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# The Exonuclease TREX1 Is in the SET Complex and Acts in Concert with NM23-H1 to Degrade DNA during Granzyme A-Mediated Cell Death

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## Summary

**Granzyme A (GzmA) activates a caspase-independent cell death pathway with morphological features of apoptosis. Single-stranded DNA damage is initiated when the endonuclease NM23-H1 becomes activated to nick DNA after granzyme A cleaves its inhibitor, SET. SET and NM23-H1 reside in an endoplasmic reticulum-associated complex (the SET complex) that translocates to the nucleus in response to superoxide generation by granzyme A. We now find the 3′-to-5′ exonuclease TREX1, but not its close homolog TREX2, in the SET complex. TREX1 binds to SET and colocalizes and translocates with the SET complex. NM23-H1 and TREX1 work in concert to degrade DNA. Silencing *NM23-H1* or *TREX1* inhibits DNA damage and death of cells treated with perforin (PFN) and granzyme A, but not of cells treated with perforin and granzyme B (GzmB). After granzyme A activates NM23-H1 to make single-stranded nicks, TREX1 removes nucleotides from the nicked 3′ end to reduce the possibility of repair by rejoining the nicked ends.**

## Introduction

Granzyme A (GzmA), an abundant serine protease in the cytolytic granules of cytolytic T cells and NK cells, activates caspase-independent cell death when it is delivered by perforin (PFN) into the target cell through the immunological synapse (Griffiths, 2003; Lieberman, 2003). GzmA does not activate the caspases or induce cleavage of most key caspase pathway substrates, such as bid or ICAD, and is able to kill target cells resistant to caspase-mediated cell death, including cells that overexpress *bcl-2* (Beresford et al., 1999; Martinvalet et al., 2005). This may be important for immune defense against cancers and viruses that have evolved strategies for evading caspase-mediated apoptosis. Target cells have all the morphological features of apoptosis. Chromatin condensation and nuclear fragmentation can be readily seen within a few hours (Beresford et al., 1999; Lieberman and Fan, 2003; Zhang et al., 2001a). A key feature of GzmA-mediated cell death is the initiation of single-stranded DNA damage, rather than the blunt double-stranded breaks characteristic of the caspase-activated DNase CAD (Beresford et al., 1999; Fan et al., 2003a; Shresta et al., 1999). These nicks produce multikilobase DNA fragments, which are not detected by assays that measure apoptotic DNA damage by production and release of short oligonucleosomal DNA fragments. However, GzmA-induced DNA damage can be detected by radiolabeling free single-stranded ends with TUNEL or the Klenow fragment of DNA polymerase or by separating the two strands of nicked chromosomal DNA using alkaline gel electrophoresis (Beresford et al., 1999, 2001; Fan et al., 2003a; Zhu et al., 2006).

DNA damage by GzmA is mediated by the 270–420 kDa SET complex (Beresford et al., 2001). Not all the components of this complex are known. The SET complex is mobilized during the cellular response to oxidative damage and is postulated to participate in the oxidative stress response (Martinvalet et al., 2005). When GzmA is delivered into a target cell, mitochondria are damaged to generate superoxide, and the SET complex, normally associated with the endoplasmic reticulum, translocates to the nucleus (Martinvalet et al., 2005). The SET complex contains both the endonuclease that makes the characteristic single-stranded cuts in GzmA-treated cells as well as a specific inhibitor of the nuclease. The GzmA-activated endonuclease is NM23-H1, which is also a tumor metastasis suppressor and nucleoside diphosphate kinase. Its inhibitor is the nucleosome assembly protein and GzmA substrate, SET (Fan et al., 2003a). When GzmA cuts SET, the NM23-H1 endonuclease is activated. The importance of NM23-H1 and SET in GzmA-mediated cell death was confirmed by finding increased DNA damage and cell death in cells that overexpress *NM23-H1* or have silenced *SET* and, conversely, by finding less cell death in targets with silenced *NM23-H1* or enhanced *SET* expression (Fan et al., 2003a). The SET complex also contains 2 additional GzmA substrates—the apurinic endonuclease responsible for base excision repair, Ape1 (Fan et al., 2003b), and the DNA-binding protein HMG-2 (Fan et al., 2002). pp32, a tumor suppressor protein that inhibits protein phosphatase PP2A, is also a component of the SET complex (Beresford et al., 2001).

Following GzmA treatment, the purified SET complex shows significant exonuclease activity on supercoiled plasmid DNA (Beresford et al., 2001; Fan et al., 2003a). Although NM23-H1 may have some 3′-to-5′ exonuclease activity, this activity has only been demonstrated on single-stranded DNA or double-stranded DNA with significant 3′ overhangs (Ma et al., 2004; Yoon et al., 2005). We therefore postulated that the SET complex might contain an exonuclease. Here we show that the 3′-to-5′ exonuclease TREX1/DNase III (Höss et al., 1999; Mazur and Perrino, 1999) is a component of the SET complex. Inclusion of TREX1 in the SET complex is specific, since the

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TREX1 homolog TREX2 (44% identity) (Mazur and Perrino, 1999) is not in the complex. TREX1, like NM23-H1, is not a GzmA substrate. On its own, TREX1 does not damage intact DNA. It acts in concert with NM23-H1 to destroy DNA during GzmA-mediated cell death. After NM23-H1 nicks one strand, TREX1 removes bases from the free 3′ end to enhance the damage and prevents DNA end reannealing and rapid repair.

## Results

### The SET Complex Contains the Exonuclease TREX1

The SET complex was previously identified and purified from cytoplasmic lysates by sequential binding to immobilized enzymatically inactive S-AGzmA (mutated at the active site Ser→Ala), followed by gel filtration on a Sephacryl 400 column (Beresford et al., 1997, 2001). To increase the yield of the purified complex, we took advantage of the homotypic binding of SET (Miyaji-Yamaguchi et al., 1999), to replace the GzmA affinity-purification step with binding to immobilized recombinant SET. The known components of the SET complex, including SET, pp32, and NM23-H1, coeluted in a single broad peak between ~250 and 440 kDa (Figures 1A–1C). Like the previously purified SET complex (Beresford et al., 2001), the S400 fractions also degraded plasmid DNA on prolonged incubation (Figure 1B). After 48 hr, the intact supercoiled plasmid band began to disappear and was replaced by a more rapidly migrating smear of shorter nicked DNA. During the 2 day incubation, the inhibitor SET may have transiently dissociated from some NM23-H1 molecules, allowing some nicking to occur. Although the endonuclease NM23-H1 is reported to have limited exonuclease activity on single-stranded DNA and double-stranded DNA with significant 3′ overhang substrates (Ma et al., 2004; Yoon et al., 2005), we investigated whether the purified SET complex might contain other exonucleases. The 3′-to-5′ exonuclease TREX1/DNase III coelutes in fractions 68–72 with NM23-H1, SET, and pp32 (Figure 1C). TREX1 was also present in the SET complex eluted from the S-AGzmA affinity column (data not shown). TREX2, a TREX1 homolog, is not part of the SET complex. Another exonuclease, Exo1, also was not present in the complex.

### NM23-H1 Is Not the SET Complex Exonuclease

The intact SET complex has limited nuclease activity that is detected only by prolonged (~2 day) incubation with plasmid DNA (Fan et al., 2003a) (Figure 1B). We previously showed that the nuclease activity of the intact complex is blocked by the SET protein, which inhibits the NM23-H1 endonuclease. The SET complex nuclease can be separated from the acidic SET and pp32 proteins by passage through an anion exchange Q column. We verified this result, as shown by Figure 1D. SET and pp32 bind to the column, while NM23-H1, Ape1, and HMG-2 are in the Q flowthrough (QFT) (Fan et al., 2003a) (Figure 1D). Although TREX1 binds to some extent to the Q column and is eluted with SET in the Q column eluate (QE), most TREX1 is in the QFT (Figure 1D). Because TREX1 is an exonuclease and NM23-H1 has also been reported to have exonuclease activity (Ma et al., 2004; Yoon et al., 2005), we wanted to determine which protein is responsible for the exonuclease activity of the QFT fractions of the SET complex. We therefore used immunodepletion with immobilized anti-NM23-H1 to separate NM23-H1 and TREX1 in the QFT (Figure 1D). TREX1 remained in the supernatant and did not coprecipitate with NM23-H1, suggesting that the two proteins do not interact directly. Control IgG beads did not deplete either TREX1 or NM23-H1. The control IgG supernatant that contains both TREX1 and NM23-H1 had unaltered QFT exonuclease activity. However, neither the NM23-H1 immunoprecipitate, lacking TREX1, nor the NM23-H1 supernatant, containing TREX1 but lacking NM23-H1, demonstrated exonuclease activity on plasmid DNA (Figure 1D). The NM23-H1 immunoprecipitate, however, did produce a band corresponding to nicked plasmid DNA. These results show that NM23-H1 is not the SET complex exonuclease. They suggest that TREX1 is the SET complex exonuclease but that TREX1 by itself is inactive against a circular plasmid and requires NM23-H1 to generate a nicked substrate for its activity.

Because TREX1 copurifies with the SET complex, we wanted to verify the association of SET and TREX1 within cells by coimmunoprecipitation experiments. K562 cell lysates immunoprecipitated with SET peptide antisera also reacted with antisera to TREX1, but not TREX2 (Figure 1E, top). To determine whether TREX1 interacts directly with SET, we attempted to coimmunoprecipitate the recombinant proteins. Recombinant SET coprecipitated with recombinant TREX1, but not TREX2 (Figure 1E, left), suggesting that TREX1 interacts directly with SET. However, the SET-TREX1 binding may be weak, since only a fraction of the TREX1 input material was brought down by the SET antisera. This suggests that other components of the SET complex may also interact with TREX1 and stabilize the interaction. TREX1 did not bind directly to recombinant pp32 or recombinant NM23-H1, demonstrating the specificity of the SET-TREX1 interaction (Figure 1E, right; data not shown). These results suggest that TREX1 is a component of the SET complex, potentially responsible for its exonuclease activity.

### TREX1 Is Not a GzmA Substrate

Since TREX1 is contained in a GzmA-interacting complex, it might be a GzmA substrate like the SET complex proteins SET (Beresford et al., 2001), Ape1 (Fan et al., 2003b), and HMG-2 (Fan et al., 2002). Alternatively, it might be resistant to GzmA and play a role in GzmA-mediated cell death like the endonuclease NM23-H1 (Fan et al., 2003a). K562 cells were loaded with increasing concentrations of GzmA and incubated for different times. TREX1 was not degraded using GzmA concentrations as high as 1 μM or incubation for as long as 2 hr, whereas SET cleavage was seen with 10-fold less GzmA and within 15 min (Figure 2A). Furthermore, a molar excess of GzmA does not cut recombinant TREX1 in vitro (Figure 2B). Under the same conditions, GzmA cuts SET at low concentrations.

### SET Does Not Inhibit the TREX1 Exonuclease

In our previous study, we found that SET inhibits the endonuclease activity of NM23-H1 (Fan et al., 2003a). Since SET interacts directly with TREX1, we wanted to determine whether SET affects TREX1 exonuclease