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When are the BET factors the most sensitive to bromodomain inhibitors?

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The recent publication of two detailed studies of mouse spermatogenesis, either after chemical inhibition of the BET bromodomains, or in the context of genetic alterations of one specific BET member, Brdt, provides the unique opportunity to assess the functional impact of BET bromodomain inhibitors.

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Abbreviations: BET, Bromodomain and Extra Terminal; BD1, bromodomain 1; BD2, bromodomain 2; TSS, transcriptional start site

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Introduction

Bromodomain-containing proteins have received increasing interest ever since bromodomains were discovered to bind acetylated histones.[1] Bromodomain is a conserved structural module present in 61 copies in 46 distinct human proteins.[2] These factors combine one or multiple bromodomains to specific activities such as histone acetyltransferase, ATPase and histone lysine methyltransferase.[3] Thanks to its associated activities, bromodomains are essential relays in translating signals initiated by histone acetylation. Inhibition of the bromodomain-acetylated histone interaction therefore constitutes an elegant strategy to block "at their source" specific signaling pathways triggered by histone acetylation. This strategy has proven to be the most effective in case of a specific class of bromodomain factors, all containing two adjacent bromodomains and a highly conserved domain named extra terminal (ET). They have thus been called BET for Bromodomain and Extra Terminal.[4] In mammals, four paralogs exist, known as Brd2, Brd3, Brd4 and Brd6 or Brdt (bromodomain testis-specific), which, in contrast to the other members, is specifically expressed in male germ cells.

Potent inhibitors of BET have been developed that show very promising therapeutic applications.[3] These inhibitors, however, show no remarkable specificity toward particular members of the BET family, which are involved in various and probably unrelated cellular activities.[5]

Recent and parallel studies of spermatogenesis, either under general inhibition of BET bomodomains by BET inhibitor JQ1[6] or in the context of mouse genetic mutants for Brdt,[7] the testis-specific BET member, provided insight into the functions of the different BET members and, in the case of Brdt, into how the two bromodomains are involved in its activities.

Spermatogenesis is Directed by a Specific Gene Expression Program

Male germ cell differentiation involves peculiar features due to the specific requirement for male gametes to leave the parent organism and to confront a harsh environment. Accordingly, unique events drive the re-packaging of the male genome in order to make it resistant to the assaults that may endanger the integrity of the male genome and hence the perpetuation of species.[8] Specific genes that are locked in a repressive state in all somatic and in female germ cells become active in the haploid male germ cells in a step-dependent manner to drive the spectacular metamorphosis of the cells, which involves the wrapping of the male genome into a "swimming package," equipped with all the fuel to travel long distances and to deliver its genome to the egg. Nothing similar happens in any somatic

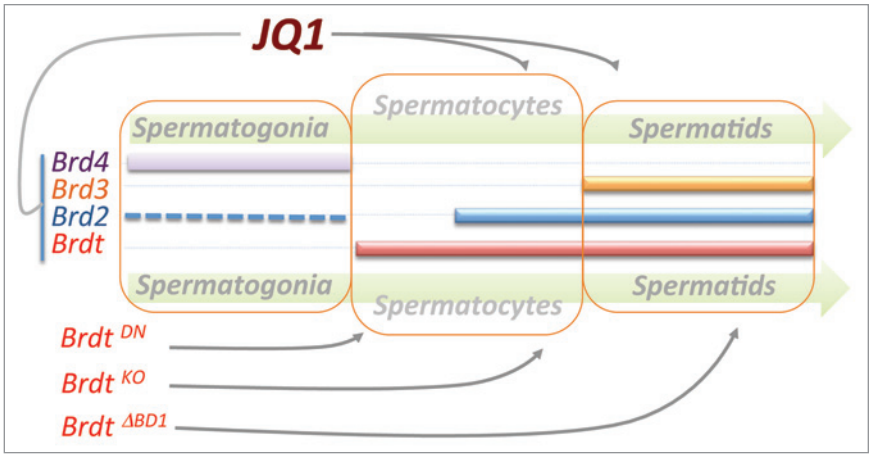

Figure 1. Effect of JQ1 treatment and genetic alterations of *Brdt* on spermatogenesis. Spermatogenic cells express all members of the BET family at different stages of their maturation. The scheme represents the timing of the expression of each member, adapted from Shang et al.[14] JQ1, although inhibiting the bromodomains of all BET members, affects spermatogenesis in spermatocytes and round spermatids.[6] Dramatic impairment of spermatogenesis is observed at different stages depending on the genetic alterations of *Brdt*.[7] A dominant negative non-functional *Brdt* (*Brdt*^{DN}) induces massive apoptosis of early spermatocytes. A complete absence of Brdt (*Brdt*^{KO}) induces an arrest of meiosis before the first meiotic division. Finally, the absence of Brdt first bromodomain (*Brdt*$^{\Delta BD1}$) leads to an arrest of spermatogenesis due to the impairment of histone removal in elongating spermatids.

cells or even in the female germ cells and, therefore, the corresponding genetic program needs an exclusive instruction to be turned on.

Brdt: A Coordinator of Meiotic and Post-Meiotic Male-Specific Gene Expression Program

Brdt is a typical BET member with restricted male germ cell expression. Background information on its physiological activities was scarce until very recently.[9] Interest in this factor increased about a decade ago, when it was proposed that Brdt reorganizes hyperacetylated chromatin preceding histone replacement in elongating spermatids. This work led to the first molecular[10] and structural[11] characterizations of the protein.

Meanwhile, more information on Brdt in spermatogenic cells was produced following the generation of mice expressing a mutated form of the protein deleted for its first bromodomain, leading to male infertility associated with post-meiotic defects.[12,13]

Recently, using this mouse strain, as well as two additional mouse models—one carrying a Brdt knockout (KO) and another one expressing a non-functional protein (a dominant negative mutant)—and through molecular analyses including genome-wide approaches, a comprehensive molecular portrait of Brdt functions could be painted in the physiological setting of spermatogenesis. These studies showed that Brdt is a powerful driver of spermatogenic differentiation and male genome programing, combining its ability to recruit RNAP II kinase pTEFb and histone acetylation marks readout to target critical meiotic and post-meiotic genes. These studies additionally showed that Brdt mediates a chromatin acetylation-dependent exchange of histones by sperm-specific transition proteins, through the ability of its first bromodomain to recognize hyperacetylated H4 and its polymerization on acetylated chromatin fibers.[7]

A parallel study considering mouse spermatogenesis after the administration of the BET inhibitor JQ1 also revealed a severe impairment of spermatogenesis.[6] The comparison of the phenotypical and transcriptomic analyses reported in this work with the molecular data reported in the above mentioned publication is rich in information on the specificities and redundancies of the activities of BET members, all expressed in spermatogenic cells,[14] and on how the action of the two bromodomains in Brdt could be distinguished.

Genetic Modulation of Brdt Activity vs. the Chemical Inhibition of BET Bromodomains

Treatment of mice with JQ1 showed depletion of spermatocytes and post-meiotic cells. Analyses of gene expression in adult mouse whole testis after six weeks of JQ1 treatment compared with control testis suggested that genes that are normally expressed in meiotic and post-meiotic cells are downregulated. These studies also showed increased expression of genes normally active in progenitor cells, spermatogonia, probably due to depletion of the cells at later stages and subsequent enrichment of the analyzed samples with spermatogonia.[6] Overall, JQ1 treatment seems to essentially affect meiotic and post-meiotic gene expression with no effect on genes active in spermatogonia. Interestingly, despite the presence of other BET members in these cells,[14] these data point to Brdt as the major target of JQ1 (**Fig. 1**). Gaucher and colleagues transcriptomic analyses of developing testes from *Brdt*^{KO} mice and *Brdt*$^{\Delta BD1}$ (lacking its first bromodomain) mice, before the appearances of observable phenotypes, showed that Brdt is required for the activation of a meiotic and post-meiotic gene expression program.[7] More interestingly, both studies independently showed that *Ccna1* encoding cyclin A1 is drastically downregulated by JQ1 treatment as well as in the absence of Brdt. *Ccna1* is a critical gene and its non-expression is sufficient to explain the meiotic phenotypes observed in both cases. However, spermatocytes expressing a truncated Brdt lacking its first bromodomain (ΔBD1) show an almost normal level of *Ccna1* expression.[7] Gaucher and colleagues' ChIP-Seq study also shows that, in contrast to what was observed for many Brdt-regulated genes, there was no significant Brdt binding at the *Ccna1* transcriptional start site (TSS). This observation could either be due to the fact that the antibody could not detect Brdt bound to this promoter or that *Ccna1* expression is indirectly controlled by Brdt. In either case, considering all the data together, it is possible to conclude that the direct

or indirect regulation of *Ccna1* by Brdt involves the second bromodomain of Brdt, since *Ccna1* expression did not require the BD1 but was sensitive to JQ1, which also inhibits the BD2.

The second interesting concordant observation concerns spermatogonia, which are insensitive to JQ1[6] and have been shown by Gaucher and colleagues not to express Brdt, further suggesting that Brdt could indeed be the major target of JQ1.

It is important to note that two observations by Gaucher and colleagues strongly argue in favor of the existence of a compensation of Brdt functions, to some extent, by other BET members. First, in the absence of Brdt (KO), the major Brdt-dependent gene expression occurs at 20 d postpartum (d.p.p), while at 17 d.p.p. only a small fraction of these genes are affected, despite Brdt being expressed at both ages in wild type cells. This observation suggests that either Brdt has no marked functions before the pachytene stage or that other BET members could compensate for Brdt functions in its absence in early spermatocytes. The second observation from the Gaucher study is that mice expressing a non-functional Brdt showed a more dramatic phenotype than mice lacking Brdt, with defects occurring at the time of appearance of the first meiotic cells. This suggests that Brdt is functionally active in early meiotic cells and that, in the case of mice expressing the non-functional form of Brdt, no compensation could occur because of the dominant negative nature of the expressed Brdt. The lack of remarkable defects in gene expression in these early meiotic cells in the absence of Brdt strongly argues in favor of compensation by other BET members (**Fig. 2**).

Additionally, the Gaucher study demonstrated that only half of the genes bound by Brdt at their TSS shows a Brdt-dependent activity (downregulated in the absence of Brdt). This observation strongly suggested that, in the absence of Brdt, other BET members could take over and maintain the expression of genes normally regulated by Brdt.

The molecular dissections reported by Gaucher and colleagues indicated that Brdt should also have very specific target genes, mainly meiotic and post-meiotic,

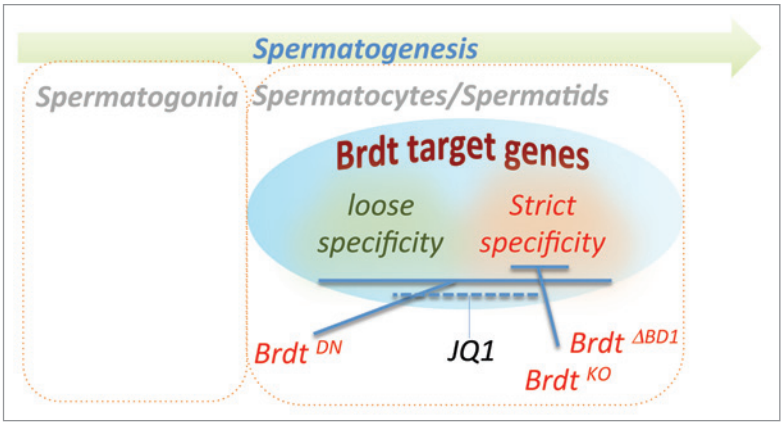

Figure 2. Specific and redundant roles of Brdt in regulating testis-specific gene expression and partial effect of JQ1 treatment. The combined analysis of Brdt-bound genes and Brdt-dependent transcriptomic data and of the effect of a *Brdt*^{DN} mutant,[7] allowed to propose the existence of different categories of genes according to their regulation by Brdt during spermatogenesis: genes strictly requiring Brdt for their expression (late meiotic and post-meiotic genes) and genes, mostly expressed in early spermatocytes when Brdt is first activated, whose expression is not affected by the absence of Brdt or a Brdt lacking its first bromodomain, but affected by the expression of a *Brdt*^{DN} mutant. The effects of JQ1 on spermatogenesis are however much milder than the effects of *Brdt*^{DN} or *Brd*^{KO}, suggesting that either JQ1-dependent bromodomain inhibition is partial or that bromodomain-independent functions prevail in Brdt and the other BETs.

not seen by other BETs. The whole picture of Brdt-regulated genes appears as a sum of genes exclusively regulated by Brdt and genes interchangeably using Brdt or other BET members. Accordingly, the prediction is that JQ1 should affect the expression of all these genes by preventing any compensation and produce effects similar to the dominant negative Brdt mutant. A careful comparison of the phenotypes of the three Brdt mouse models used in the Gaucher and colleagues work with those observed after a JQ1 treatment shows that, in general, the effect of JQ1 is much milder than what was observed in the genetic models. This observation argues in favor of the existence of important bromodomain-independent functions of Brdt, which could not be inhibited by JQ1, or suggests the inability of JQ1 to completely inhibit BET bromodomains in vivo (**Fig. 2**). These observations are also in line with the fact that, at least in the case of Brd4, Brd3 and Brd2, important regulatory functions of the corresponding proteins have been shown to be independent of the bromodomains.[15] Additionally, and in support of these conclusions, it has been reported that the expression of some genes is suppressed by the downregulation of BET factors but not after BET bromodomain inhibition.[16] All together, these observations indicate that BET inhibitors affect only a subset of the regulatory functions of BET factors.

When are the BET Factors the Most Sensitive to Bromodomain Inhibitors?

Taking into account the data reported in these two studies, a striking observation is that, despite the presence of all BET members in spermatogenic cells and the fact that JQ1 has no marked selectivity toward these factors, Brdt seems to be predominantly affected in its function by the inhibitor (**Fig. 1**). This observation could give a clue on how BET inhibitors could act and, more interestingly, on when BET bromodomains are required in their function.

Brdt becomes active in early spermatocytes and helps turning on a strictly specific set of genes in later stages, which cannot be regulated by the other BET members. Interestingly these data parallel those published by Nicodeme and colleagues.[16] This study of pro-inflammatory genes, which are induced in bone marrow macrophages in response to lipopolysaccharide (LPS) treatment, showed that a BET inhibitor treatment had no significant effect on the basal gene expression including

that of the housekeeping genes, but that most of the BET inhibitor sensitive genes belonged to the secondary response genes, induced during late macrophage activation. Interestingly, these genes had a particular epigenetic and promoter structure characterized by low level of active marks and lower amounts of CpG, suggesting that specific help is required to turn these genes ON and that BET bomodomains are critical in the process, probably following histone acetylation by co-activators.

It can now be hypothesized that transcriptional activation of a particular category of epigenetically defined silent genes, such as late meiotic and post-meiotic genes, specifically requires the bromodomains of BET members and that this is why the BET inhibitors have little effects on the steady-state gene expression in many differentiated cells of an adult organism. It is however clear that BET members are critical factors for basal cellular functions,[17,18] but the rather limited effects of BET inhibitors suggest that these functions are mostly bromodomain independent. It is therefore likely that BET bromodomains are essential in the regulation of specific categories of epigenetically defined genes, functioning in particular gene expression programs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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