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AUTHOR'S VIEWS

Sp3-cificity of TNF-α expression promotes the Smac mimetic-mediated killing of cancer cells

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ABSTRACT

A genome-wide small-interfering RNA-based screen identified the transcription factor Specificity Protein 3 (SP3) as a critical factor for Second mitochondrial-derived activator of caspase (Smac) mimetic-mediated killing of cancer cells. In concert with Nuclear Factor kappa B (NF-κB,) SP3 is required for the expression of the cytokine Tumor Necrosis Factor alpha (TNF-α) under basal and Smac mimetic-stimulated conditions.

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The Cellular Inhibitor of APoptosis 1 (cIAP1) and 2 (cIAP2) family members, encoded by the *BIRC2* (Baculoviral IAP Repeat-Containing 2) and *BIRC3* genes, respectively, are two highly similar oncogenic proteins that are essential for Tumor Necrosis Factor Receptor Super-Family (TNFRSF) signaling .[1] Both cIAP1 and cIAP2 function as E3 ubiquitin ligases, modifying key signaling components within the TNFRSF pathways, such as Receptor Interacting serine/threonine Kinase 1 (RIPK1) and MAP3K14 (Kinase Mitogen-activated Protein Kinase Kinase Kinase 14; best known as NIK, or NF-κB-Inducing Kinase), resulting in the activation of the classical or alternative NF-κB gene regulatory networks, respectively. Small molecule antagonists of cIAP1 and 2, called Second mitochondrial-derived activator of caspase (Smac) mimetics (referred to as SMs hereafter), induce the autoubiquitination and proteasomal-mediated degradation of cIAP1 and cIAP2, thereby altering TNFRSF signaling in both cancer and normal cells. The SM-induced loss of the cIAPs in cancer cells shunts Tumor Necrosis Factor alpha (TNF-α) signaling towards the generation of RIPK1-containing death complexes, forming either the ripoptosome (consisting of RIPK1, FADD (Fas-Associated Via Death Domain) and CASP8 (Caspase 8)) or the necrosome (consisting of RIPK1, RIPK3 and MLKL (Mixed Lineage Kinase Domain Like Pseudokinase)). These complexes result in the RIPK1- and TNF-dependent death of cancer cells (e.g.[2]). On the other hand, SM-induced loss of cIAP1 and cIAP2 in immune cells leads to stabilization of the kinase NIK and subsequent activation of the alternative NF-κB pathway that provides survival or co-stimulatory signals to T cells and dendritic cells. These signals help mount adaptive immune responses against cancer cells. The dual mechanism of SM anticancer effects, affecting both target cancer cells and effector immune cells, is likely responsible for the robust anti-tumor effects of SMs observed in immunocompetent animal cancer models or in co-cultures of cancer and immune cells (e.g.[3–5]).

To further understand the mechanism of SM-mediated effects on cancer cells, we undertook an unbiased whole-genome small-interfering RNA (siRNA)-based screening approach of the human triple-negative breast cancer cell line, MDA-MB-231 (which produces TNF-α in an autocrine fashion and is highly sensitive to SM-induced death) to identify tumor-intrinsic factors important for this death process .[6] The screen identified several hundred hits that could potentially rescue the triple-negative breast cancer line from SM-induced killing. Secondary screens and validation studies of the top 200 hits showed that many of the hits failed to reconfirm or that several hits non-specifically targeted RIPK1 in an 'off-target' manner. However, one of the validated hits was the transcription factor, Specificity Protein 3 (SP3). This hit caught our attention due to the limited knowledge of SP3, its reported broad transcriptional effects and its lack of prior association with the mechanism of action of SMs.

The effects of SP3 were not specific to triple-negative breast cancer, as multiple other cancer lines of different origins, including glioblastoma, ovarian adenocarcinoma, melanoma and pancreatic carcinomas, were resistant to SM-induced death in the absence of SP3. Analysis of SP3-deficient cells demonstrated that intracellular and extracellular TNF-α levels were decreased and this likely could explain why the cells are resistant to SM-induced killing. SP3, as well as the highly similar transcription factor SP1, bind to identical GC-box gene promoter elements to affect the transcription of numerous genes. Sequence analysis of the *TNF-α* promoter revealed several putative SP1/SP3 consensus binding sites. Chromatin immunoprecipitation (ChIP) experiments with anti-SP3 antibodies revealed binding of SP3 specifically to

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two of the proximal GC-boxes in the *TNF-α* promoter region, which were adjacent to putative NF-κB responsive elements (NREs). Electrophoretic mobility shift assays (EMSAs) with radiolabeled DNA probes and DNA-binding ELISAs with anti-NF-κB transcription factor antibodies identified that NF-κB binding to a consensus NF-κB probe that was enhanced in the presence of SP3. In addition, ChIP experiments demonstrated that SP3 was required for the binding of NF-κB factors to the *TNF-α* promoter to the two critical regions identified, suggesting cooperative binding of these factors and thereby cooperative enhancement of *TNF-α* gene transcription (Figure 1).

Cancer cells, as compared to normal cells, demonstrate a unique sensitivity to TNFα-induced death under conditions in which cIAP1 and cIAP2 proteins are lost but the reason for this susceptibility is not clear. Many factors, such as kinases, ubiquitin ligases, deubiquitinases and the CASP8 inhibitor, cFLAR (CASP8 and FADD Like Apoptosis Regulator gene encoding cFLIP, Cellular FLICE-Like Inhibitory Protein), have been identified to modulate the activity of TNF-α-RIPK1 signaling axis to NF-κB signaling or to alter the formation of death complexes. However, it is still unclear if any of those factors explain the differential sensitivity to TNF-α between normal and cancer cells. We

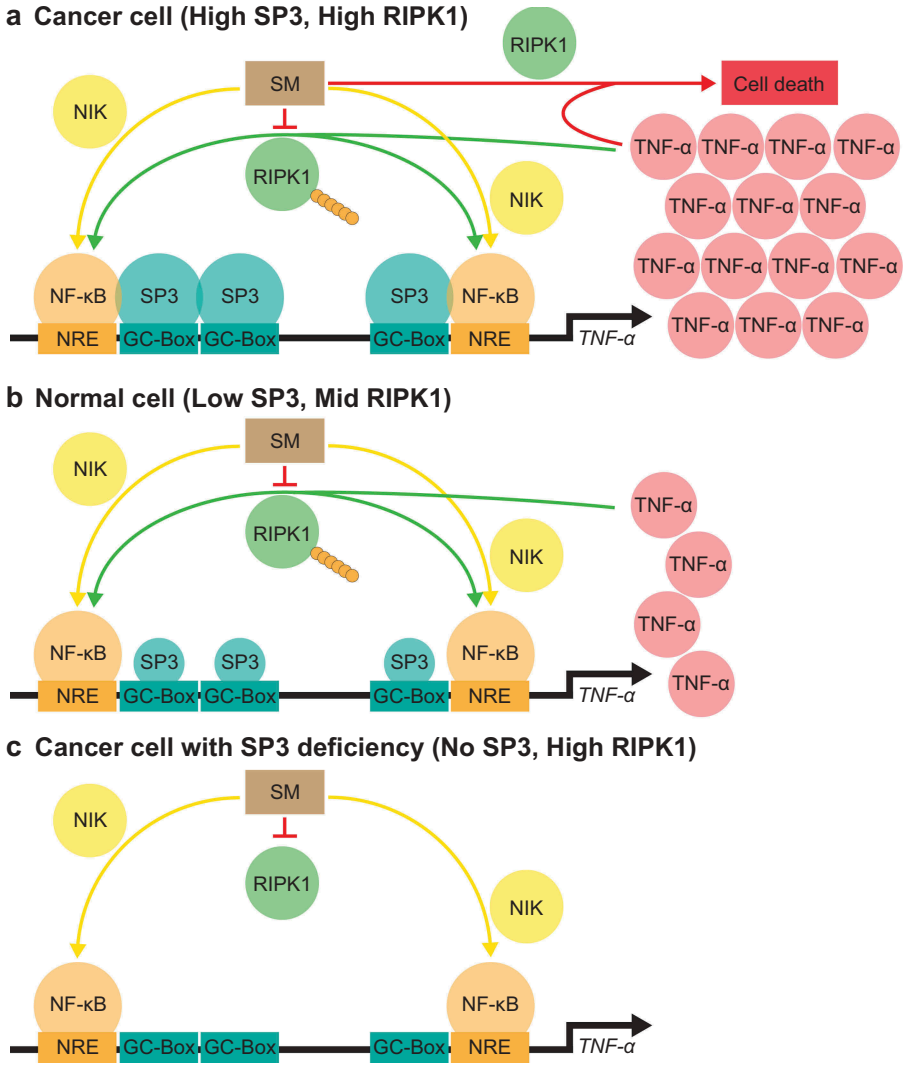

Figure 1. The transcription factor SP3 is an essential factor for expression of *TNF-α* and Smac mimetic (SM) killing of cancer cells. (a) In cancer cells, high expression of Specificity Protein 3 (SP3) cooperates with the classical (green arrows) or alternative (yellow arrows) Nuclear Factor kappa B (NF-κB) pathway to induce strong and sustained expression of *TNF-α* by binding to the respective DNA recognition sites, GC-boxes (for SP3) and NF-κB responsive elements (NREs). The classical NF-κB pathway is activated by the binding of TNF-α to its cognate receptor 1, TNF-R1, in a ubiquitinated-Receptor Interacting serine/threonine Kinase 1 (RIPK1) dependent manner, which causes a feed-forward loop of *TNF-α* expression. In addition, the alternative NF-κB pathway is activated through SM treatment, resulting in accumulation of the NF-κB inducing kinase, NIK, which also transcriptionally induce *TNF-α* expression. Both the classical and alternative NF-κB family members bind to the same NRE promoter elements. Since SM treatment results in RIPK1 no longer being ubiquitinated, RIPK1 is free to associate with a ripoptosome death complex upon TNF-α stimulation, and thereby leads to the death of cancer cells (red arrows). (b) The levels of SP3 in normal cells are lower, which leads to less overall production of TNF-α as a result of non-optimal NF-κB activity at the *TNF-α* promoter. As well, SMs do not induce the death of normal cells. (c) In SP3-deficient cancer cells targeted by specific small interfering RNAs, TNF-α is not produced since SP3 is absolutely required for *TNF-α* expression. Consequently, the cancer cells are not susceptible to SM-induced cell death.

noticed that the cancer cell lines that were sensitive to SM-induced killing had higher levels of SP3 protein compared to the 'resistant' normal cell lines. We then undertook an *in silico* analyses of *SP3* mRNA levels in tumors versus normal tissue using The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases. We found that *SP3* was overexpressed in 11 out of 31 cancer types. This difference was also seen in low- and high-grade gliomas and pancreatic cancers for which we see sensitivity to SM-induced killing *in vitro* and protection with downregulation of SP3. Interestingly, the RNA expression profile for nine factors related to TNF-α production and activity, consisting of TNF-α, RIPK1, SP1, SP3, PIAS1 (Protein Inhibitor of Activated STAT 1, a SUMO ligase that regulates SP3 activity), and the NF-κB factors, NF-κB1 (also known as p105), NF-κB2 (also known as p100), RELA (RELA Proto-Oncogene, NF-KB subunit), RELB (RELB Proto-Oncogene, NF-KB subunit), showed that this nine-gene signature (revealed via t-distributed stochastic neighbor embedding (t-SNE) clustering) can distinguish between glioma cells and brain tissues. Hence, SP3 levels may help to explain the differential activity towards SMs between normal and cancer cells. The high level of SP3 is required for the full engagement of NF-κB factors to produce TNF-α, either basally or upon SM stimulation, in order to overcome a threshold of TNF-α and TNFR1 signaling needed for redirecting RIPK1 towards death complex formation following cIAP1/2 loss (Figure 1).

A whole-genome Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based screen performed by another group on KBM7 leukemia cells (rendered FADD deficient to promote necroptosis) also identified SP1 and SP3 as factors required for SM-induced death of those cancer cells .[7] We tested if the SP3 similar transcription factor, SP1, also prevented SM-induced death in our model system and failed to find evidence for this. However, others have found involvement for SP1 in TNF-α production or in SM action .[8,9] An RNA expression profile analysis of L3.6pL pancreatic cancer cells (rendered singly deficient in Specificity Proteins, SP1, SP3 or SP4, by siRNA) noted a more pronounced effect by SP3 at inducing *TNF-α* expression as well as TNF-α and NF-κB-driven gene expression of target genes such as *BIRC3* .[10] Hence, SP3 may be an important enhancer of the NF-κB-mediated induction of the inflammatory cytokine TNF-α with consequences not only for cancer progression, but also for immunity and inflammation. Our study opens to door to a better understanding of the pathways and mechanisms leading to the NF-κB-induction of TNF-α expression. Understanding SP3 regulation and activity could shed light on the biomarkers and mechanisms responsible for differential sensitivity to TNF-α-induced killing between normal and cancer cells.

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