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BRD4 regulates the induction and maintenance of cancer stem cells in squamous cell carcinoma

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Comment on: Fisher ML, Balinth S, Hwangbo Y, *et al.* BRD4 Regulates Transcription Factor $\Delta Np63\alpha$ to Drive a Cancer Stem Cell Phenotype in Squamous Cell Carcinomas. *Cancer Res* 2021;81:6246-58.

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Non-melanoma skin cancer (NMSC) is one of the most frequently diagnosed cancers across the world (1). Greater than 70% of NMSCs are basal cell carcinomas (BCC) and about 25% are squamous cell carcinomas (SCCs). Both forms have good prognosis if detected and treated early (2). The majority of NMSC-related deaths (~75%) are due to SCC, which has a mortality rate (MR) of up to about 10%; MR in BCC is about 0.02% (1,2). SCC represents an aggressive group of cancers that arise due to uncontrolled growth of squamous epithelial cells in the head and neck region, oral cavity, esophagus, lungs, bladder or cervix. SCC has considerable metastatic potential and high risk of recurrence owing to the presence of highly proliferative and invasive squamous cells (2). Besides surgery, cryosurgery and radiotherapy, different combinations of therapeutic agents including cisplatin, 5-fluorouracil, doxorubicin, epidermal growth factor receptor inhibitors (cetuximab, panitumumab and matuzumab) as well as anti-programmed cell death protein 1 (PD-1) immune checkpoint inhibitors (cemiplimab and pembrolizumab) are used to treat various forms of SCC. However, the therapeutic efficacy of these drugs remains limited by side effects, chemoresistance and, perhaps most importantly, increased risk of recurrence (3). The possibility that drugs in use to treat SCC often miss the very small but all-important cancer stem cell (CSC) populations has been previously entertained as one of the reasons behind chemotherapy-related relapse in SCC (4). Therefore, investigating whether SCCs harbor CSCs and

identifying targets of potential therapeutic utility against such small cell populations is of paramount importance.

In this context, a recent study by Fisher *et al.* (5) revealed that CSCs displaying an aggressive phenotype with significant migration, invasion and growth potential can be isolated from spheroid cultures of SCC cell lines. The enriched CSC population they identified expressed high levels of the bromodomain and extra terminal (BET) domain family member BRD4, the enhancer of zeste homologue 2 (EZH2), the P53 family member P63 splice variant $\Delta Np63\alpha$, the SRY-box transcription factor 2 (SOX2) and the octamer-binding transcription factor 4 (OCT4) among others. The authors used small molecule inhibitors and clustered regularly interspaced short palindromic repeats (CRISPR)-based gene depletion and rescue approaches to investigate the role of BRD4, EZH2, the signal transducer and activator of transcription 3 (STAT3) and P63 in the induction and maintenance of CSCs both *in vitro* and in nude mice xenografted with genetically-manipulated SCCs. The study showed that BRD4 regulates the transcriptional activity of $\Delta Np63\alpha$ in SCC cells with a stem cell (SC)-like phenotype through the recruitment of EZH2/STAT3 complexes, presumably to the promoter regions of stemness-related genes. The characterization of this stemness-promoting BRD4-dependent regulatory pathway in SCC is of particular interest given that SCC associates with high levels of recurrence that is likely driven by the presence of CSC populations in SCC masses.

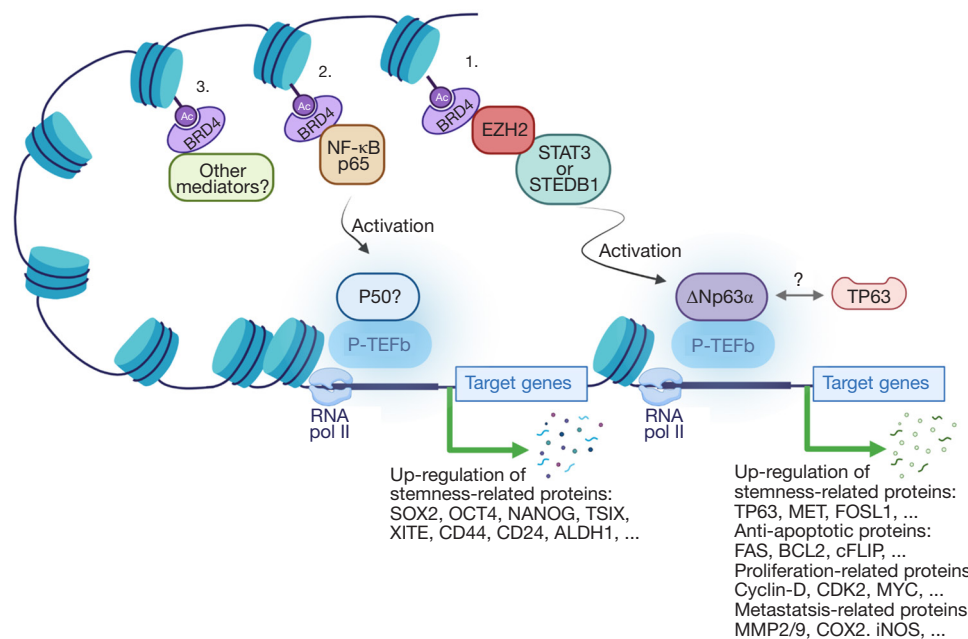


Figure 1 BRD4-dependent regulation of CSC transcriptional activities in SCC. CSC, cancer stem cell; SCC, squamous cell carcinoma.

The key conclusion made by Fisher *et al.* (5), builds upon a considerable body of experimental evidence documenting the roles of BRD4 as a super-enhancer (SE) and P63 as a transcription factor (TF) in SC biology. BRD4 has been previously recognized as a critical regulator of the embryonic SC (ESC) transcription factors homeobox transcription factor Nanog (NANOG) (6) and OCT4 (7), which sustain ESC self-renewal and stemness properties. Additionally, several studies have suggested that $\Delta Np63$ is indispensable for the maintenance of SC self-renewal potential in numerous normal and tumorous tissues including adult epithelia and glandular tissues (8,9) by enhancing the expression of the fibroblast growth factor receptor 2 (FGFR2) and the jagged canonical notch ligand 2 (JAG2) (10) among others. Ectopic expression of $\Delta Np63$ in the human breast carcinoma cell line MCF-7 was reported to increase the percentage of CSCs and to enhance cancer cell clonogenicity (9) possibly by engaging the sonic hedgehog signaling pathway (8).

BRD4, through its two tandem bromodomains (BD1, BD2), binds acetylated lysine residues on H3 and H4 histones and subsequently recruits mediator complexes, the positive transcription elongation factor b (P-TEFb) and RNA polymerase II to promote the initiation and elongation steps in target gene transcription (Figure 1). Greater than 70 genes involved in normal and tumor cell survival, growth and differentiation are known to be regulated by BRD4 (11).

In fact, the role of BRD4 in cancer extends beyond transcriptional and epigenetic regulation as it contributes to checkpoint activation, telomere homeostasis and DNA damage repair (11). Numerous studies have established that BRD4 is overexpressed in SCC and that its overexpression associates with poor prognosis and reduced patient survival (12). The BET inhibitor JQ1 was reported to significantly reduce the ability of BRD4 to bind H3K27 acetylation-enriched residues in the matrix metalloproteinase 2 (*MMP2*) gene and hence epigenetically suppress oral squamous cell carcinoma (OSCC) proliferation, migration and invasion (13). While inhibition of BRD4 in primary and transformed skin SCCs was shown to inhibit proliferation and induce apoptosis and cell cycle arrest, its overexpression reversed these effects and promoted tumor growth (14). Further investigation into the role of BRD4 in skin SCCs showed that it is required for the expression of cyclin D1, BCL-2 and MYC oncogenes (14). Similar studies have also shown that BRD4 collaborates with YAP1 (15) or JOSD1 (16) to promote the tumorigenesis of head and neck squamous cell carcinoma (HNSCC). Therefore, BRD4 is broadly recognized as an important therapeutic target in SCC; the fact that over 30 targeted inhibitors of various BET family members including BRD4 are currently in preclinical and clinical trials is testament to its perceived importance.

The interaction between BRD4 and EZH2 has been previously documented in different forms of SCC. Previous

work has shown that BRD4 is a key upstream regulator of EZH2 in bladder cancer and that BRD4 inhibition diminishes C-MYC recruitment to the EZH2 promoter (17). This is in agreement with the Fisher group (5) observation that BRD4 regulates CSC markers via C-MYC. The ubiquitin-specific processing proteases 17 (DUB3), which is overexpressed in OSCC tissues and cell lines, was reported to promote cell proliferation and inhibit apoptosis by modulating the expression/processing of caspase-3, PARP, p21 and cyclin D1 genes (18). Interestingly, DUB3 expression in OSCC was found to positively correlate with and facilitate the production of EZH2. Further investigation into how DUB3 regulates EZH2 expression identified BRD4 as an intermediary in this process as evidenced by the observation that downregulation of DUB3 promoted BRD4 ubiquitination and that inhibition of BRD4 repressed DUB3-induced EZH2 production. EZH2, which methylates histone H3K27 along with another set domain-containing oncogenic methyltransferase (SET domain bifurcated 1; SETDB1) that methylates histone H3K9, were recently reported to play a critical role in the proliferation of HSC-5, FaDu, and Cal33 SCC cell lines (19); the same cell lines used in the Fisher *et al.* study (5). Both EZH2 and SETDB1 were also shown to be highly expressed in an aggressive CSC sub-population of SCC (19). Interestingly, while SETDB1 was found to regulate the SCC-derived CSC population through cooperation with $\Delta Np63\alpha$, the upstream EZH2 mediator activated the SETDB1/ $\Delta Np63\alpha$ pair by repressing the tumor suppressor RUNX3 (19). Another recent study by Dong *et al.* (4) showed that BRD4 recruits NF- $\kappa Bp65$ to form SEs at cancer stemness-related genes including TP63, MET and FOSL1 along with multiple mediators and oncogenic transcripts instead of MYC. The Dong *et al.* study also showed that disruption of SEs by the BET inhibitor JQ1 suppressed HNSCC invasive growth and metastasis by abolishing B cell-specific Moloney murine leukemia virus integration site (BMI1+) CSCs as well as proliferating non-stem tumor cells (4). It is worth noting that the BMI1 functions as a ring finger protein in the polycomb repressive complex 1 (PRC1) and that EZH2 is a functional enzymatic component of PRC2. This possibly points to the involvement of PRCs in CSCs in a way that parallels PRCs' involvement in SC lineage commitment. Additionally, the observation that BRD4 recruits NF- $\kappa Bp65$ to regulate the expression of stemness-related genes including TP63 (4) and that $\Delta Np63\alpha$ is a splice variant of P63 (20), speaks of a possible BRD4-orchestrated loop whereby NF- $\kappa Bp65$ -induced TP63 may feed into the

“EZH2-dependent stemness pathway” (Figure 1).

The role of $\Delta Np63\alpha$, one of the two original N-terminal splice variants of P63 (20), in SCC tumorigenesis and metastasis is well established. Overexpression of $\Delta Np63\alpha$, which has been documented in SCC of the lung, cervix, head and neck and esophagus, associates with poor prognosis and reduced patient survival (21). $\Delta Np63\alpha$ has been shown to promote tumor growth, stemness, and metastasis by blocking the tumor suppressor activity of P53 family members (P53, P63 and P73) (22). $\Delta Np63\alpha$ was reported to suppress apoptosis in SCC through its ability to interact with histone deacetylase 1 (HDAC1) and HDAC2 (23). Previous work has also shown that high $\Delta Np63\alpha$ expression in HNSCC cell lines correlates with increased expression of the metastasis-promoting heat-shock protein 70 (HSP70) gene (24). $\Delta Np63\alpha$ was also shown to regulate HSP70 gene transcription by recruiting additional TFs including the CCAAT binding factor in the case of HNSCC (25) and E2F1 transcription factor 1 in the case of bladder cancer (24). The involvement of $\Delta Np63$ in the initiation and maintenance of CSCs can also be discerned by the observation that its expression is regulated by the Hedgehog and the epidermal growth factor receptor (EGFR)/STAT3 stem-cell renewal-related pathways (8) and the observation that $\Delta Np63\alpha$ and STAT3 signaling is required for CSCs maintenance (26).

The preceding discussion points to the presence of a BRD4-EZH2- $\Delta Np63\alpha$ axis that promotes CSC self-renewal and potency in SCCs (Figure 1) and which can be further interrogated to identify new therapeutic targets in chemoresistant and recurrent SCCs. Additionally, with the versatile transcriptional and non-transcriptional roles BRD4 plays in cell survival and growth in mind, the realization that it is a player in this stemness axis promises to unveil scores of new players in CSC biology (Figure 2). That said, there are a number of concerns that need to be considered as research work in this area progresses: (I) both the Fisher *et al.* study (5) and the Balinth *et al.* study (19), used SCC cell lines to enrich for CSCs. It is true that the Fisher *et al.* study showed that patient-derived SCC tumor arrays are positive for BRD4, P63, EZH2 and SOX4 but this is hardly convincing as evidence of the presence of CSCs in patient samples. For one thing, the frequency of CSCs in any cancer mass is presumably small so how can such low frequency cells account for such high levels of intensity as presented in their study. Furthermore, as shown in Figure 2, co-expression analysis of publically available SCC transcriptomic datasets showed that HNSCC, as a representative of SCC, is highly positive for these and other

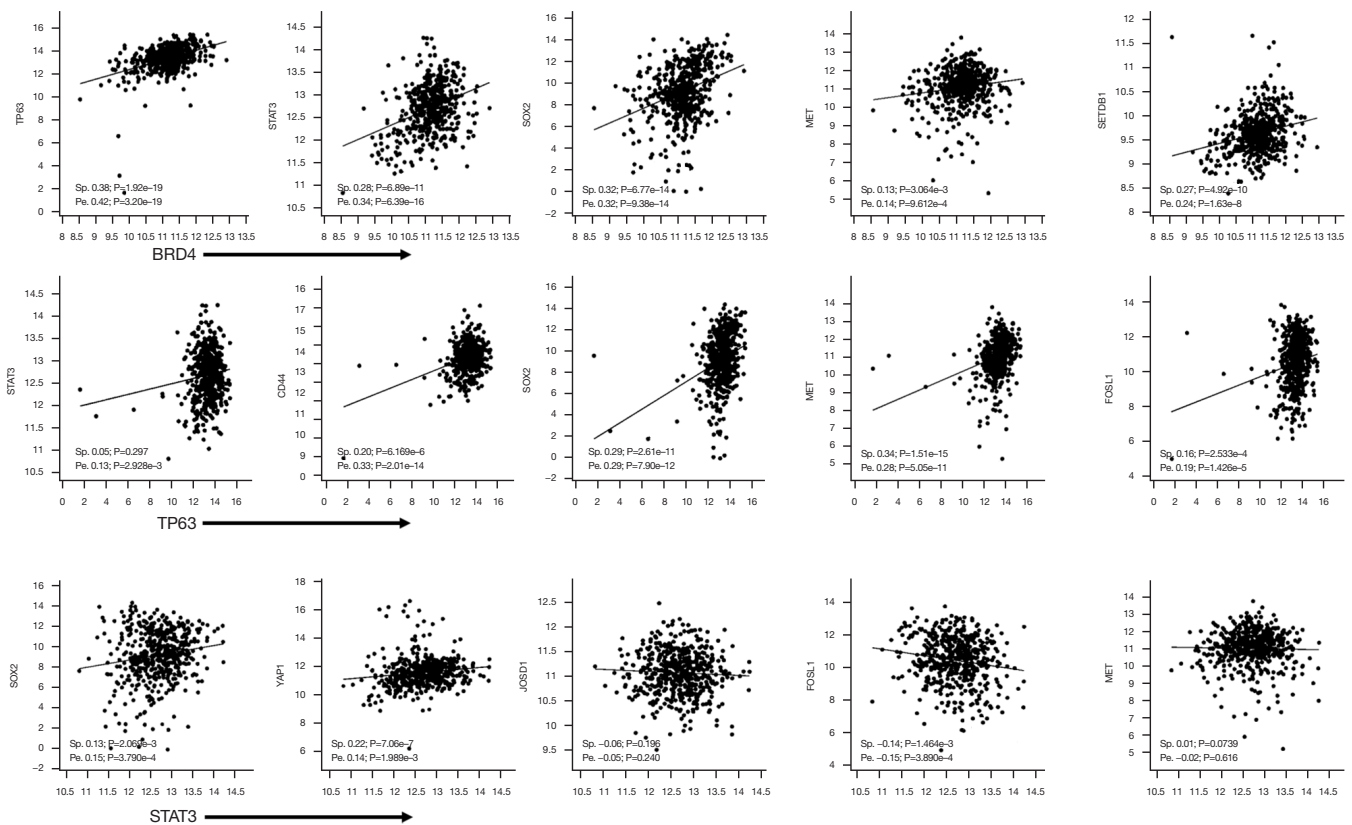


Figure 2 Co-expression analysis of BRD4, TP63 or STAT3 versus key stemness-related genes in HNSCC. (TCGA, Firehose Legacy) (528 patients/530 samples) from cBioPortal cancer genomics database (<https://www.cbioportal.org/>). HNSCC, head and neck squamous cell carcinoma; TCGA, The Cancer Genome Atlas.

SC markers. Therefore, additional high-resolution evidence documenting the presence of CSCs in clinical samples of SCC need to be furnished should the observation that SCCs do indeed harbor CSCs be broadly accepted. (II) A deeper understanding of the role of the tumor microenvironment (growth requirements, immune surveillance, etc.) in the rise and preservation of CSCs is in order. (III) The potential involvement of BET members other than BRD4, mediators other than EZH2 and TFs other than $\Delta Np63\alpha$ in inducing and/or maintaining CSCs in SCC is also worth considering. (IV) As suggested earlier (4), one is tempted to believe that SCC recurrence is at least partially precipitated by the presence of CSCs that are missed by anti-SCC drugs in current use. But this is just an assumption which still needs to be further investigated. But should this be the case, what is next? Are there any available drugs to target CSCs? Is there a need to develop new classes of drugs tailor-made for this purpose? Will there be a need to devise new patient management protocols to deal with the tumor itself and the

CSC populations it harbors? Will such CSC populations prove to be homogenous or will they be heterogeneous and how will that bear on future efforts to deal with them? In short, the plot is thickening, stay tuned!

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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