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Regulation of Tankyrase activity by a catalytic domain dimer interface

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Abstract

Tankyrases (TNKS and TNKS2) are enzymes that catalyze poly-ADP-ribosylation (PARsylation) of their target proteins. Tankyrase-mediated PARsylation plays critical regulatory roles in cell signaling, particularly in the Wnt/β-catenin pathway. The sterile alpha motif (SAM) domain in tankyrases mediates their oligomerization, which is essential for tankyrase function. The oligomerization regulates the catalytic activity of tankyrases, but the underlying mechanism is unclear. Our analyses of crystal structures of the tankyrase catalytic domain suggest that formation of a head-to-head dimer regulates the catalytic activity. Our activity assays show that residues in the catalytic domain dimer interface are important for the PARsylation activity of tankyrases both in solution and cells. The dimer is weak and may only form in the context of the SAM domain-mediated oligomers of tankyrases, consistent with the dependence of the tankyrase activity on the SAM domain.

Keywords

Tankyrase; dimerization; oligomerization; PARsylation

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1. Introduction

Tankyrases 1 and 2 (TNKS and TNKS2) are two highly homologous members of the poly-ADP-ribosylation polymerase (PARP) family. PARPs catalyze the transfer of the ADP-ribose group from NAD^+ to their target proteins for PARsylation, a post-translational modification that is involved in regulating many cellular processes, including telomere maintenance, DNA repair and Wnt/β-catenin signaling [1]. Dys-regulation of TNKS and TNKS2 by altered expression levels or mutations is associated with malignant tumors [2]. Inhibitors of TNKS and TNKS2 are currently being developed for targeting colorectal cancer and other cancer types [3].

PARsylation of the target proteins often leads to their degradation through the ubiquitin-proteosomal system [4, 5]. One of the PARsylation substrates of tankyrases are the Axin proteins (Axin1 and 2), negative regulators of the Wnt/β-catenin pathway [6]. Axin is a core components of a multi-protein complex referred to as the destruction complex, which also contains adenomatous polyposis coli (APC) and glycogen synthase kinase 3α/β (GSK3 α/β) [7, 8]. In the absence of Wnt, the destruction complex constitutively drives the degradation of β-catenin, keeping the downstream pathways off. Tankyrases promote Wnt/β-catenin signaling by PARsylation of the Axin proteins thereby inducing degradation of Axin proteins and lifting the suppressive action of the destruction complex on signaling [6]. Consequently, levels of β-catenin are increased, which enters the nucleus to promote gene transcription. Tankyrases also undergo auto-PARsylation, which promotes their own destruction through the ubiquitin-proteasome pathway [6, 9].

Tankyrases are distinct members in the PARP family in that they contain several unique regulatory domains in addition to the conserved catalytic domain. There are five N-terminal ankyrin repeat clusters (ARCs), which are responsible for substrate recruitment by recognizing “RXXXDG” (single-letter amino acid code) motifs in substrate proteins [10, 11]. Another unique domain in tankyrases is the Sterile Alpha Motif (SAM) domain located between the ARCs and the catalytic domain [12, 13]. The SAM domain mediates tankyrase oligomerization by forming a left-handed spiral with six subunits per turn [14–16]. The positive regulation of Wnt signaling by tankyrases is dependent on the SAM domain-mediated oligomerization, which has been shown to both control tankyrase subcellular localization and promote substrate recruitment.

Disruption of the SAM domain-mediated oligomerization has also been shown to cause diminished auto-PARsylation of tankyrases, suggesting that the oligomerization is required for the catalytic activity ([12, 14, 15]). The mechanism by which the oligomerization regulates the catalytic activity remains unclear. From the analyses of crystal structures of the catalytic domains of tankyrases in the database, we found a reoccurring dimer that is correlated with the open conformation of the substrate-binding site in the catalytic domain. Our mutational analyses support the notion that the dimerization of the catalytic domain in the context of the SAM mediated-oligomers promotes the catalytic activity of tankyrases.

2. Materials and Methods

2.1. Protein expression and purification.

The catalytic domain of human TNKS (residues 1105–1313) cloned into a modified pET28 vector (Novagen) was expressed and purified as described previously [17]. The SAM-catalytic domains (residues 1017–1315) were cloned into the same vector and expressed in a similar manner. The SAM-catalytic domains were purified using a similar procedure as for the catalytic domain and stored at −80°C before use. Mutations were introduced by site-directed mutagenesis.

2.2. In vitro auto-PARsylation Assay and Histone PARsylation assay.

Two methods were used to detect the auto-PARsylation of TNKS SAM-catalytic domains. In the first purified proteins were run on SDS-PAGE and transferred to PVDF membrane (Millipore). Ponceau staining was used to check loading of proteins. Auto-PARsylation of proteins that has occurred during expression in E. Coli was detected by Western blot with an anti-PAR polyclonal antibody (Trevigen, #4336-APC-050). In the second protocol, purified proteins, both WT and mutants, were mixed with 6-biotin-17-NAD (TREVIGEN) in the reaction buffer (50 mM Hepes pH7.5, 150 mM NaCl, 2 mM $MgCl_2$) at 30°C for 90 minutes. Reaction products were resolved on SDS-PAGE and detected by Western blot using HRP conjugated anti-biotin antibody (Cell Signaling Technology, #D5A7). To check loading of protein, the same membrane was stripped and probed with anti-His antibody (Takara, #631212). Histone PARsylation assay was conducted as described in HT Universal Color PARP Assay Kit w/ Histone Coated Strip Wells (Trevigen, #4677–096-K) [17].

2.3. Analytical Ultracentrifugation.

Various concentrations of wild-type TNKS catalytic domain (140 μM, 70 μM, 37 μM, 11.6 μM and 3.7 μM) were loaded into the “sample” side of dual-sectored charcoal-filled Epon centerpieces, while the “reference” sectors were loaded with the centrifugation buffer (10 mM Tris, pH8.0, 150 mM NaCl and 1 mM DTT) of the same volume. Filled cells were loaded into an An50Ti rotor and equilibrated for 2 hours under vacuum in a Beckman Coulter Optima XL-I ultracentrifuge at 4°C prior to centrifugation. Data were acquired at 50,000 rpm at 4°C via absorbance at 305 nm and interference optics. Data were analyzed using the c(s) methodology in the program SEDFIT [18]. The figure featuring c(s) distributions were rendered with the program GUSSI [19].

2.4. Thermodenaturation assay.

The TNKS catalytic domain in a buffer containing 10 mM Trish, pH8.0, 150 mM NaCl and 1 mM DTT was mixed with the protein thermal shift dye from Applied Biosystems (#4461141) to reach the final protein concentration of 30 μM. The samples (20 μl) in 96-well plate were subjected to thermodenaturation by using a CFX96 Real-time PCR machine (Bio-Rad). Fluorescence signal was recorded during temperature increase from 4°C to 85°C.

2.5. Structural and sequence analyses.

The PyMOL software package (http://www.pymol.org) was used for structure analyses and figure rendering. The sequence alignment was rendered using ESPript [20].

2.6. Immunoprecipitation and Western blot.

The entire coding human TNKS cDNA sequence was fused to the IgG Fc domain by ligating into the pcDNA3 EK FC vector using flanking restriction sites introduced by PCR. Human TNKS-FLAG DNA constructs harboring various mutations were engineered using site-directed mutagenesis. HEK293 or COS1 cells were transfected with the corresponding cDNA construct using Effectene (Qiagen) according to the manufacturers protocol. To chemically inhibit TNKS, cells were treated with the corresponding inhibitor for 24 hours. For immunoblotting, cells were lysed in 1X protein sample loading buffer diluted from Biorad 4X Laemmli protein sample buffer (#1610747), and proteins were separated on SDS-PAGE. For immunoprecipitation or IgG pull-down studies, cleared lysates were mixed with Protein A agarose beads in the presence or absence of 2 μg of desired antibody and rotated for 4 hours at 4°C. Beads were then washed three times with lysis buffer (1% NP40 in PBS). Bound proteins were eluted using 2x protein sample loading buffer and separated on SDS-PAGE. Antibodies were acquired from the following sources: anti-FLAG (Sigma, #A2220), anti-TNKS1/2 (Santa Cruz Biotechnology, #sc-8337), anti-NPT (Millipore, #06-747), and anti-ACTIN (Sigma, #A1978).

3. Results

3.1. Catalytic activity of tankyrases is dependent on the SAM domain-mediated oligomerization.

Previous studies have shown that loss of the SAM domain-mediated oligomerization reduces self-PARsylation of tankyrases from mammalian cells [14, 15]. We found that TNKS SAM-catalytic domains purified from E. Coli is auto-PARsylated (Figure 1). We made two oligomerization-impaired SAM-catalytic mutants, V1056G and Y1073A, based on the crystal structures of the SAM domain tankyrases [14, 15]. Consistent the previous studies, the mutants showed much lower levels of PARsylation than the wild-type protein (Figure 1B). These observations suggest that the SAM domain-mediated oligomerization promotes the catalytic activity of tankyrases. Alternatively, the diminished self-PARsylation of the mutants may be caused by impaired PARsylation *in trans* of tankyrase molecules that cannot oligomerize, rather than reduced catalytic activity *per se*. To distinguish these two possibilities, we examined the PARsylation activity of the SAM-catalytic domains of TNKS by using histone as a substrate. The results show that wild-type SAM-catalytic domains PARsylated histone much more efficiently than the V1056G and Y1073A mutants (Figure 1C). The isolated catalytic domain was even less active (Figure 1C). These results support the notion that the catalytic activity of tankyrases is dependent on the SAM domain-mediated oligomerization.

3.2. A catalytic domain dimer in crystal structures of TNKS and TNKS2.

To understand how the oligomerization regulates the PARsylation activity of tankyrases, we analyzed crystal structures of the catalytic domains of tankyrases in the PDB database. Interestingly, we found a catalytic domain dimer that is present in most of the structures of TNKS and in several structures of TNKS2, crystallized in different conditions and space groups (Table S1). The repeated occurrence of this dimer of both TNKS and TNKS2 suggests that it may represent a functionally relevant state rather than a crystal packing artifact. The following descriptions of the dimer will refer to the TNKS catalytic domain structures of PDB ID 3TOS [17].

The two protomers in the dimer interact in a head-to-head fashion, burying ~3100 $Å^2$ solvent accessible area (Figure 2). The dimer interface is formed by helix α2 and a number of neighboring loops in the catalytic domain. One of the loops in the dimer interface is that connecting helix α3 and strand β4, which is referred to as the D-loop and a major structural element of the binding site for the NAD^+ substrate. The interactions with the dimer partner appear to pull the D-loop away from the active site, resulting in a conformation that is more open for the access of the NAD^+ substrate. In contrast, in crystal structures of tankyrases where the dimer is not present, the D-loop adopts more closed conformations that obstruct NAD^+ binding (Figure 2D). Based on these analyses, we hypothesize that the formation of the head-to-head dimer of the catalytic domain stabilizes the D-loop in the open conformation, which promotes NAD^+ binding and thereby enhances the catalytic activity.

The dimer interface is predominantly composed of complementarily charged residues from each subunit, indicating weak but specific interactions. For example, Glu1199 in the D-loop and Glu1298 in the loop between strands β8 and β9 make charge-charge interactions with Arg1296 from the dimer partner (Figure 2C). Arg1200 in the D-loop also interacts with Glu1172 in helix α2 from the dimer partner. A sequence alignment of tankyrases and other PARP family members shows that residues in the dimer interface are conserved among tankyrases from diverse species, but not conserved in other PARPs (Figure S1). Residues in the dimer interface are mostly surface exposed in the catalytic domain in the monomeric state, suggesting that their conservation is not for maintain the structural stability of the protein. As a comparison, C1163, and Q1166 in helix α2, which do not contribute to the dimer interface but located in close proximity to some of the interface residues, are not conserved. This conservation pattern is consistent with the idea that residues in the dimer interface are not required for the catalytic activity of the PARP family enzymes in general, but conserved for mediating the formation of the catalytic domain dimer in tankyrases.

3.3. Catalytic domain of TNKS is monomeric in solution.

To examine whether the catalytic domain dimer forms in solution, we analyzed purified catalytic domain of TNKS using sedimentation velocity analytical ultracentrifugation (AUC). The TNKS catalytic domain at concentrations as high as 140 μM showed a sedimentation coefficient of 2.3 s, corresponding to a monomeric species of ~24 kDa (Figure S2). These data show that the catalytic domain does not dimerize on its own, in agreement with the notion that the dimer is weak as suggested by the polar nature of the dimer interface. The formation of the dimer in crystals is likely driven by high protein

concentrations in the crystallization conditions. In the SAM domain-mediated oligomers of tankyrases, the catalytic domains are brought together to reach very high local concentrations, favoring the formation of the head-to-head dimer that induces the active conformation of the catalytic domain. This model therefore provides a putative explanation for the dependence of the catalytic activity of tankyrases on the SAM domain-mediated oligomerization.

3.4. Disruption of the catalytic domain dimer impairs tankyrase auto-PARsylation.

We used the auto-PARsylation assay of the SAM-catalytic domains of TNKS to test the role of the catalytic domain dimer in regulating the catalytic activity. We introduced charge-reversal mutations of Arg1200, Arg1296 and Glu1298, which are located at the core of the dimer interface but away from the active site, into the SAM-catalytic domains. These mutations are expected to disrupt the dimerization of the catalytic domain, but not affect the oligomerization of the SAM domain. The mutants showed dramatically reduced auto-PARsylation compared to the wild-type protein (Figure 3A). As a control, we compared the stability of the wild-type catalytic domain and the dimer interface mutants by using a thermo-denaturation assay. This assay is based fluorescence increase of the environmentally sensitive dye SYPRO Orange upon binding to hydrophobic residues exposed during protein unfolding [21]. The results show that the wild-type and mutants denatured at very similar temperature (T_m within the range of 48–50 °C), confirming that the mutations did not affect the stability of the protein (Figure 3B). These analyses together support the notion that the enzymatic activity of tankyrases is promoted by the formation of the catalytic domain dimer within the SAM domain-mediated oligomer.

3.5. The catalytic domain dimer is required for tankyrase activity in cells.

We next addressed the relevance of the catalytic dimer interface to the function of tankyrases in cells. We confirmed prior reports that suppression of tankyrase PARsylation activity induces the accumulation of tankyrase protein in COS1 cells using three distinct classes of tankyrase inhibitors (IWR-1, IWR-3 and XAV939) (Figure 4A) [17]. Using this assay as an indicator of tankyrase activity in cells, we examined the effects of two of the catalytic dimer interface mutants, R1200E and R1296E. The results showed that both mutants were more abundant compared to wild-type TNKS (Figure 4B), suggesting that disruption of the catalytic domain dimer impairs tankyrase activity in cells. Co-immunoprecipitation experiments using HEK293 cells showed that IgG Fc-tagged TNKS could pull down FLAG-tagged TNKS, confirming the SAM domain-mediated oligomerization in cells (Figure 4C). The R1296E mutant was also pulled down by wild-type TNKS (Figure 4C), suggesting that this mutation does not affect the SAM domain-mediated oligomerization. Taken together, these observations support the model that the catalytic domain dimerization in the context of SAM domain-mediated tankyrase oligomers is important for the PARsylation activity in cells (Figure 4D).

4. Discussion

In humans, there are 17 different PARP family members [22]. Recently, the mechanisms of activity control for some PARP family members have been revealed. For example, PARP-1,

which is the most abundant enzyme in this family and involvement in DNA damage repair, undergoes an over 500-fold activity increase upon binding of DNA strand breaks [23, 24]. PARP-1 catalytic domain has a helical domain (HD), which inhibits the activity by blocking the active sites. The HD unfolds when PARP-1 encounters a DNA break, which releases the autoinhibition and allows NAD^+ binding to the catalytic site [23, 24]. The HD domain is present in PARP 1, 2 and 3, but not in tankyrases. Our model based on the dimerization of the catalytic domain provides a distinct mechanism for the regulation of tankyrases. In this model, the D-loop is an element in controlling substrate access to the active site. The sequence of the D-loop is conserved among tankyrases from different species but not other PARPs (Figure S1), suggesting that it may have evolved to serve as a unique regulatory element for tankyrases. The basal activity of the isolated tankyrase catalytic domain is extremely low, ~1000 fold lower compared with PARP-1 [25]. The activity of tankyrase is dramatically elevated by the SAM domain-mediated polymerization. Our model provides an explanation for the dependence of the catalytic activity on the SAM domain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights:

- The catalytic domain of tankyrases forms a head-to-head dimer that is correlated with the open conformation of the active site.
- The catalytic domain dimer promotes the catalytic activity of tankyrases.
- The catalytic domain dimer forms in the context of the SAM domain-mediated oligomer of tankyrases.

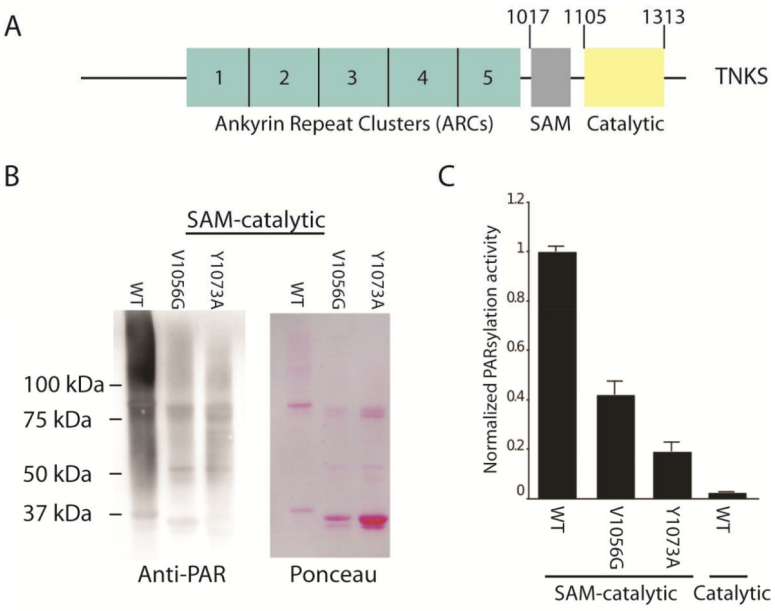

Figure 1. Dependence of the catalytic activity of TNKS on the SAM domain-mediated oligomerization.

(A) Domain architectures of human TNKS. (B) Disruption of the SAM domain-mediated oligomerization impairs auto-PARsylation in the TNKS SAM-catalytic domains. (C) Efficient PARsylation of Histone by the TNKS is dependent on the SAM domain-mediated oligomerization.

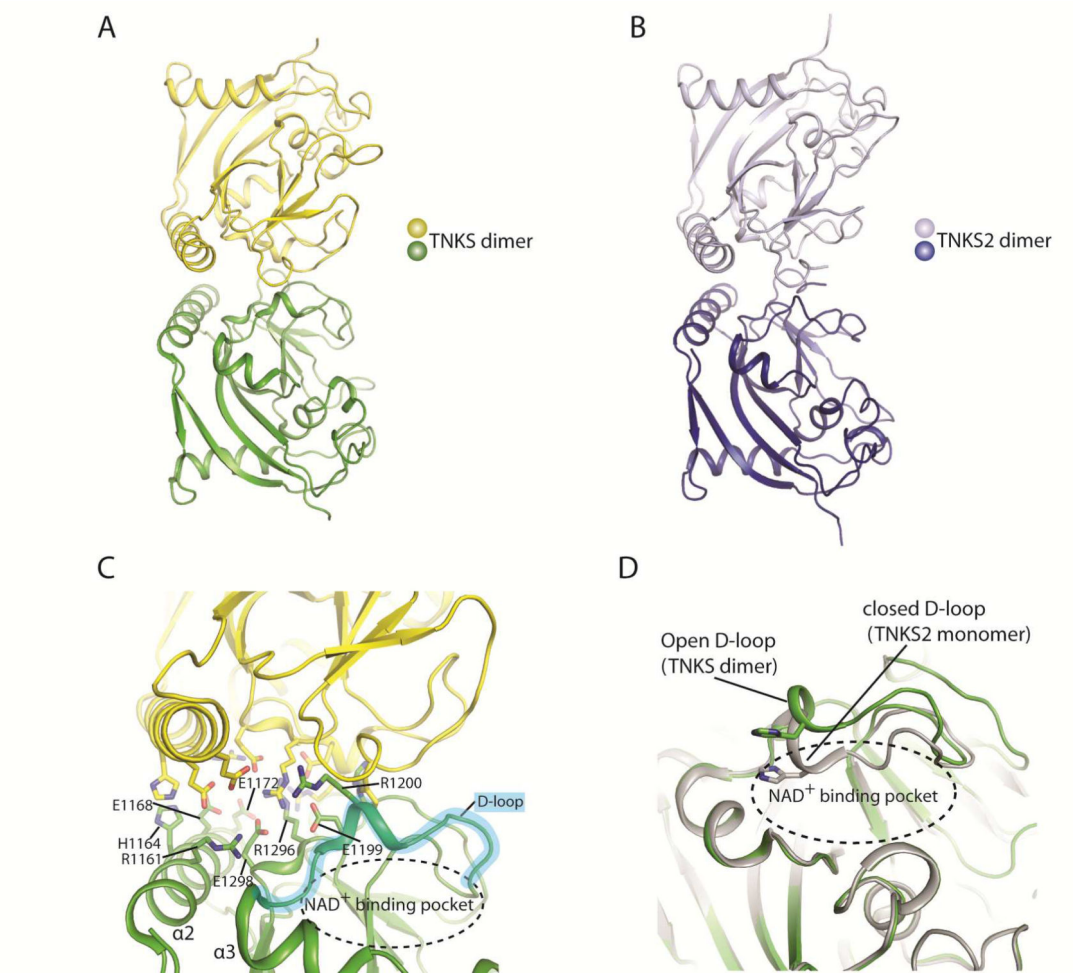

Figure 2. Head-to-head dimer of the TNKS catalytic domain.

(A) and (B) Overall structure of the head-to-head dimers formed by TNKS (PDB ID: 4TOR) and TNKS2 (PDB ID: 5FPF), respectively. (C) Detailed view of the dimer interface of TNKS. (D) Comparison of the D-loop in the structures of TNKS dimer in green (PDB ID: 4TOR) and TNKS monomer in gray (PDB ID: 3KR8).

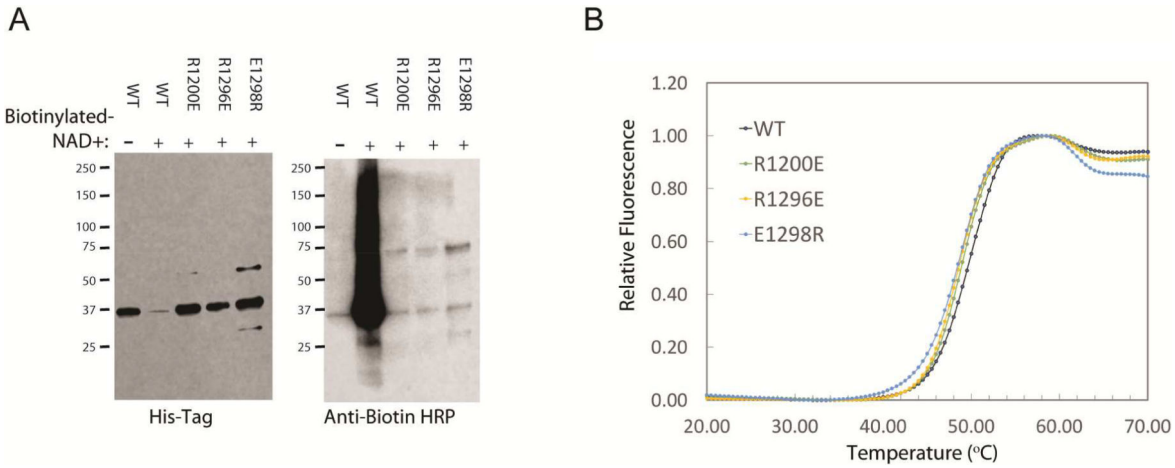

Figure 3. Dependence of auto-PARsylation of the TNKS SAM-catalytic domains on the catalytic domain dimer interface.

(A) Auto-PARsylation of the TNKS SAM-catalytic domains wild-type and catalytic domain dimer interface mutants. The right panel uses anti-biotin as a readout for biotin-labelled PARsylation of the proteins. The left panel shows equal protein loading with anti-His western blot. Note that the same amount of wild-type protein was used in lanes 1 and 2. Heavy PARsylation of the protein in lane 2 caused smearing of the protein in the entire lane, which led to the apparent weaker band in the left panel. (B) Thermodenaturation assays showing that the catalytic domain mutants have similar stability as the wild-type protein.

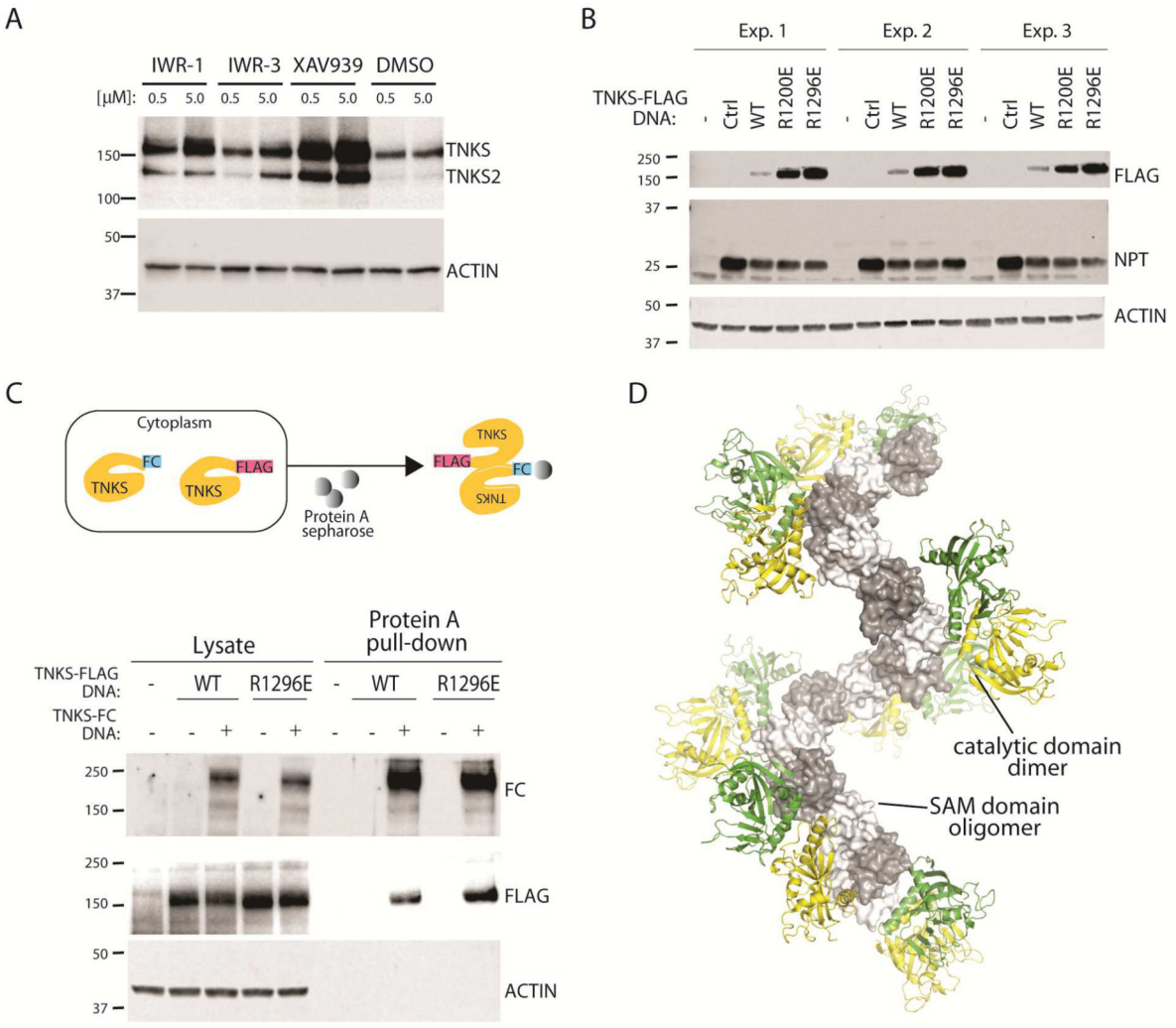

Figure 4. Mutations in the catalytic domain dimer interface compromise TNK activity in cultured cells.

(A) The catalytic activity of TNKS suppresses its own abundance. Exposure of COS1 cells to inhibitors binding to the adenosine- or nicotinamide-binding pocket or both (IWR-1, XAV939, and IWR-3, respectively) induces accumulation of TNKS and TNKS2. (B) Mutations in the catalytic domain dimer interface result in increased TNKS abundance in COS1 cells. NPT=neomycin phosphotransferase II antibody (transfection control). (C) Oligomerization of TNKS in HEK293 cells. TNKS-Fc protein could pull-down both the wild-type and R1296E mutant, suggesting that the mutation did not affect SAM domain-mediated oligomerization of TNKS. (D) Model of the TNKS catalytic domain dimer in the context of the SAM mediated oligomer (PDB ID: 5KNI).