The formin-binding protein 17, FBP17, binds via a TNKS binding motif to tankyrase, a protein involved in telomere maintenance

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Abstract In acute myelogenous and lymphoid leukemias, rearrangements involving the MLL (mixed lineage leukemia) gene at chromosome 11q23 are frequent. The truncated MLL protein is fused in-frame to a series of partner proteins. We previously identified the formin-binding protein 17 (FBP17) as such an MLL fusion partner. In this study, we explored in vivo physiological interaction partners of FBP17 using a two-hybrid assay and found tankyrase (TNKS), an ADP-ribose polymerase protein involved in telomere maintenance and mitogen-activated protein kinase signaling. We demonstrate that FBP17 binds via a special TNKS-binding motif to tankyrase. The physiological relevance is indicated by co-immunoprecipitation of endogenous proteins in 293T cells.

Key words: Acute myeloid leukemia; Mixed lineage leukemia fusion; Formin-binding protein 17; Yeast two-hybrid; Tankyrase

1. Introduction

The chromosomal region 11q23 containing the MLL (mixed lineage leukemia) gene locus is frequently involved in chromosomal translocations associated with hematological malignancies. *MLL* functions as the mammalian counterpart of *Drosophila* trithorax (trx), which positively maintains the expression of homeobox genes during development. The translocations examined so far resulted in an in-frame fusion of the partner to the N-terminus of MLL. The fusion proteins retained several functional MLL domains and are assumed to disrupt critical patterns of homeobox gene expression in hematopoietic progenitor cells contributing to either myeloid or lymphoid acute leukemia [1].

In a case of acute myeloid leukemia showing an ins-(11;9)(q23;q34)inv(11)(q13q23) we were able to identify the human formin-binding protein 17 (FBP17) as a novel MLL fusion protein [2]. The members of the FBP family were isolated several years ago by screening mouse limb bud expression libraries with a formin-derived probe [3]. Formins are nuclear phosphoproteins required for proper limb and renal development. The physiological relevance of this interaction remains to be proved and in previous experiments we were unable to demonstrate the interaction between formin and wild type FBP17 or formin and the leukemic MLL/FBP17 fusion protein [2]. Thus, we speculated that there may be other interaction partners of FBP17. Herein, we demonstrate that FBP17 binds to tankyrase (also termed tankyrase 1, TNKS), a protein with diverse biological functions, e.g. mitogen-activated protein kinase signaling in the cytoplasm and telomere regulation in the nucleus [4,5].

2. Materials and methods

2.1. Cloning, homologous recombination and sequencing

We used polymerase chain reaction (PCR)-based cloning methods for the production of all fusion constructs. To serve as template for the FBP17 PCRs we obtained from the Kazusa DNA Research Institute (www.kazusa.or.jp/en/) clone KIAA0554 containing a 5383 bp cDNA fragment of FBP17. For amplification of full length FBP17 (wild type, WT, GenBank accession number AF265550) we used primers FBP17-F1 and FBP17-R1 (Table 1). For functional analyses truncated constructs were generated by combining the primers F1/R2, F1/R3 and F2/R1. All PCR reactions were performed with the proofreading polymerase Pfu (Stratagene, La Jolla, CA, USA) and a standard PCR protocol (60°C annealing, 35 cycles). The cloning of the fragments into vector pcDNA3.1DV5-His-TOPO (Invitrogen, Carlsbad, CA, USA) was performed according to the manufacturer's instructions.

For cloning of FBP17 in the pLexA vector we chose a recombination-based method as described in [6].

The wild type TNKS insert was generated by PCR amplification of the full length TNKS cDNA (GenBank accession number AF082556; primers TNKS-F and TNKS-R, Table 1) from a cDNA clone provided by T. de Lange and S. Smith. Truncated TNKS constructs (T1 and TS) were obtained by PCR amplification of the yeast two-hybrid prey clone insert (primers T1-F/T1-R and TS-F/pB42AD-R respectively; Table 1) and subsequent cloning into pcDNA3.1D-V5-His-TOPO.

GFP/TNKS and GFP/SNX2 fusions were produced by PCR-based cloning of the prey plasmid inserts into the pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) according to the manufacturer's instructions. FBP17 was fused to the red fluorescent protein by cloning into pDsRed1-N1 (Clontech, Palo Alto, CA, USA; restriction sites used: *Eco*RI and *Bam*HI).

All constructs were verified by sequencing using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI373A sequencer (ABI Biosystems, Foster City, CA, USA). Further analyses of the sequences were done with the program OMIGA 2.0 (Oxford Molecular), BLAST search algorithms (www.ncbi.nlm.nih.gov/blast/) and the Prosite database at www.expasy.ch/prosite/.

2.2. Mutagenesis

For examination of the putative tankyrase-binding motif of FBP17 we used the QuikChange™ Multi Site-Directed mutagenesis system

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Table 1
Primer sequences (5′→3′)

Forward primer for PCR		Reverse primer for PCR	
FBP17-F1	aggagctgcgagccggaggagg	FBP17-R1	ctaggaatctttggcatttttgtccaaacag
		FBP17-R2	ctagtcctgggcgcagttgttgactgtg
FBP17-F2	ggagagtccatgaagacatatgcag	FBP17-R3	ctatgaaaggctgttatctgacacagtgcg
TNKS-F	atggcggcgtcgcgtcgctctcagcatcatc	TNKS-R	ctaggtcttctgctctgcggctgttgcggtctggg
T1-F	gaattcgcggccgcgtcgactgttg	T1-R	ttaactcagaatctgctgcactgcttc
TS-F	gagagtacacctatacgtacttc	pB42-R	ggtagacaagccgacaaccttgattgg
Mutagenesis primer for FBP17			
R577A+D581A	gcgcccaggacgctgagagcccagctggcagttacac	577del582	caactgcgcccaggacagttacacagaggagc

Inserts with restriction sites, myc or FLAG tags were produced by adding a corresponding sequence to the primer.

(Stratagene) and primers R577A+D581A and 577del582 (Table 1). The myc-FBP17-WT construct served as template for the mutagenesis reaction according to the manufacturer's instructions.

2.3. Yeast two-hybrid screening

For our yeast two-hybrid library screen we used the Matchmaker LexA Two Hybrid System (Clontech) with the yeast indicator strain EGY48[p8op-lacZ]. To serve as bait FBP17 (F1/R1) was fused to the C-terminus of the LexA DNA-binding domain. We screened a human kidney cDNA library in vector pB42AD obtained from Clontech and tested for false positive clones according to the manufacturer's instructions. The identification of the insert sequences of true positive clones was done with the help of the BLAST search algorithms.

2.4. Cell culture and transfection

293T cells were cultured in Dulbecco's modified Eagle's medium containing 4500 mg/l glucose, 862 mg/l L-alanyl-L-glutamine, 110 mg/l Na-pyruvate, 50 000 U/l penicillin, 50 mg/l streptomycin and 10% (v/v) fetal calf serum. The cells were grown in a humidified incubator at 37°C and 5% CO_2. For transfection we seeded 6×10^6 cells in a 100 mm culture dish at least 24 h prior to the procedure. For each transfection a total amount of 20 μg DNA was used following a standard calcium phosphate transfection protocol [7]. The cells were harvested 48 h after transfection. For microscopy the cells were trypsinized and examined for development of fluorescence in a confocal laser scanning microscope (Zeiss).

2.5. Preparation of mammalian and yeast cell extracts

We lysed 293T cells using a hypotonic lysis buffer (20 mM HEPES pH 7.5, 10 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 10% glycerol, 4 mM dithiothreitol [DTT], 0.5 mM sodium vanadate, 2 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 4 μl/ml protease inhibitor mix [Sigma]). After centrifugation the supernatant representing the cytosolic fraction was used for co-immunoprecipitations and is designated the cytoplasmic extract. Yeast cell extracts were produced by a standard urea/sodium dodecyl sulfate (SDS) protein extraction protocol [8].

2.6. Co-immunoprecipitation and Western blotting

Precleared lysates were incubated with 2 μg polyclonal immunoprecipitation antibody (Table 2) for 2.5 h under constant motion at 4°C with addition of 30 μl protein A/G agarose after 1.5 h. Afterwards the beads were washed seven times with phosphate-buffered saline (PBS) containing 0.25% bovine serum albumin and 2 mM DTT and one time with PBS. After centrifugation the supernatant was removed, leaving about 20 μl, and a corresponding volume of sample buffer (Pierce, Rockford, IL, USA) was added. The samples were incubated at 90°C for 5 min and electrophoresed on an SDS polyacrylamide gel. The proteins were detected following a standard Western blotting chemiluminescence protocol (SuperSignal™ West Pico System, Pierce). For details on detection antibodies used in this study see Table 2. For stripping blots were incubated with Restore™ Western Blot stripping buffer (Pierce) for 15 min at 37°C.

3. Results

3.1. Yeast two-hybrid screen

For our yeast two-hybrid experiments we fused residues 48–2042 of the human FBP17 mRNA sequence (GenBank accession number AF265550) to the C-terminus of a LexA DNA-binding domain. The expression of the fusion protein was confirmed by Western blotting showing a strong signal at the expected molecular weight of 109 kDa (data not shown). Since FBP17 shows high expression levels in the kidney (www.kazusa.or.jp/huge/grpage/KIAA0554), we decided to screen a human kidney cDNA library for interaction partners of FBP17. We obtained a commercially available library in which the cDNAs were fused to the C-terminus of a B42 activation domain. The library consisted of 3.5×10^6 independent clones which were screened for LacZ activation. After 5 days of cultivation on induction medium lacking tryptophan, histidine, uracil and leucine we picked 288 blue colonies, of which 44 clones were considered true positives. Two different sequences were obtained of 450 bp and 2850 bp, respectively. These plasmids gave a similarly strong positive result in comparison to various controls (Fig. 1A,B). We identified the insert sequences as encoding sorting nexin 2 (SNX2) [2] and TNKS (GenBank accession number AF082556). Since the sequences among the various clones were identical, the clones are probably derived from a single library clone each. However, the TNKS insert resembled neither the already described splicing variant TT7 nor the variant TT9 (GenBank accession numbers AF082558 and AF082559) but represented

Table 2
Antibodies used in this study

Antibody	Source	Used for	Manufacturer
Anti-c-myc	rabbit polyclonal against c-Myc tag	immunoprecipitation	Sigma
Anti-c-myc	mouse monoclonal, clone 9E10	detection	Sigma
Anti-FLAG	mouse monoclonal, M2	detection	Stratagene
Anti-TNKS	goat polyclonal (N-20)	immunoprecipitation	Santa Cruz
Anti-TNKS	rabbit polyclonal (H-350)	detection	Santa Cruz
Anti-FBP17	rabbit polyclonal antiserum against epitope NH_2-KQLESSKRRFERDC-COOH	detection	Eurogentec
Secondary antibodies			
Anti-mouse IgG	goat, Fab-specific, HRP conjugate		Sigma
Anti-rabbit IgG	goat, HRP conjugate		Santa Cruz

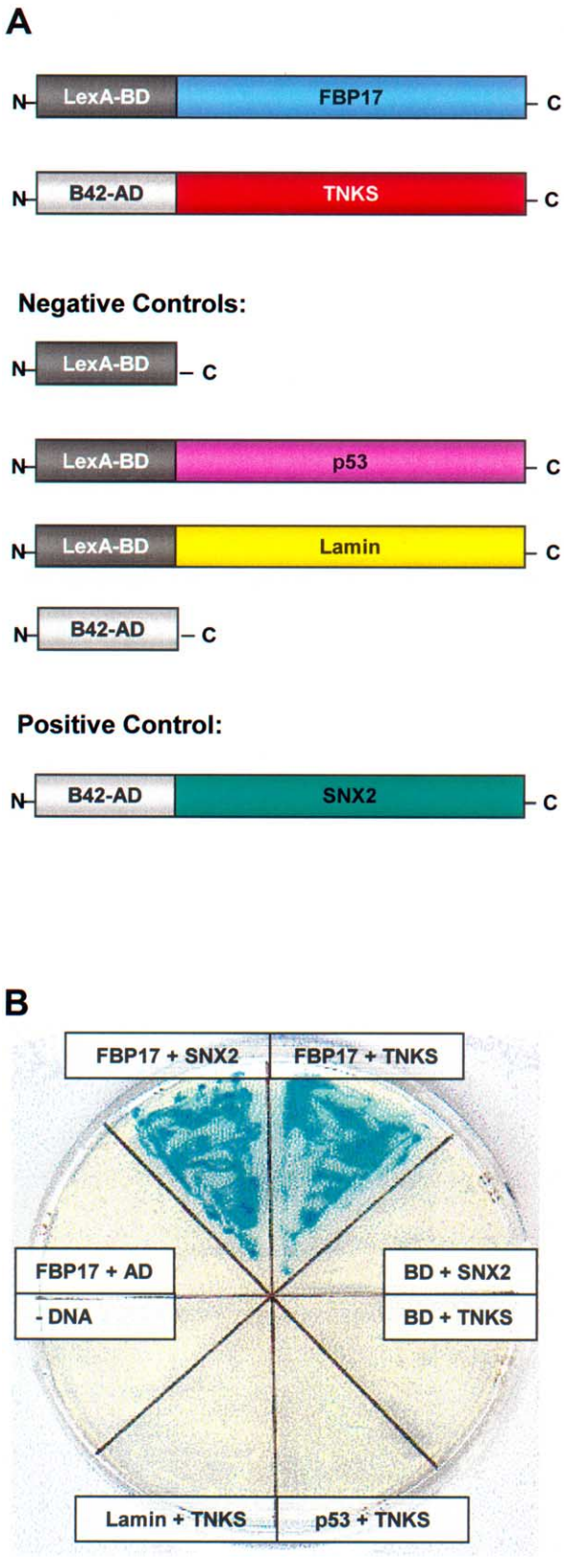


Fig. 1. A: Constructs used in the yeast two-hybrid interaction trap. LexA-BD: DNA-binding domain of the LexA protein, B42-AD: activation domain of the B42 protein. B: Two-hybrid screen results, showing colonies exhibiting FBP17/TNKS interaction in comparison to positive (FBP17/SNX2) and negative controls (lamin C/TNKS, p53/TNKS, co-transformations with empty vectors).

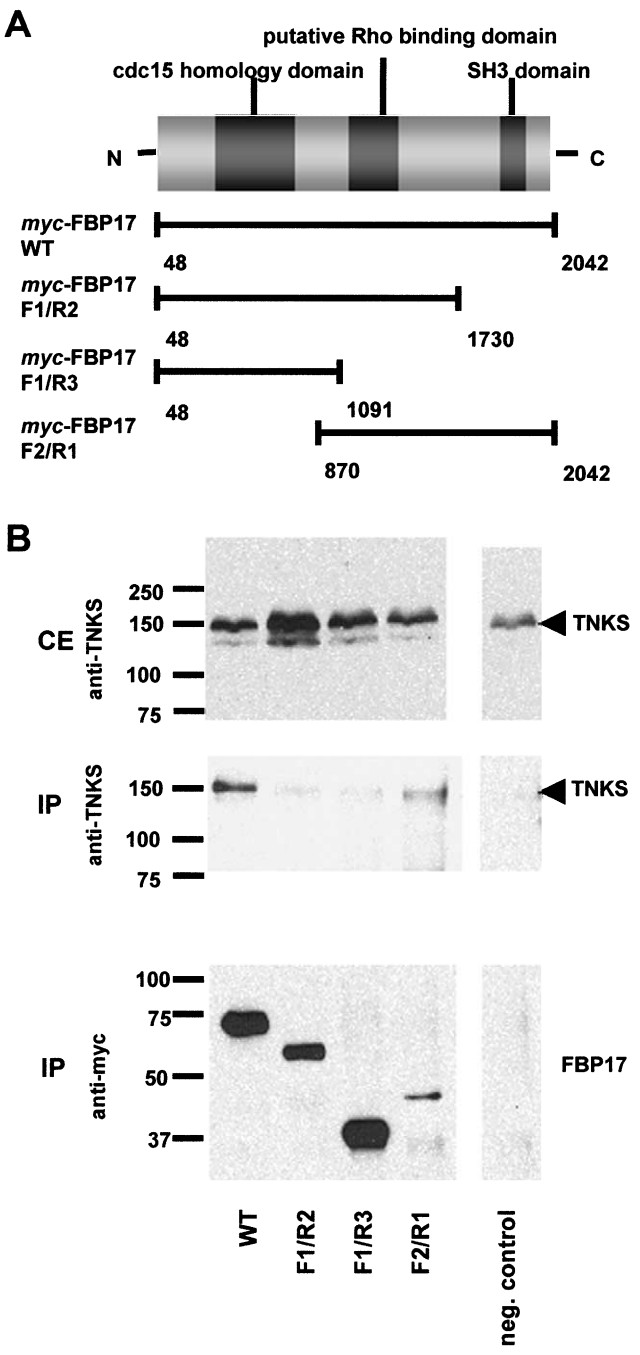


Fig. 2. A: Graphical overview of the FBP17 deletion constructs. Numbers refer to nucleotides in mRNA. B: Co-immunoprecipitation assays of TNKS with the myc-FBP17 deletion constructs. CE: cytoplasmic extracts of transfected 293T cells, IP: immunoprecipitate. Immunoprecipitation was performed with polyclonal anti-myc antibody. Top panel: Verification of TNKS expression in the cytoplasmic extracts with anti-TNKS antibody at approx. 150 kDa. Middle panel: TNKS detection with anti-TNKS in immunoprecipitates. Strong signal of TNKS in the FBP17 wild type (WT) immunoprecipitate and a weaker TNKS signal in the F2/R1 immunoprecipitate. No TNKS signal in the F1/R2 and F1/R3 immunoprecipitates. No signal in the immunoprecipitate of the solely TNKS-transfected cells (neg. control). Bottom panel: Confirmation of the correct myc-FBP17 construct expression and immunoprecipitation with anti-myc antibody. Molecular weights of the constructs: WT = 73 kDa, F1/R2 = 62 kDa, F1/R3 = 38 kDa, F2/R1 = 42 kDa.

exons 2–23 (encoding aa 231–1051) of tankyrase. The wild type tankyrase protein contains an ankyrin repeat region (aa 205–943), a sterile α module (aa 1030–1089) with the ability to mediate both homotypic and heterotypic protein–protein interactions [9] and a poly(ADP-ribose) polymerase (PARP) domain (aa 1176–1327) [10] (Fig. 3A).

3.2. Verification of the interaction

The interactions were verified by co-immunoprecipitations. FBP17 was tagged at its N-terminus with a myc epitope (NH_2-EQKLISEEDL-COOH). In all cases the expression was driven by a cytomegalovirus promoter. The constructs were transfected into 293T cells. Myc-FBP17 was isolated from cytoplasmic extracts with a polyclonal anti-myc antibody. Only in samples of cells co-transfected with FBP17 and TNKS was the TNKS protein detectable at approximately 150 kDa, whereas the control transfection solely with FLAG-TNKS showed no signal in the corresponding area. Correct expression of myc-FBP17 at 73 kDa was confirmed by probing with monoclonal anti-myc antibody (Fig. 2B, left lane).

3.3. Involvement of the protein domains in the interaction

For a more precise identification of the domains involved in the FBP17/TNKS interaction we produced a series of FBP17 deletion mutants. The first mutant, termed myc-FBP17 F1/R2, contained nucleotides 48–1730 of the mRNA sequence and therefore represented FBP17 without its SH3 domain leading to the expression of a 62 kDa protein. The second, myc-FBP17 F1/R3, consisted of residues 48–1091, producing a FBP17 protein lacking the SH3 domain as well as the putative rho-binding domain with an apparent molecular weight of 38 kDa. The third mutant, myc-FBP17 F2/R1, contained residues 870–2042 representing FBP17 without the N-terminal Cdc15 homology domain, producing a 42 kDa protein (Fig. 2A). After co-transfection with TNKS in 293T cells only wild type FBP17 and construct F2/R1 gave positive results in the co-immunoprecipitation assay, although construct F2/R1 was poorly expressed (Fig. 2B). Thus, the C-terminal part of FBP17 is necessary for its binding to tankyrase.

The regions of tankyrase responsible for the interaction were examined by bisecting tankyrase. The fragments represented aa 231–639 (T1) and aa 640–1051 (TS) of the wild type protein (Fig. 3A) and after co-transfection only fragment T1 was able to bind FBP17 (Fig. 3B). Since the ankyrin repeats in tankyrase are organized in clusters [11], fragment T1 contains the complete ankyrin repeat cluster II and parts of clusters I and III.

3.4. Mutational analysis of the tankyrase-binding motif

Closer examination of the C-terminus of FBP17 revealed a putative tankyrase-binding motif (RxxPDG) also present in the known tankyrase interaction partners IRAP, TAB182, the telomeric repeat-binding factor-1 (TRF1) and slightly varied in Mcl-1 [12,13]. In FBP17 this motif is located at nucleotides 1729–1746 encoding RESPDG. Site-directed mutagenesis produced the mutants AESPDG (R577A), AESPAG (R577A+D581A) and a deletion mutant lacking the binding motif (577del582). After co-transfection with tankyrase only wild type FBP17 was able to bind tankyrase in the co-immunoprecipitation assay efficiently. The point mutations im-

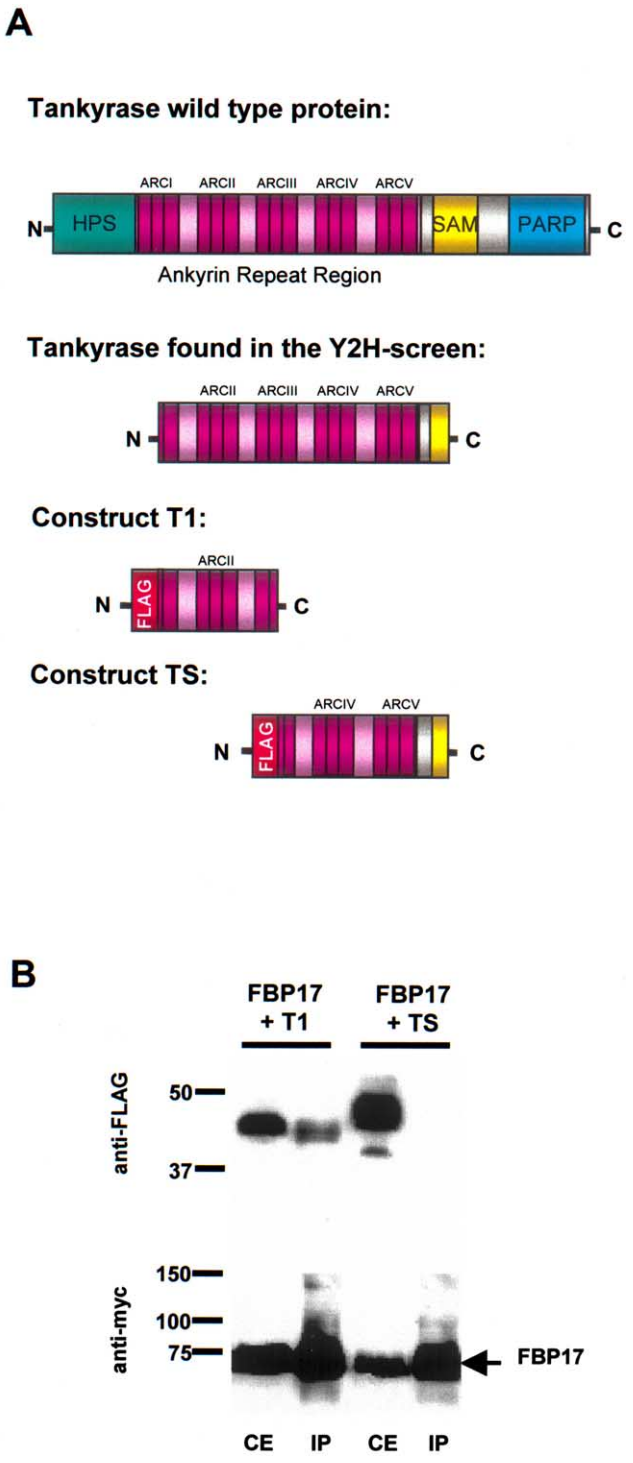
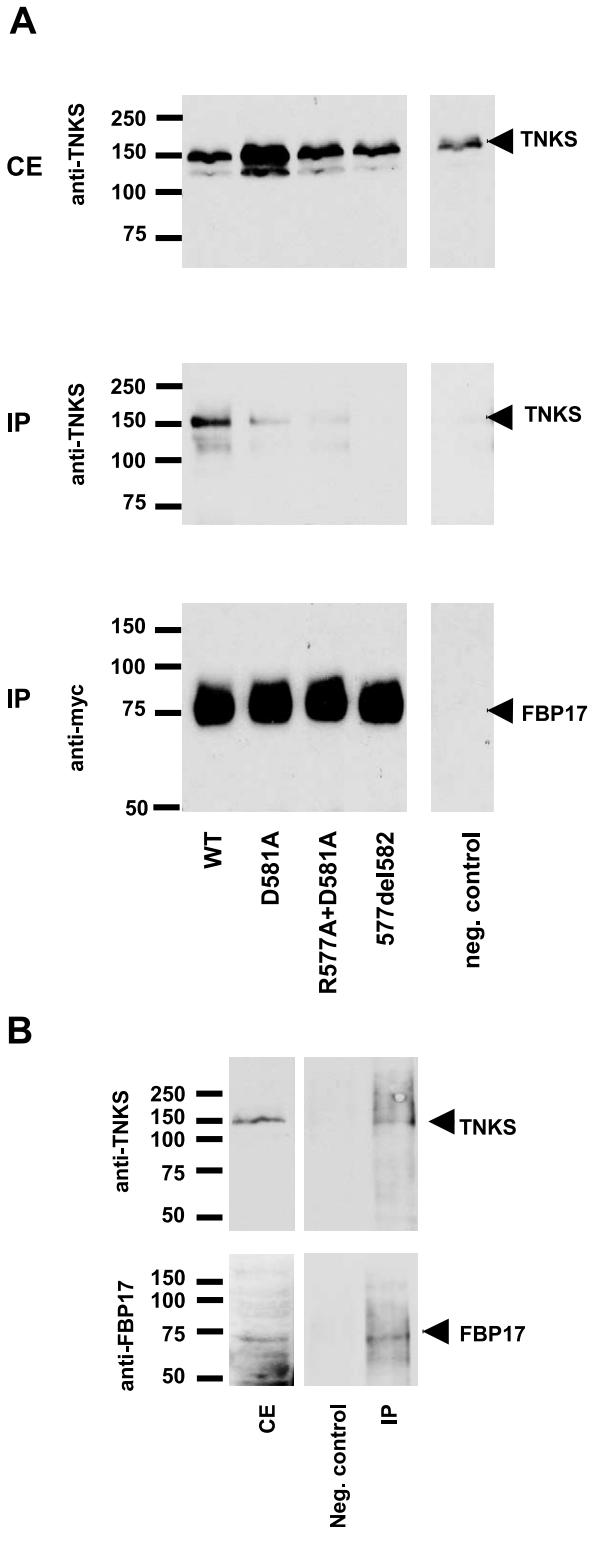


Fig. 3. A: Schematic representation of tankyrase wild type protein, the shortened tankyrase found in our library screen and the tankyrase deletion constructs T1 and TS. PARP = PARP domain, HPS = histidine-, proline-, serine-rich domain, SAM = sterile α module, ARC: ankyrin repeat cluster. B: Co-immunoprecipitations of TNKS deletion constructs with myc-FBP17. CE: cytoplasmic extracts of transfected 293T cells; IP: immunoprecipitate. Immunoprecipitation was performed with polyclonal anti-myc antibody. Upper panel: Detection of TNKS constructs with anti-FLAG antibody. Only construct T1 is detectable in the myc-FBP17 immunoprecipitate of the cotransfected cells. Lower panel: Confirmation of the correct myc-FBP17 expression and immunoprecipitation with anti-myc antibody at 73 kDa.

paired the binding abilities and deletion of the RESPDG motif completely abolished the interaction (Fig. 4A).

3.5. Interaction of endogenous FBP17 and TNKS

To test whether FBP17 and TNKS interact in their physiological context we performed a co-immunoprecipitation assay by immunoprecipitating endogenous TNKS from the cytoplasmic extracts of human embryonic kidney 293T cells. Detection of FBP17 with a specific antiserum was possible only in the cytoplasmic extracts and the tankyrase immunoprecipitate but not in the negative control without precipitation antibody (Fig. 4B).



3.6. Confirmation of the cytoplasmic localization of FBP17 and TNKS in the cell

To confirm the cytoplasmic localization of FBP17 and TNKS as well as SNX2 (positive control) in the cell, we transfected 293T cells with DsRed/FBP17 and GFP/TNKS or GFP/SNX2 fusion constructs. Seventy-two hours after transfection 293T cells showed strong expression of the fluorescent fusion proteins in the laser scanning confocal microscope. All proteins tested were localized exclusively in the cytoplasm, as has previously been reported for exogenously expressed tankyrase [14]. After overlay a localization of the proteins at the margin of the cytoplasm was observed (Fig. 5).

4. Discussion

In previous studies, FBP17, an MLL fusion partner in acute leukemias, was found to interact with the sorting nexin SNX2 [2,3,15], a molecule that co-immunoprecipitates with the receptor tyrosine kinases for epidermal growth factor, platelet-derived growth factor and insulin [16]. Herein, we extend these observations and report that FBP17 also interacts with tankyrase, a protein that has different functions in both the nucleus and the cytoplasm where the majority of tankyrase molecules can be found [4,10,13]. This interaction was mediated by an RESPDG hexapeptide in FBP17 that is retained in the MLL/FBP17 fusion protein found in leukemias. The physiological relevance of these findings was emphasized by the demonstration of an interaction between endogenous FBP17 and cytoplasmic tankyrase (Fig. 4B).

Wild type FBP17, like several other MLL fusion proteins, e.g. AF6, MSF or hCDCrel, is physiologically localized in the cytoplasm [2,17–19]. After its fusion to MLL, the chimeric MLL/FBP17 oncoprotein is found in the nucleus [2]. It has therefore been hypothesized that MLL fusion partners might lose their physiological binding partners and that this process contributes to leukemogenesis. As long as the exact function of cytoplasmic tankyrase is unclear, such a role for the FBP17–tankyrase interaction remains speculative.

Tankyrase belongs to the ankyrin protein family, whose

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Fig. 4. A: Co-immunoprecipitation assay of TNKS with wild type myc-FBP17 and FBP17 constructs with mutated tankyrase-binding motif. Top panel: Verification of TNKS expression in the cytoplasmic extracts (CE) of transfected 293T cells with anti-TNKS at approx. 150 kDa. Middle panel: TNKS detection in FBP17 immunoprecipitates (IP). Immunoprecipitation was performed with polyclonal anti-myc antibody. TNKS binds to wild type FBP17 and much more weakly to the D581A mutant containing one point mutation in the binding motif. Introduction of a second point mutation (R577A+D581A) impairs the binding even more. Deletion of the binding motif (577del582) abolishes the binding completely. Bottom panel: Confirmation of equal FBP17 immunoprecipitation with anti-myc. No signal in the solely TNKS-transfected negative control. B: Co-immunoprecipitation of endogenous FBP17 by TNKS. TNKS was immunoprecipitated with a polyclonal anti-TNKS antibody from cytoplasmic extracts of 293T cells. FBP17 was detected with an anti-FBP17 antiserum in the cytoplasmic extract (CE) as well as in the immunoprecipitation (IP) but not in the negative control (IP without anti-TNKS).

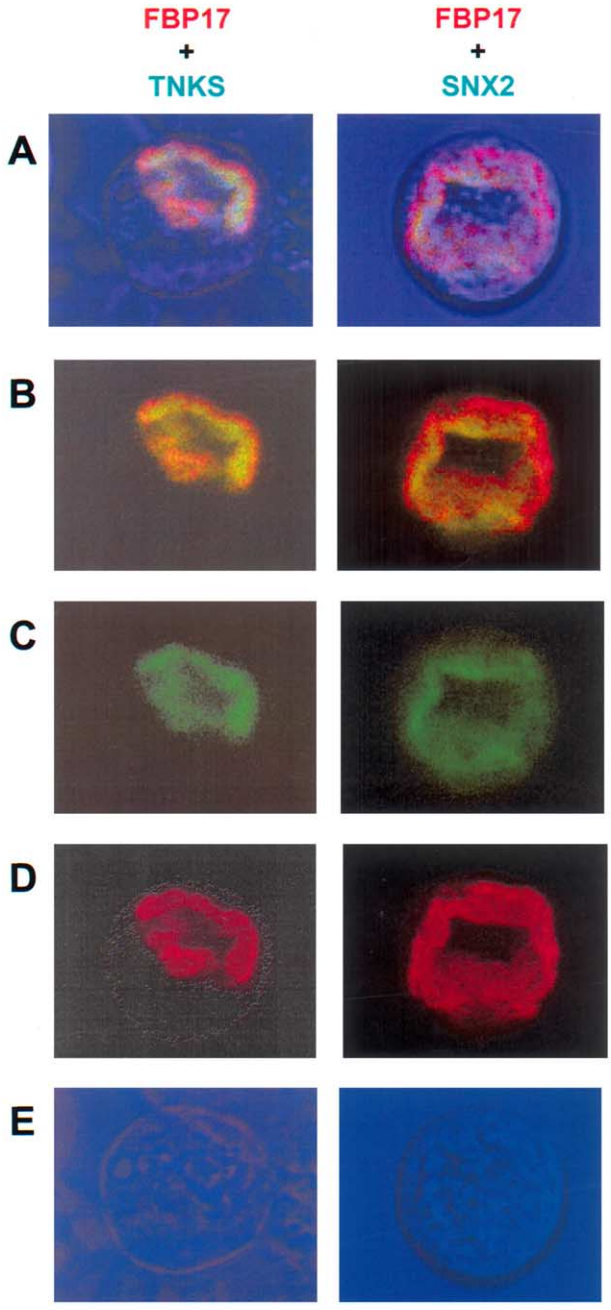

Fig. 5. Confocal laser scanning microscopy of transfected 293T cells showing the cytoplasmic localization of transfected FBP17 and TNKS. Fusion proteins used for transfection: DsRed-FBP17, GFP-TNKS, GFP-SNX2 (positive control). A: Merge of fluorescence and transmission. B: Merge of fluorescence. C: Green fluorescence caused by GFP fusion proteins. D: Red fluorescence caused by DsRed-FBP17. E: Transmission.

members function as linkers that couple diverse proteins via ankyrin motifs to the underlying cytoskeleton [20–22]. This family is characterized by tandem repeats of a 33 aa motif (ANK repeats). Tankyrase possesses 24 ANK repeats as well as a sterile α module, responsible for protein–protein interactions, and a PARP homology domain [10]. All known tankyrase interaction partners (TRF1, IRAP, TAB182 and Mcl-1) bind to the ankyrin repeat domain which is organized in five repeat clusters separated by highly conserved LLeaaK/R motifs. The fact that the partners are able to bind to overlapping ankyrin repeats suggests a competition between the different interacting proteins with regulative potential [4,10,11,13,23].

The PARP domain enables tankyrase to ADP-ribosylate itself and specific substrates like TRF1 [10,11]. Thus, tankyrase is involved in proper regulation of human telomere maintenance. A recently described tankyrase 2, which is highly homologous to tankyrase, lacks the N-terminal histidine-, proline- and serine-rich domain (HPS) but shows a very similar behavior to tankyrase, with which it is able to oligomerize [24–26]. In addition two splicing variants of tankyrase have been described (GenBank accession numbers AF082558 and AF082559), both lacking the sterile α module and the PARP domain and therefore probably limited in their regulative abilities. The truncated protein discovered in our library screen also lacks the PARP and HPS domains, indicating that these domains are dispensable for the interaction with FBP17. The absence of intrinsic PARP activity abolishes the ADP-ribosylation capabilities of tankyrase and therefore impairs the regulation of telomeric function [10].

In addition tankyrase might also exhibit a role in regulation of cell survival and apoptosis. As has been shown recently tankyrase down-regulates Mcl-1 protein expression, an anti-apoptotic Bcl-2 protein family member. Of special note, the down-regulation of Mcl-1 proteins by tankyrase does not depend on ADP-ribosylation [13].

In further studies the mode of interaction between tankyrase and FBP17 should be examined. FBP17 could serve as a poly(ADP-ribosyl)ation substrate for tankyrase or mediate secondary interactions with substrates or effectors. In sum, these data provide a link between an MLL fusion partner and a possible deregulation of telomere function and/or apoptosis in human cells.

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