A (PRC)1-(RNF)2 knock-out punch for cancer

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Shedding light on epigenetic mechanisms controlling anti-tumor immune responses, a new study demonstrates that the tumor-intrinsic Ring Finger Protein 2 (RNF2), the catalytic subunit of the Polycomb Repressor Complex 1 (PRC1), acts as a negative regulator of a collaborative NK and CD4$^+$ T cell anti-tumor immune response against breast cancer.

Several tumor-intrinsic factors have been reported to modulate the composition of the tumor microenvironment (TME) and thus the anti-tumor immune response[1]. Tumor-intrinsic epigenetic regulators are of particular interest, due to their druggable nature and the natural plasticity of epigenetic modifications. Inhibitors of the histone methyltransferase Enhancer Of Zeste Homolog 2 (EZH2), part of the Polycomb Repressive Complex 2 (PRC2), have recently been approved to treat cancers with hyperactivated EZH2[2]. Interestingly, preclinical studies indicate that EZH2 inhibition in cancer cells may also enhance the immune response against tumor cells, similar to the findings described here for PRC1[3]. The genes encoding subunits of PRC1 are typically not mutated in cancer, but their dysregulation or genetic amplification has been described for many cancer types, including breast cancer[4]. Recent findings indicate that amplification or overexpression of the PRC1 core factor *RNF2* is associated with reduced overall survival in patients with triple negative breast cancer (TNBC)[4,5]. In this issue of *Nature Cancer*, Zhang et al.[6] demonstrate that this association is likely the result of RNF2 functioning to prevent effective anti-tumor immunity. *Rnf2* deletion in two pre-clinical TNBC models (4T1 and EMT6) led to durable immune-mediated tumor rejection by increasing the genomic accessibility and expression of immune response-associated genes. This increased the infiltration, activation, and function of both CD4$^+$ T cells and NK cells in the TME, which collaboratively controlled the tumor. From an epigenetic and immunological perspective, the findings of Zhang et al.[6] have several far-reaching implications for the treatment of TNBC.

Rnf2 encodes the RNF2/RING1B protein, which functions as an E3 ubiquitin ligase within PRC1. RNF2/RING1B mediates mono-ubiquitylation of K119 of histone H2A (H2AK119Ub), a modification associated with gene repression[7]. Zhang et al.[6] showed by immunoprecipitation of RNF2 in TNBC cells that RNF2 predominantly interacts with subunits of canonical PRC1 (cPRC1). Indeed, disruption of *Bmi1*, another cPRC1 complex subunit, or the downstream H2AK119Ub reader protein, *Rsf1*, similarly disrupted tumor growth in immunocompetent mouse tumor models. ATAC-sequencing of *Rnf2* KO cells revealed many genomic regions that became more accessible, but also many less accessible regions. This fits with recent reports demonstrating that PRC1 can play opposing roles in regulating chromatin accessibility by not only closing chromatin, but in

certain contexts, by binding to and promoting transcription of cancer-specific enhancers[4]. In estrogen receptor positive (ER+) breast cancer, RNF2 functions as a cell-intrinsic suppressor of metastasis, and high levels of RNF2 are associated with improved survival[4]. Here, focusing on TNBC, Zhang et al.[6] showed opposing effects, whereby *Rnf2* deficiency enhances tumor control. Importantly, *Rnf2* KO and *Rnf2*-WT TNBC tumors grew equally in immune compromised mice, indicating that *Rnf2* did not act intrinsically and played a role in altering cancer immune interactions. This difference may be the result of RNF2 associating with different transcriptional regulators in TNBC compared to ER+ breast cancers[4]. Together, these findings suggest that the role of RNF2 in the regulation of breast cancer is highly context-specific.

To test whether the E3 ligase activity of RNF2 was needed for its role in tumor control, Zhang et al.[6] developed a TNBC cell line containing a catalytically dead RNF2 mutant. Compared to control cells, tumor growth was not affected, indicating that suppression of the anti-tumor immune response was not dependent on RNF2's E3 ligase activity. This was surprising, since RNF2 deletion in both 4T1 and EMT6 cells significantly reduced H2AK119Ub levels and deletion of the H2AUb reader protein *Rsf1* also led to tumor suppression. However, these findings are in line with previous studies in embryonic stem cells demonstrating that the ability of RNF2 to ensure a compact chromatin state does not require its histone ubiquitination activity[8,9]. Furthermore, RNF2 might not be the only critical histone H2A ubiquitin ligase in TNBC. RING1A was shown to be more enzymatically active towards histone H2A than RING1B in another TNBC cell line, the human line MDA-MB-231[4]. Additionally, human breast cancer cell lines often exhibit amplified 17q23, which contains *TRIM37*, another E3 ubiquitin ligase for H2AK119 with oncogenic potential[10]. Alternative ubiquitin ligases may explain the fact that Zhang et al.[6] found *Rsf1* deletion to also be critical for cancer immunity. Further investigation, for example by performing H2AK119Ub ChIP-sequencing in TNBC cell lines, will be required to determine the importance of H2AK119Ub in regulating the anti-tumor immune response.

Zhang et al.[6] demonstrated that the genomic regions that gained accessibility after *Rnf2* KO were enriched in immune-related gene signatures and that the genes in these regions were more highly expressed. Among the genes upregulated and shown to be bound by RNF2 using ChIP were the MHC class II genes *H2-Ab1* and *H2-Eb1*[4]. Knockout of *H2-Ab1* and *H2-Eb1* in *Rnf2* KO 4T1 tumor cells restored tumor growth, indicating that RNF2 affects the anti-tumor response by preventing tumor cell direct recognition by $CD4^+$ T cells. Tumor directed cytotoxicity of $CD4^+$ T cells has recently been described in both preclinical mouse models and in patient-derived $CD4^+$ T cells[11]. Indeed, $CD4^+$ T cells displayed tumoricidal activity against *Rnf2* KO tumor cells. In addition, increased recognition of tumor cells by $CD4^+$ T cells also enhanced the anti-tumor NK cell response because $CD4^+$ T cell depletion reduced NK cell infiltration, activation, and function. Interestingly, NK cell depletion also affected the anti-tumor $CD4^+$ T cell response, suggesting a cooperative role for NK cells and $CD4^+$ T cells in the rejection of *Rnf2* KO tumors. *In vitro* co-culture

experiments showed mutual activation of $CD4^+$ T cells and NK cells upon *Rnf2* loss and indicated that their activation was driven by interferon γ (IFNγ), but not IL-2, produced by $CD4^+$ T cells and NK cells. In agreement with this, IFNγ neutralizing antibodies restored growth of *Rnf2* KO tumors *in vivo*. Importantly, IFNγ did not act directly on tumor cells, such as by increasing MHC class II expression, because IFNγ receptor deletion in *Rnf2* KO cells did not prevent immune-mediated cancer rejection. Finally, re-challenge experiments of cured mice showed that $CD4^+$ T cells were uniquely required for anti-tumor memory responses. Similar interplay of NK cells and $CD4^+$ T cells has been described before. For example, eradication of MHC class I-low tumors has been described to occur by NK cell-mediated killing of tumor cells, which results in the release of tumor antigens that can induce tumor-specific $CD4^+$ T cells[12]. Similarly, Zhang et al.[6] found that NK cells acted early to control tumor growth and $CD4^+$ T cells played a dominant role later in controlling tumors and establishing anti-tumor immune memory. Further experiments will be required to fully appreciate this dynamic T-NK crosstalk, such as identifying the additional factors mediating their communication, the location of these interactions (TME vs. elsewhere), and the timing of the interactions during the anti-tumor response.
Surprisingly, while $CD4^+$ T cell and NK cell depletion both abrogated *Rnf2* KO tumor control, $CD8^+$ T cell depletion did not have a similar effect. This is an unexpected finding, since $CD8^+$ T cells have been demonstrated to be involved in the anti-tumor immune response against TNBC cell lines expressing RNF2, and because high numbers of $CD8^+$ tumor infiltrating lymphocytes are associated with better survival in TNBC[13]. The absence of $CD8^+$ T cell control of *Rnf2* KO tumors could be related to the downregulation of MHC class I proteins in *Rnf2* KO cells, but this needs further exploration. Nevertheless, these results suggest that mutated or dysregulated chromatin modifiers can fundamentally alter the type of immunological response waged against cancer.

In view of translating the findings of Zhang et al.[6] into therapeutic approaches for cancer, the observation that the catalytic activity of RNF2 is dispensable for its effect on tumor growth is important. A small molecule inhibitor of RNF2, PRT4165, which inhibits its E3 ubiquitin ligase activity, exists[14]. However, based on this study, it is unlikely that an enzymatic inhibitor of RNF2 will have the desired effect. As an alternative approach, RNF2-containing enhancers in MDA MB-231 cells have been demonstrated to require the activity of bromodomain-containing protein 4 (BRD4), making BET-inhibitors an enticing strategy to explore in future studies[4]. Finally, the effect of *Rnf2* disruption on the immune compartment needs to be considered. For instance, in mouse macrophages, in addition to H2A ubiquitination and chromatin compaction, RNF2 has been demonstrated to promote STAT1/STAT2 dissociation from DNA, thereby negatively regulating interferon-stimulated gene expression[15]. It is likely that RNF2 also plays important roles in NK cells and $CD4^+$ T cells and additional studies investigating how RNF2 disruption impacts the activation and function of distinct immune cell populations are needed. In conclusion, since current immunotherapies are largely focused on enhancing the $CD8^+$ T cell response, the work of Zhang et al.[6] beckons the investigation of new

therapeutic approaches targeting chromatin-regulating complexes to awaken other immune cell subsets that can attack cancer.

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Figure 1: RNF2 blocks a collaborative NK and CD4$^+$ T cell anti-tumor immune response. *The immune response against triple negative breast cancers expressing the canonical PRC1 complex subunits RNF2, BMI1, and the ubiquitin reader protein RSF1 engages CD8$^+$ T cells, which alone are insufficient for tumor control. Disruption of either Rnf2, Bmi1, or Rsf1 leads to increased genomic accessibility and expression of immune response-associated genes, including MHC class II genes. This leads to direct recognition and killing of tumor cells by CD4$^+$ T cells and NK cells.*

Further, CD4^{+} T cells and NK cells both enhance each other's activity, in part by producing IFNy.