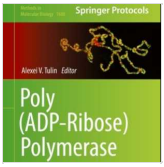

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Validation of Tankyrase Binding and PARylation in Full-Length Protein Context

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

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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

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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].

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

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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.

- "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

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- 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 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

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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).

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].

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- 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):

(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.

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- 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.

3.3 Validation of Tankyrase Binding and PARylation in Full-Length Protein Context

1

This final section outlines a step-by-step approach to assess and validate the full-length candidate protein interaction with full-length tankyrase and the extent of its tankyrase-dependent PARylation. Mammalian expression constructs for MYC_2-tagged TNKS2 (pLP-dMYC SD-TNKS2) and FLAG- or $FLAG_3$-tagged candidate interacting proteins (pCMV-FLAG-MERIT40 and pLP-tripleFLAG SD-TRF1) are generated. Standard site-directed mutagenesis is performed to mutate the Gly at position 6 of the FP-validated TBMs to Arg. The FLAG-tagged candidate proteins and corresponding TBM mutant derivatives are next used as “baits” in co-immunoprecipitation experiments, to assess their binding to and their PARylation by MYC_2-TNKS2, using catalytically inactive TNKS2 (G1032W) as control [31].

3.3.1 Co-expression of TNKS2 and MERIT40 or TRF1 in HEK293T Cells

2 Grow a sufficient number (here two) 15 cm cell culture plates of HEK293T cells to a cell density of ≈90% confluence in standard DMEM media supplemented with 10% FBS.

3 

Remove the media and gently rinse the cell culture plates with **10 mL Versene** to remove residual media, which could impede the efficient dissociation of the cells by trypsin.

4 

2m

Add **3 mL trypsin-Versene** to each plate and place the plates back in the incubator for **00:02:00** . Gently tap the side of the plates to help the cells detach from the plate and dissociate from each other.

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5

Add **10 mL DMEM media with 10% FBS** to each plate; the FBS will inactivate the trypsin. Pipette the cell suspension up and down a few times to ensure proper dissociation of cell clumps into a homogenous cell suspension.

6 2m

Measure the cell density, using either a hemocytometer or automated cell counter. Add 6×10^6 cells each to the required number of 10 cm cell culture plates for the co-transfection of different DNA constructs (Table 3). Add **10 mL DMEM media with 10% FBS** to each plate and incubate cells **Overnight** .

A	B	C	D	E	F	G	H
	empty FLAG vector	MERIT40 WT	MERIT40 G33R	MERIT40 G53R	MERIT40 GG33/53RR	TRF1 WT	TRF1 G18R
empty MYC2 vector	–	+	–	–	–	+	–
TNKS2 WT	++	+	+	+	+	+	+
TNKS2 G1032W	–	+	–	–	–	+	–

Table 3
Chart for co-transfection of HEK293T cells for co-immunoprecipitation. The indicated FLAG/$FLAG_3$-tagged constructs (5 µg) are used as baits in co-immunoprecipitation with the indicated MYC_2-tagged TNKS2 constructs (5 µg). The G-to-R mutation at position 6 of the TBM efficiently abolishes the ARC–TBM peptide interaction [7]. A G1032W mutation abolishes both poly- and mono(ADP-ribosyl)ation by TNKS2 [31]. Each "+" sign denotes a single co-transfection setup

7 15m

The next day, co-transfect cells with 5 µg of each DNA construct (thus 10 µg total) per plate (Table 3):
Pipette **5 µg each DNA construct** in a 15 mL Falcon tube, add **250 µl 2 M CaCl2** followed by **1.75 mL H2O** and mix thoroughly. Subsequently, add **2 mL 2× HBS** , mix the solution thoroughly by pipetting up and down and incubate for **00:05:00** – **00:10:00** at **Room temperature** .

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

8

Replace the growth media from the 10 cm plate with **15 mL fresh growth media** supplemented with **[M]25 Micromolar (µM) chloroquine** . Chloroquine inhibits degradation of endocytosed DNA and can increase transfection efficiency [21].

9 1d

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Gently add the transfection mix (4 mL each, dropwise) to each plate and incubate transfected cells for **24:00:00** .

10 5m

The next day, remove the media and add **10 mL ice-cold PBS** . Harvest the cells by gently scraping them off the plate.

> 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.

Collect the scraped cell suspension in a 15 mL Falcon tube and pellet the cells by centrifugation at **300 x g, 4°C, 00:05:00** . Discard the supernatant; snap-freeze cell pellet in liquid nitrogen and store at **-80 °C** until further use.

3.3.2 Co-immunoprecipitation 2h 20m 6s

11 5m

Add **1 mL ice-cold RIPA buffer** (with freshly added DTT and protease inhibitors) to each cell pellet and resuspend cells **On ice** . The freeze–thaw and the detergents will break open the cells. Transfer the lysates to 1.5 mL microcentrifuge tubes and incubate **On ice** for **00:05:00** for extraction.

12 6s

Sonicate lysates for **00:00:06** **On ice** , using a small tip sonicator at 25% amplitude output to shear chromatin.

13

Clear cell lysates by high-speed centrifugation at **18000 x g, 4°C, 00:15:00** and transfer supernatants to new 1.5 mL microcentrifuge tubes.

14 5m

Transfer **30 µl each cleared lysate** to a new microcentrifuge tube (**input samples**) and add **10 µl 4× SDS sample buffer** . Boil samples at **95 °C** for **00:05:00** , collect by brief centrifugation , and store at **-20 °C** until analysis, if required.

15 2h

Pre-equilibrate a sufficient amount of anti-FLAG M2 Agarose resin (**25 µl packed-volume resin** per immunoprecipitation sample plus a minimum of 10% dead volume) with RIPA buffer and incubate resin with cleared lysates for **02:00:00** on a rotating wheel at **4 °C** .

16

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Gently settle the resin by centrifugation at **800 x g, 4°C, 00:05:00** and remove supernatant using a vacuum pump.

17 5m

Wash the resin by adding **1 mL RIPA buffer** to each sample and incubating microcentrifuge tubes for **00:05:00** on a rotating wheel at **4 °C** .

18 Settle the resin as before (step 16: Gently settle the resin by centrifugation at **800 x g, 4°C, 00:05:00** and remove supernatant using a vacuum pump.) and repeat the wash step four more times:

18.1

Gently settle the resin by centrifugation at **800 x g, 4°C, 00:05:00** and remove supernatant using a vacuum pump. (Wash 1/4)

18.2

Gently settle the resin by centrifugation at **800 x g, 4°C, 00:05:00** and remove supernatant using a vacuum pump. (Wash 2/4)

18.3

Gently settle the resin by centrifugation at **800 x g, 4°C, 00:05:00** and remove supernatant using a vacuum pump. (Wash 3/4)

18.4

Gently settle the resin by centrifugation at **800 x g, 4°C, 00:05:00** and remove supernatant using a vacuum pump. (Wash 4/4)

19 5m

After the last wash step, gently remove as much of buffer as possible and add **25 µl 2× SDS sample buffer** . Boil samples at **95 °C** for **00:05:00** , collect briefly by centrifugation and store at **-20 °C** until analysis, if required (immunoprecipitate (IP) samples).

3.3.3 SDS-PAGE and Immunoblotting 3h 30m

20 Resolve **10 µl boiled input** (step 14 above) and IP (step 19 above) samples on a pre-cast 4–15% Tris–glycine polyacrylamide gradient gel for SDS-PAGE analysis.

21 Transfer proteins from the gel onto a nitrocellulose membrane using a wet-transfer blotting system. Ponceau S can be used to assess the transfer quality.

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22 1h

Incubate the nitrocellulose membrane for at least **01:00:00** at **Room temperature** on a horizontal shaking platform in 5% dry milk powder in PBS to block the membrane to reduce nonspecific binding of antibodies during the subsequent immunodetection steps.

23 1h

Incubate the membranes with the required antibody at the appropriate dilution in **5 % (v/v) milk/PBS** **Overnight** at **4 °C** on a horizontal shaking platform. (Antibody dilutions: anti-FLAG HRP-conjugated antibody, 1:1000; anti-MYC HRP -conjugated antibody, 1:1000; anti-PAR, 1:1000) .

24 Wash the membrane three times for **00:05:00** with copious amounts PBS + 0.1% Tween 20 on a horizontal shaking platform: 5m

24.1 5m

Wash the membrane for **00:05:00** with copious amounts PBS + 0.1% Tween 20 on a horizontal shaking platform. (Wash 1/3)

24.2 5m

Wash the membrane for **00:05:00** with copious amounts PBS + 0.1% Tween 20 on a horizontal shaking platform. (Wash 2/3)

24.3 5m

Wash the membrane for **00:05:00** with copious amounts PBS + 0.1% Tween 20 on a horizontal shaking platform. (Wash 3/3)

25 1h

As required, incubate the membrane with the matching HRP-coupled secondary antibody, here diluted 1:5000 in **5 % (v/v) milk/PBS** for **01:00:00** at **Room temperature** on a horizontal shaking platform (*see* **Note 20**).

26 Repeat wash: Wash the membrane three times for **00:05:00** with copious amounts PBS + 0.1% Tween 20 on a horizontal shaking platform: 5m

26.1 Wash the membrane for **00:05:00** with copious amounts PBS + 0.1% Tween 20 on a horizontal 5m

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shaking platform. (Wash 1/3)

26.2 Wash the membrane for **00:05:00** with copious amounts PBS + 0.1% Tween 20 on a horizontal shaking platform. (Wash 2/3) 5m

26.3 Wash the membrane for **00:05:00** with copious amounts PBS + 0.1% Tween 20 on a horizontal shaking platform. (Wash 3/3) 5m

27 Develop Western blot by incubating membrane with ECL Western blotting substrate following the manufacturer's instructions.

28

Detect chemiluminescence signal by exposing membrane to X-ray film in a dark room and develop the film with an X-ray film developer.

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

Fig. 4 shows the TBM-dependent interaction of FLAG3-TRF1 and FLAG-MERIT40 with MYC2-TNKS2, and the TNKS2-dependent PARylation of both TNKS2 interactors, in addition to TNKS2 auto-PARylation.

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.

Both MERIT40 TBMs contribute to tankyrase binding (Fig. 4b).

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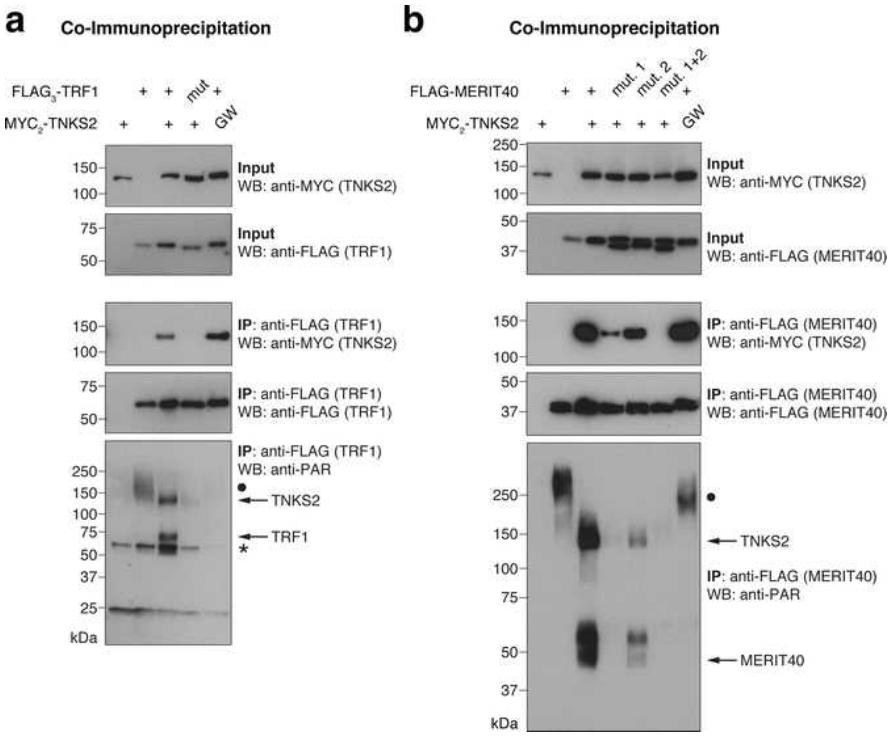

Fig. 4
Assessing tankyrase binding and PARylation by tankyrase in the full-length protein context. (**a**) $FLAG_3$-TRF1, either in its wild-type form or as a G18R TBM mutant ("mut."), was co-expressed with the indicated MYC_2-TNKS2 constructs, either in wild-type form or as a G1032W PARP-inactive mutant ("GW") [31]. FLAG-TRF1 was immunoprecipitated and input and immunoprecipitate (IP) samples analyzed by SDS-PAGE and Western blotting as indicated. "•" in the anti-PAR blot labels a high-molecular-weight PARylated species that appears to be antagonized by MYC_2-TNKS2 overexpression. "*" in the anti-PAR blot denotes a nonspecific band. (**b**) Same analysis as in (**a**) with FLAG-MERIT40, either in wild-type form or as a G33R ("mut. 1"), G53R ("mut. 2"), or GG33/53RR ("mut. 1 + 2") TBM mutant. MERIT40 appears as a doublet, most likely reflecting differentially phosphorylated species [32]. *See* **Note above** on attributing PAR signals to candidate proteins.

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