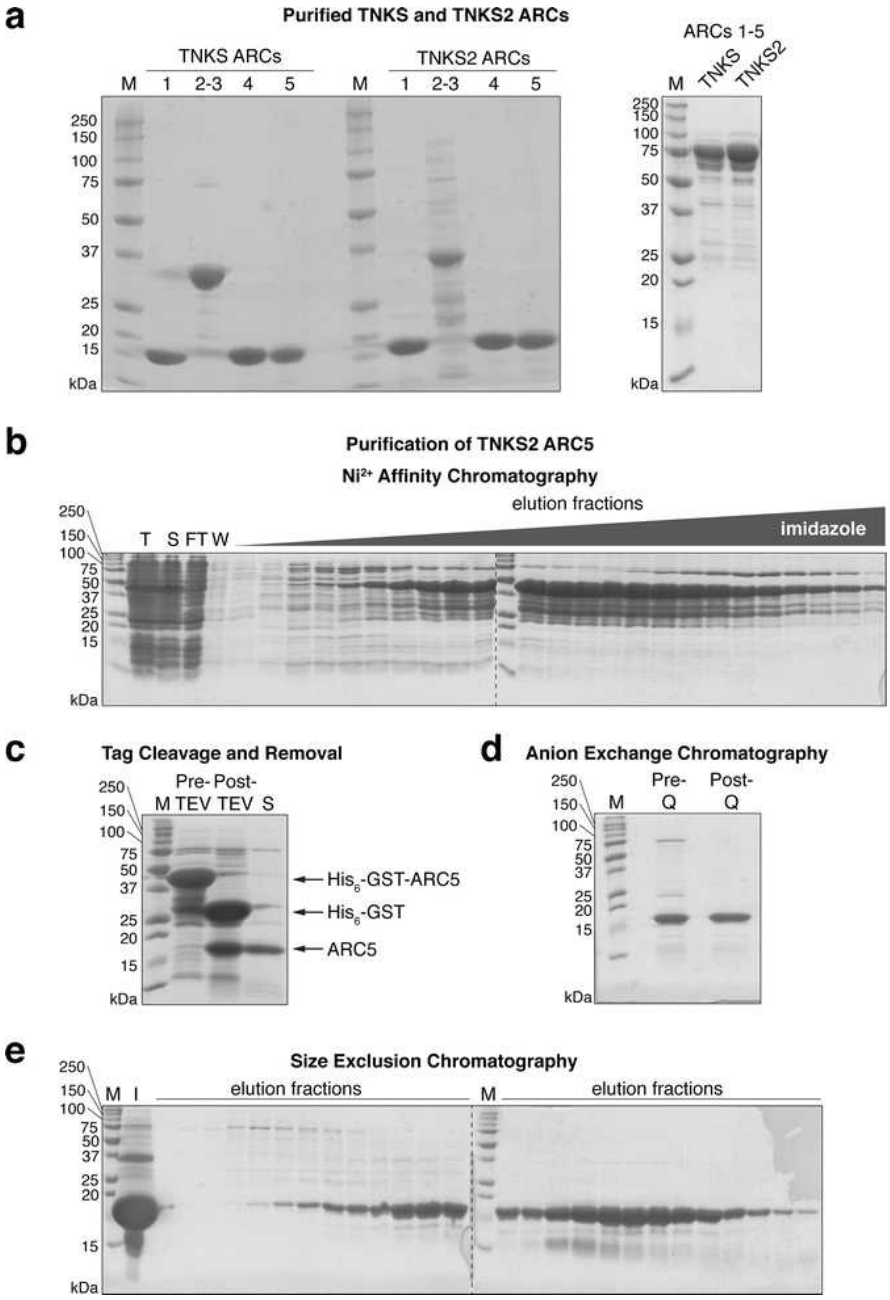

Fig. 2
Purification of tankyrase ARCs. (**a**) 5 µg purified TNKS and TNKS2 ARCs were resolved on 15% Tris–glycine polyacrylamide gels for SDS-PAGE and the gels stained with Coomassie. M, marker. *See* Table 1 for construct boundaries. (b)-(e) Step-by-step purification of a representative ARC, TNKS2 ARC5. (**b**) Ni^{2+} affinity purification of His_6-GST-TNKS2 ARC5 fusion protein. T, total lysate; S, soluble lysate; FT, flow-through; W, wash. (**c**) Removal of the His_6-GST affinity tag by TEV cleavage and a second Ni^{2+} affinity purification step. Pre-TEV, protein before TEV cleavage; Post-TEV, protein after TEV cleavage; S, pooled protein after subtraction of affinity tag. (**d**) Anion exchange chromatography . Pre-Q, protein before anion exchange chromatography; Post-Q, protein after anion exchange chromatography. (**e**) Size exclusion chromatography. M, marker; I, input

10

Dissolve protease inhibitor tablets in the required volume of lysis buffer (approximately **20 mL buffer** for every **10 g cell pellet**) and add lysozyme ([M] **100 microgram per milliliter (µg/mL)** final) in a large glass beaker containing a magnetic stirrer bar. Add frozen cell pellet and stir gently until cells are completely thawed and a homogeneous suspension is obtained.

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11 Break cells by using a large-tip sonicator at 40% amplitude output, using a **00:00:05** on, **00:00:05** off pulse, for a total of **00:05:00 "on" time** . Keep cells **On ice** continuously to avoid overheating. Take a sample of the total lysate for subsequent analysis by SDS-PAGE (**9 µl lysate** + **3 µl 4× SDS sample buffer**). 10m

> For large volumes, it may be easier to lyse cells in two batches. Alternative disruption techniques can be used, such as homogenization by an Avestin EmulsiFlex homogenizer.

12  30m

Remove cell debris and insoluble material by centrifugation at **30000 x g, 00:30:00** . If lysate is still cloudy, centrifuge for a further **00:30:00** until clear.

13 

While the lysate is being cleared, prepare a **5 mL HisTrap Ni2+ affinity column** by washing with **10 column volumes (CV) H2O** and then equilibrating with **10 column volumes (CV) buffer A** for Ni^{2+} affinity column, using a low-pressure peristaltic pump, set to a flow rate of 2 mL/min.

> We recommend using one 5 mL column per 4 L expression culture. If using larger volumes, connect additional column(s) in series. Ni^{2+} affinity columns can easily be reconstituted by stripping from and re-charging with Ni^{2+} as per the manufacturer's instructions.

14 Decant the supernatant into a beaker, and sonicate **On ice** , **00:00:05** on, **00:00:05** off for a total of **00:00:20 "on" time** to further break up genomic DNA and avoid clogging the filter in the next step. 40s

15 

Filter the supernatant through 5.0 µm syringe filter units. Take a sample of the soluble lysate for subsequent analysis by SDS-PAGE (**9 µl lysate** + **3 µl 4× SDS sample buffer**). Load the cleared lysate on the equilibrated HisTrap column using the peristaltic pump as before.

> If available with the FPLC setup, a superloop or, preferably, a sample pump can be used to load the column.

The His6-GST-tagged protein will bind to the immobilized Ni^{2+} ions. Wash the column with **20 column volumes (CV) buffer A (100 mL)** to remove unbound contaminants. Collect lysate flow-through and the wash buffer in case the protein was not bound or was released during the wash, and take samples for analysis by SDS-PAGE (**9 µl flow-through or wash** + **3 µl 4× SDS sample buffer**).

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16 Attach the HisTrap column to an FPLC system, and elute the His_6-GST-ARC protein with the following three-step protocol, at a flow rate of 2 mL/min, collecting 1.5 mL fractions in a 96-deep-well block:

16.1 step 1: **2 column volumes (CV) 100% buffer A** .

16.2 step 2: **over 20 column volumes (CV) gradient from 0–100% buffer B** .

16.3 step 3: **4 column volumes (CV) 100% buffer B** .

17 Take samples of **each** peak elution fraction (**9 µl eluate** + **3 µl 4× SDS sample buffer**). Analyze the samples taken in steps 11 and 15 (2 µL of sample for lysates and flow-through, **5 µl of sample** for wash) and the peak elution fractions (**5 µl of sample**) by SDS-PAGE (Fig. 2b).

18 Pool fractions containing ARC fusion protein. Take a sample of the pooled fractions (**9 µl protein** + **3 µl 4× SDS sample buffer**). Add TEV protease (approximately **100 µl of 5 mg/mL stock** for every **25 mL fusion protein solution**) to cleave the tag.

19 20s

Soak dialysis tubing (3500 Da MWCO) in dialysis buffer, and seal one end with a dialysis membrane clip. Transfer the protein into the dialysis tubing and seal the other end. More than one dialysis tube may be required. Dialyze **Overnight** in a 2 L glass beaker containing dialysis buffer, with gentle stirring at **4 °C** . Avoid contact between the stirrer bar and the dialysis tubing to prevent rupture.

20

Pour content of dialysis tubing into 50 mL Falcon tubes. Centrifuge at **3200 x g, 00:15:00** to remove any precipitate that may have formed. Take a sample of the protein post-TEV cleavage (**9 µl protein** + **3 µl 4× SDS sample buffer**). White precipitate often forms overnight with the change to low-salt buffer. SDS-PAGE analysis of the precipitate reveals it to be enriched in a contaminant (not shown).

21

Meanwhile, wash a **5 mL HisTrap column** with **10 column volumes (CV) H2O** . Next, equilibrate the column in **10 column volumes (CV) dialysis buffer** . Filter the supernatant through a 0.22 µm syringe filter and load it onto the equilibrated HisTrap column using the low-pressure peristaltic pump. Collect the flow-through. The untagged ARC protein will pass through, while the cleaved tag (or residual His6-GST-ARC fusion protein) will be retained on the column.

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Depending on the ARC construct and the final concentration of imidazole in the dialysis buffer, ARCs can bind weakly to the Ni^{2+} column even after tag cleavage . They can be eluted with a further imidazole gradient. While this adds one more step to the purification protocol, it enables even higher purities to be achieved.

Wash with 2 CV of dialysis buffer to fully remove all ARC protein from the column, collecting the wash. Take samples of the flow-through and wash (**9 µl protein** + **3 µl 4× SDS sample buffer**).

22

Analyze **5 µl pre-TEV sample** , post-TEV sample and flow-through/wash samples from the second Ni^{2+} column by SDS-PAGE to assess successful tag removal and separation (Fig. 2c).

23

Take the protein-containing samples (flow-through/wash) and concentrate the protein to about **20 mL** by centrifuging in a 3000 Da MWCO spin concentrator at **3200 x g, 00:30:00** at a time in a swinging bucket rotor. Mix by gently pipetting up and down to prevent a steep concentration gradient from forming in the concentrator. Take a sample of the concentrated protein (**9 µl protein** + **3 µl 4× SDS sample buffer**).

24

The following anion exchange chromatography step is optional, but was found to increase the purity of ARC proteins, especially TNKS2 ARC5, by removing a higher-molecular-weight impurity:

Using the peristaltic pump at 2 mL/min , wash a 5 mL HiTrap Q HP column with **5 column volumes (CV) H2O** , followed by **5 column volumes (CV) buffer B** for Q column, and then equilibrate with **10 column volumes (CV) buffer A** for Q column. Pass the concentrated protein over the column, and collect the flow-through. Wash the column with **2 column volumes (CV) buffer A** for Q column, also collecting the flow-through. ARCs do not bind to the Q column; however, the impurity does and will be retained on the column.

The Q column step can also be performed before concentration, directly using the flow-through from the second Ni^{2+} affinity column; however, prior concentration saves time in loading the column.

Take a sample of the combined flow-through and wash (**9 µl protein** + **3 µl 4× SDS sample buffer**).

Analyze **5 µl samples** from step 23 and this step by SDS-PAGE as above (Fig. 2d).

25

Concentrate the protein as in step 23, to ≤ **2 mL** : Take the protein-containing samples (flow-through/wash) and concentrate the protein by centrifuging in a 3000 Da MWCO spin concentrator at **3200 x g, 00:30:00** at a time in a swinging bucket rotor. Mix by gently pipetting up and down to prevent a steep concentration gradient from forming in the concentrator. Take a sample of the concentrated protein (**9 µl protein** + **3 µl 4× SDS sample buffer**).

26

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Centrifuge protein in a microcentrifuge tube at **18000 x g, 00:10:00** to remove any particles before size exclusion chromatography.

27 Load the concentrated protein onto an equilibrated size exclusion column connected to an FPLC system , using a 2 mL sample loop.

Use a Superdex 200 column for ARC1-5 constructs. All other ARC constructs are sufficiently small for the Superdex 75 column.

Elute the protein isocratically, using **1.2 column volumes (CV) size exclusion buffer** . Collect 1 mL fractions in a 96-deep-well block.

28 Take samples of the input material and peak fractions (**9 µl input** or **9 µl fraction** + **3 µl 4× SDS sample buffer**). Analyze **5 µl samples** by SDS-PAGE (Fig. 2e).

29 Combine fractions containing pure protein. Wash the membrane of a spin concentrator by spinning through one concentrator volume of H_2O to remove any stabilizing agents such as glycerol and azide. Now concentrate the protein using the pre-washed spin concentrator as described above (step 24) until a concentration of > **0.5 Milimolar (mM)** is achieved (solubility of multi- ARC constructs can be lower). The concentration can be determined by measuring the UV absorbance at 280 nm and using the molar extinction coefficient calculated by ProtParam (*http://web.expasy.org/protparam/*) [29].

30

Aliquot the protein into small volumes in thin-walled PCR tubes and flash-freeze them in a liquid nitrogen bath. Frozen protein aliquots can be stored at **-80 °C** until future use.

3.2.3 Fluorescence Polarization Assay for TBM Validation 30m

31

This assay measures the direct binding of a fluorescently labeled TBM candidate peptide to a tankyrase ARC [7]. The ARC protein is titrated at a constant peptide probe concentration, and the change in polarization (ΔFP) is measured and plotted. Plotting ΔFP values against [ARC] enables a dissociation constant (K_d) to be calculated. In the present example, we use fluorescein-based peptide labels, but the general method can be adapted for different fluorophores.

32 Ensure the plate reader is set up for fluorescence polarization, with the correct filters for the fluorophore . The fluorescein example uses a 485 nm excitation filter and two 520 nm emission filters, one parallel and one perpendicular to the plane of the linearly polarized light used for excitation.

33

Dissolve the lyophilized peptide probe in FP assay buffer, to approximately **100 Micromolar (µM)** . The concentration can be measured spectrophotometrically.

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Confirm the pH of the peptide stock solution before measuring the concentration. Acidity, for example due to residual trifluoroacetic acid from the peptide synthesis, will strongly affect fluorophore absorption.

Here, we measure the absorbance of the peptide stock at 492 nm and use a molar extinction coefficient for fluorescein of ε_{492} = 83,000/M/cm [30]. The concentrated probe can then be aliquoted into small volumes (5–10 µL) and flash-frozen in liquid nitrogen for long-term storage at **-80 °C** , to be thawed when required.

34 Determine the optimal probe concentration to use. This will depend on the sensitivity of the plate reader. FP values should be independent of the probe concentration used, and give a robust signal window for an unbound vs. protein-bound peptide. Make 2× stocks of a twofold dilution series of probe in FP buffer, from 0–800 nM (10 concentrations + no peptide). It is often easier to make a 1/100 dilution of the concentrated peptide stock to avoid pipetting small volumes. Total sample volumes per well are either 15 or 50 µL (see step 36 below).

35 Prepare a 2× protein stock solution, by diluting tankyrase ARC protein in FP buffer to **20 Micromolar (µM)** (10 µM final protein concentration). The protein concentration should be in vast excess over the peptide probe to obtain a first impression of the anticipated signal window between free and protein-bound peptide probe .

36 31m

Plate out the assay samples in technical duplicate (see Table 2 and note below). Small-volume plates will require 15 µL per well (**7.5 µl** of each stock), standard-volume plates **50 µl** per well (**25 µl** of each stock). Mix the probe stock solution **1:1** with either FP buffer for the peptide probe-only titration or protein stock for the titration in the presence of protein. Add a “blank” well with FP buffer only. A well with **5 Nanomolar (nM) fluorescein** (literature FP of 35 millipolarization units, mP) can be used as a standard to automatically set the gain adjustment of the plate reader. Temporarily seal the plate with adhesive film or a lid and spin at **1000 x g, 00:01:00** in a swinging bucket rotor with plate inserts. Incubate in the dark at **Room temperature** for **00:30:00** to ensure binding equilibrium is reached.

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A														
B		0	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	Blank	Standard
C		0	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	Blank	Standard
D		0	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	Blank	Standard
E		0	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	Blank	Standard

Table 2
Example plate layout for establishing the probe concentration in a fluorescence polarization assay. Rows B and C correspond to 3BP2 peptide titrations (here up to 400 nM) in the absence of protein, while rows D and E correspond to 3BP2 peptide titrations in the presence of protein. Numbers correspond to final fluorophore concentration (nM). Blank wells contain buffer alone. Standard wells contain 5 nM of free fluorescein. Wells along the edge of the microplate (row A and column 1) are left empty to minimize the “edge effect”

We recommend leaving wells in the outermost rows and columns empty to reduce the microplate “edge effect,” a discrepancy in readings between the central and peripheral wells [36].

37 Set the gain adjustment of the instrument. The POLARstar Omega automatically calculates the gain by scaling the FP value to 35 mP for the wells containing **5 Nanomolar (nM) free fluorescein** . 50 flashes per well is a good

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starting point to minimize fluorophore bleaching, and means each data point will be the average of 50 measurements.

38

Read the plate and calculate FP in mP. Some plate readers and software packages will automatically blank-correct and calculate the FP values. If not, subtract the averaged F_parallel and F_perpendicular readings obtained for the blank wells from the corresponding individual values of the other wells. FP values are calculated using the following equation:

$$FP(mP) = \frac{(F\_parallel - F\_perpendicular)}{(F\_parallel + F\_perpendicular)}$$

F_parallel and F_perpendicular denote the fluorescence intensities parallel and perpendicular to the plane of the linearly polarized lightused for excitation. mP, millipolarization units.

39 Export the FP data and raw fluorescence intensity values. Plot the data (average FP vs. [probe]) using GraphPad Prism or your data analysis program of choice (Fig.3a).

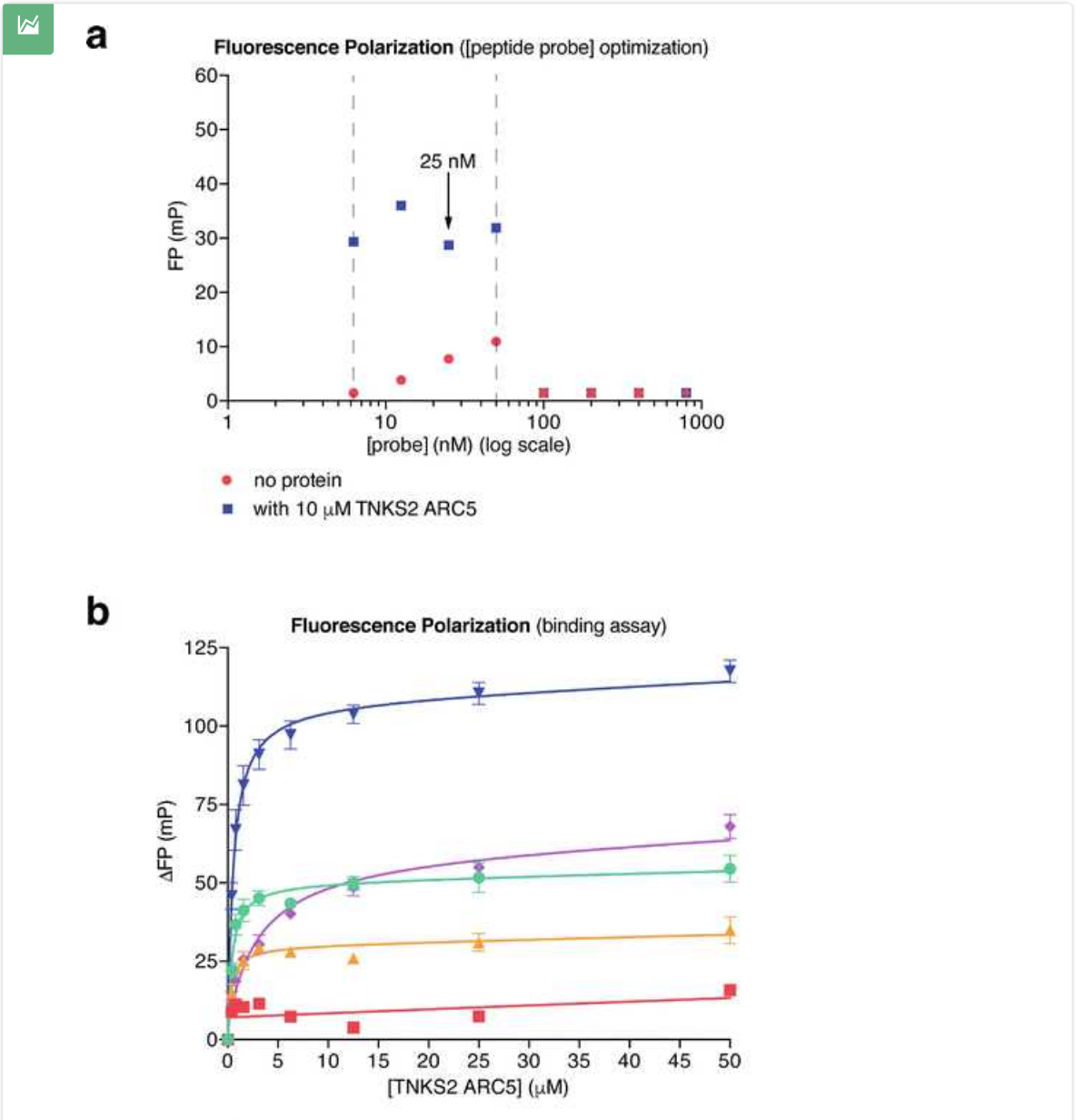

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Fig. 3
Assessing ARC–TBM peptide interactions by fluorescence polarization . (**a**) Optimization of the peptide probe (3BP2) concentration for the FP binding assay. Data are from one experiment performed in technical duplicate with mean FP values plotted. The dashed vertical lines indicate the suitable peptide probe concentration range. 25 nM was chosen for the subsequent assays. (**b**) FP binding assays for the TBM peptides from TRF1, MERIT40, and 3BP2 (WT positive control and G6R negative control). Peptide sequences (with octapeptide in bold) and affinities for TNKS2 ARC5 are indicated. n = 3 separate experiments; error bars, SEM. The error values for the dissociation constants correspond to the standard error of the fit in nonlinear regression. n.d., not determined (no binding curve)

40 Determine the minimum probe concentration required. Raw fluorescence intensity values of >10× the blank reading are necessary to calculate accurate FP values. In the example shown in Fig. 3a, fluorescence intensity values measured with probe concentrations below **[M]6.25 Nanomolar (nM)** were too low and therefore excluded.

41 Determine the maximum probe concentration. The FP values will drop sharply when the signal overloads the detectors. In this case, both detectors reach saturation, so the fluorescence intensities registered in the parallel and perpendicular channels will be identical, and hence the FP calculated will be zero. In the example shown in Fig. 3a, **[M]50 Nanomolar (nM)** is the maximum usable peptide probe concentration. FP readings between 6.25 and 50 nM probe are approximately constant.

42 Peptide probe concentrations should be kept as low as reasonably possible. Thus, the peptide concentration can be ignored in K_d calculations. 25 nM probe was chosen for the protein titration experiments shown below. Prepare a 2× probe stock solution (**[M]50 Nanomolar (nM)** in FP buffer). Prepare a twofold dilution series of tankyrase ARC protein from 0–400 µM (0–200 µM final concentration) in FP buffer. The maximum protein concentration may be lowered if B_{max} is reached at low protein concentrations, i.e., in the case of high affinity. An initial maximum [ARC] of **[M]200 Micromolar (µM)** ensures that binding can be detected for weak binders as well.

43 30m

Set up a binding experiment for K_d determination. Mix the probe and protein stock solution **1:1** to give **15 µl final well volume** (7.5 µL of each stock solution, small-volume plates). Centrifuge the plate as in step 39 (spin at **1000 x g, 00:01:00** in a swinging bucket rotor with plate inserts) above. Incubate the plate in the dark at **Room temperature** for **00:30:00** to achieve equilibrium before measurement.

44 Calculate FP as in step 38 above and export the raw fluorescence and calculated FP values.

45 Enter the individual FP values into GraphPad Prism or your data analysis program of choice.

46

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Baseline-correct by subtracting the FP value for the zero-protein well from all other values in that row, to obtain ΔFP values. Calculate the mean of the technical duplicates. Plot ΔFP against [protein]. Perform a nonlinear regression analysis, using a one-site total binding model:

$$\Delta FP = Bmax \times [ARC]/(Kd + [ARC]) + NS \times [ARC] + Background$$

B_{max} denotes the maximum specific binding in mP, where the binding curve saturates; K d is the dissociation constant ; NS denotes the slope of nonspecific binding in ΔFP/[ARC] units; Background denotes the baseline ΔFP value, which should be zero. Although it is possible to constrain the background to zero, it is advisable to retain the background term since the baseline is defined by more values than that from the zero-protein sample.

Non-binding peptides will give a straight line of nonspecific binding. Binding peptides will give a logarithmic curve (Fig. 3b). This analysis will yield a K_d value.

47 Repeat the experiment to obtain pooled data from (typically three) separate experiments . In each separate experiment, technical duplicates are combined into a single data point. Error bars (SEM) are calculated for the mean data points from the separate experiments (Fig. 3b). The peptides from 3BP2 , TRF1 , and MERIT40 (two peptides) bind TNKS2 ARC5 with the indicated affinities, while no binding is detectable for the G6R negative control peptide from 3BP2 (Fig. 3b).

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