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Article

Domain-Selective BET Ligands Yield Next-Generation Synthetic Genome Readers/Regulators with Nonidentical Cellular Functions

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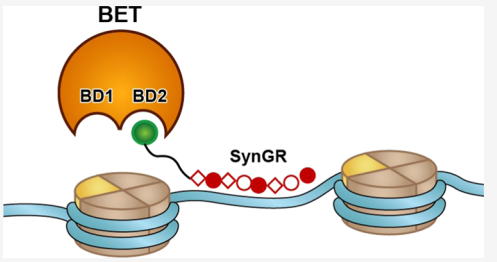
ABSTRACT: SynTEF1, a prototype synthetic genome reader/regulator (SynGR), was designed to target GAA triplet repeats and restore the expression of frataxin (*FXN*) in Friedreich's ataxia patients. It achieves this complex task by recruiting BRD4, via a pan-BET ligand (JQ1), to the GAA repeats by using a sequence-selective DNA-binding polyamide. When bound to specific genomic loci in this way, JQ1 functions as a chemical prosthetic for acetyl-lysine residues that are natural targets of the two tandem bromodomains (BD1 and BD2) in bromo- and extra-terminal domain (BET) proteins. As next-generation BET ligands were disclosed, we tested a select set with improved physicochemical, pharmacological, and bromodomain-selective properties as substitutes for JQ1 in the SynGR design. Here, we report two unexpected findings: (1) SynGRs bearing pan-BET or BD2-selective ligands license transcription at the *FXN* locus, whereas those bearing BD1-selective ligands do not, and (2) rather than being neutral or inhibitory, an untethered BD1-selective ligand (GSK778) substantively enhances the activity of all active SynGRs. The failure of BD1-selective SynGRs to recruit BRD4/BET proteins suggests that rather than functioning as "epigenetic/chromatin mimics," active SynGRs mimic the functions of natural transcription factors in engaging BET proteins through BD2 binding. Moreover, the enhanced activity of SynGRs upon cotreatment with the BD1-selective ligand suggests that natural transcription factors compete for a limited pool of nonchromatin-bound BET proteins, and blocking BD1 directs pan-BET ligands to more effectively engage BD2. Taken together, SynGRs as chemical probes provide unique insights into the molecular recognition principles utilized by natural factors to precisely regulate gene expression, and they guide the design of more sophisticated synthetic gene regulators with greater therapeutic potential.



INTRODUCTION

Targeted control of gene expression with rationally designed small molecules has been a long-standing goal at the interface of chemistry, biology, and medicine. Chemical control of gene expression is typically achieved by inhibiting enzymes that act on the transcriptional machinery/chromatin or by perturbing protein−protein interactions that drive gene expression.[1−3] Such chemical interventions have broad, often undesired, transcriptome-wide consequences, which constrain therapeutic applications. The promise of targeting specific genomic loci by design has fueled the exploration and development of different classes of sequence-selective DNA-binding molecules. Among these, pyrrole-imidazole polyamides have emerged as a versatile class of molecules that can be rationally designed to target nearly every permutation of the DNA sequence observed in the human genome.[4] Not only does this class of synthetic DNA-binding molecules display sequence selectivity and affinity properties that are comparable to mammalian DNA-binding transcription factors (TFs), but they can also access binding sites in nucleosomes and heterochromatin.[1,5−10] Moreover, polyamides retain their sequence specificity when further conjugated to other small molecules or peptides.[5,11−13] Based on the modular architecture of eukaryotic TFs, we and others have developed synthetic transcription factors by tethering DNA-binding polyamides to different ligands that bind the transcriptional machinery.[14−23] These heterobifunctional molecules, much like their natural counterparts, leverage the principle of induced proximity[24,25] to "recruit" specific cellular machinery to targeted genomic loci. This form of chemically induced proximity enables the recruited proteins to perform targeted genomic transactions.

Function-based modular assembly enabled us to generate SynTEF1, a synthetic transcription elongation factor composed of a polyamide (PA1) tethered to JQ1 (Figure 1A). PA1 was designed to selectively bind the $^{5'}$WWGWWGWWG$^{3'}$ (W = A or T) DNA sequence, while JQ1 bound to the tandem bromodomains of the bromo- and extra-terminal domain (BET) family of proteins.[6,19] In cells derived from Friedreich's ataxia patients, SynTEF1 effectively enriches at disease-causing

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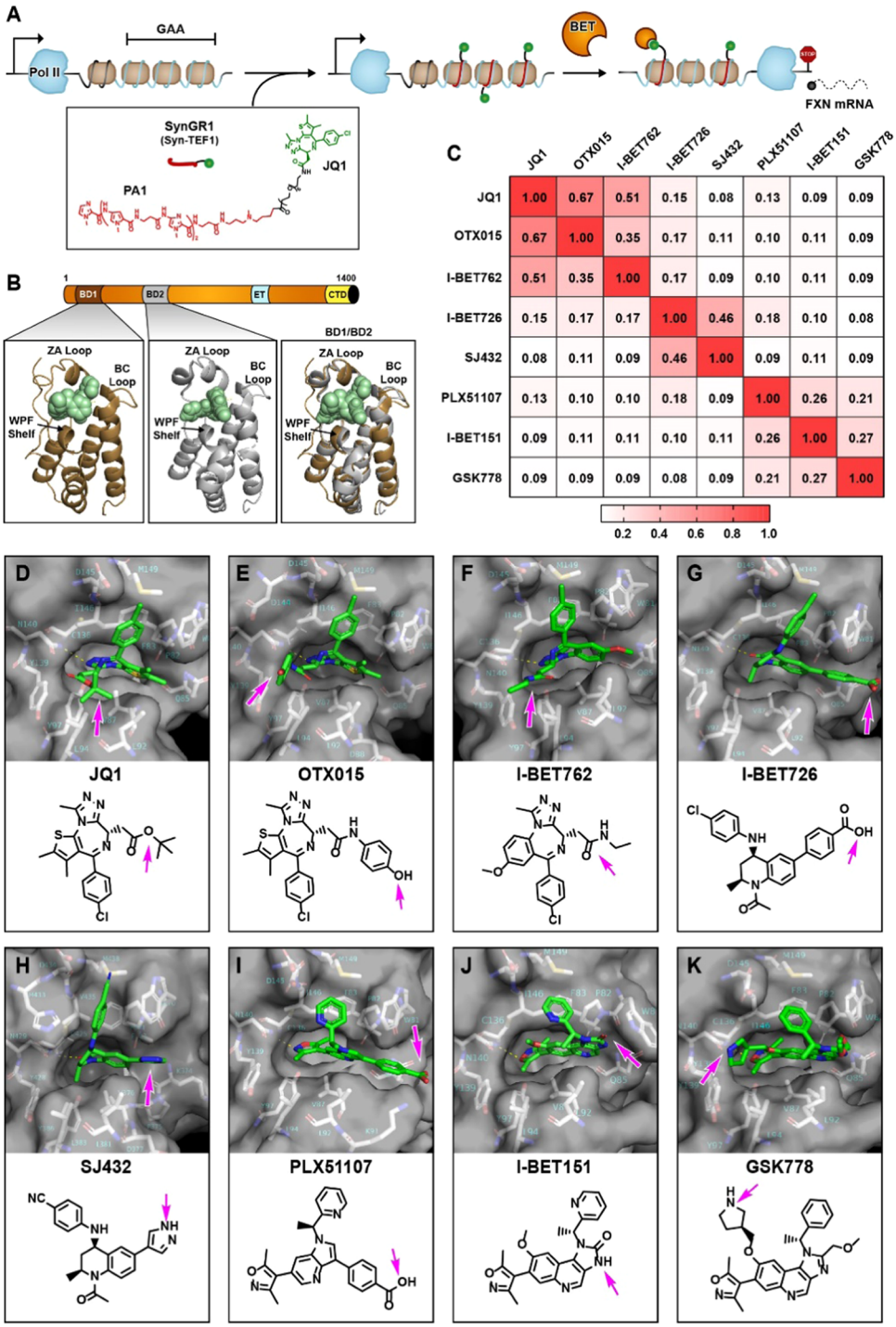

Figure 1. Design of next-generation SynGRs for disease-causing GAA repeat expansions within the *FXN* locus. (A) A model of the cascade of interactions and reactions initiated by SynGR1 (SynTEF1) to restore transcription at the frataxin (*FXN*) locus in Friedreich's ataxia cells. Pol II, RNA polymerase. (B) Schematic of the BET family member BRD4 protein and cocrystal structures of BD1 (bronze) and BD2 (silver) domains each bound to JQ1 (PDB: 3MXF, 3ONI). ET, extra-terminal domain; CTD, C-terminal domain. (C) Tanimoto similarity scores of the BET ligands used in the construction of SynGRs. (D–K) Chemical and cocrystal structures of BET ligands (green sticks) JQ1 (D), OTX015 (E), I-BET762 (F), I-BET726 (G), SJ432 (H), PLX51107 (I), I-BET151 (J), and GSK778 (K) in complex with either BRD2-BD2 (H) or BRD4-BD1 (all others). PDB: 3MXF, 5WMD, 3P5O, 4BJX, 6DDJ, 5WMG, 3ZYU, and 6SWN, respectively. Magenta arrows indicate the linker attachment site for coupling to PA1.

GAA trinucleotide repeat expansion within the frataxin (*FXN*) gene and recruits BET proteins, including BRD4, to that locus. SynTEF1-bound BRD4 and its associated cellular proteins facilitate *FXN* synthesis by RNA polymerase II (Pol II) through a repressive chromatin environment established by expanded GAA repeats in patient cells (Figure 1A). Remarkably, as a component of SynTEF1, JQ1 facilitates transcription elongation at the targeted gene, whereas when untethered, JQ1 performs exactly the opposite function: globally blocking transcription elongation by binding bromo-

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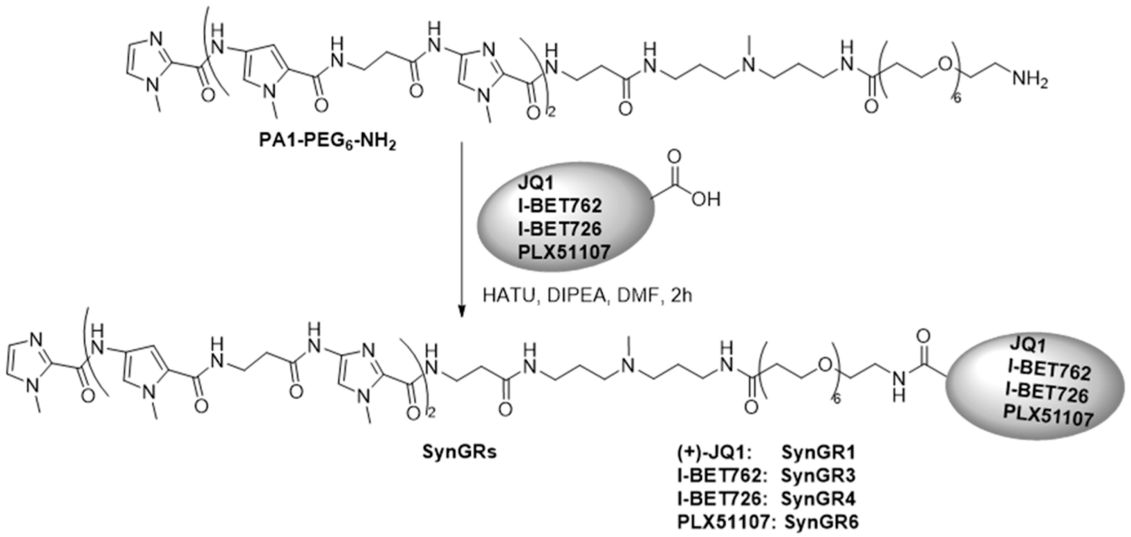
Scheme 1. Synthesis of SynGR1, SynGR3, SynGR4, and SynGR6



domains of BRD4/BET proteins and releasing these factors from actively transcribed regions of the genome.[26−28] In other words, induced proximity and targeted presentation convert JQ1 from an orthosteric inhibitor of BRD4/BET to a chemical prosthetic that substitutes for acetyl-lysine residues on proteins to effectively recruit BRD4/BET in lieu of the natural biological partners.

JQ1 binds the tandem bromodomains (BD1 and BD2) of BRD4 with relatively high affinity[26] (49 and 90 nM, respectively). These ∼110 residue domains each comprise four helices (Zα, Aα, Bα, Cα) that form a left-handed helical bundle.[29−31] (Figure 1B). The loops between the Z and A helices and the B and C helices as well as conserved residues and ordered water molecules form a pocket that preferentially binds diacetylated lysine residues on histone tails.[32] The sequence and structural homology across bromodomains, especially among BD1 (or BD2) domains of all four BET family members (BRD2−4 and BRDT), is such that most ligands function as pan-BET binders and inhibitors. The success of first-generation pan-BET inhibitors such as JQ1 in blocking cancer cell proliferation has led to the development of a structurally diverse range of highly effective BET bromodomain-binding ligands.[33−35] More recently, as the functional distinctions between BD1 and BD2 have become clearer, ligands that selectively bind one domain or the other have been developed.[36−41] Each generation of BET ligands has improved the pharmacological and functional properties.

We set out to test whether newly disclosed BET ligands could be used to develop a broader repertoire of synthetic genome readers/regulators (SynGRs) that would enable us to examine the effects of selectively targeting individual bromodomains. Among the wealth of available BET ligands, we chose eight that represent three structural classes based on the Tanimoto similarity matrix[42] (Figure 1C). Even within each class, ligands displayed varied physicochemical properties and BET bromodomain selectivities.[34,43−45] In choosing this set of ligands, we also prioritized the synthetic tractability for conjugation to the DNA-binding polyamide. Like JQ1, OTX015 and I-BET762 contain a triazolodiazepine core and exhibit comparable binding affinities to both BD1 and BD2 (Figure 1D−F); both OTX015 and I-BET762 have improved pharmacokinetic properties in vivo and are in clinical trials for cancer treatment.[46−50] I-BET726[51] and SJ432 are members of the tetrahydroquinoline class (Figure 1G,H); while the domain selectivity of I-BET726 has not been evaluated, it lacks pyrazole and nitrile groups on 4-aminobenzene that contribute to the selectivity of SJ432 for BD2 over BD1.[40] Finally, azaindole PLX51107 and imidazoquinolinones I-BET151 and GSK778 exhibit the opposite selectivity, with increasing preference for BD1 over BD2 (Figure 1J,K).[36,52−54] Examining the cocrystal structures, we chose conjugation sites that protruded out of the ligand-binding pocket of the bromodomain and thus would not be expected to affect binding affinity (marked by an arrow in Figure 1D−K). Moreover, for several ligands, the sites of conjugation have been previously used to conjugate E3-ligase-binding ligands without compromising domain specificity or binding affinity.[55−58]

Conjugating polyamide PA1 to these BRD4/BET ligands, we synthesized seven new SynGRs that, together with SynTEF1 (referred to here as the prototype SynGR1), enabled us to delineate the affinity range for BET factors that elicit biological activity and more interestingly dissect the effects of selectively targeting individual BET bromodomains. To our surprise, we found that SynGRs that selectively target BD1 were ineffective in licensing transcription at the diseased *FXN* gene, while those that bound BD2 were highly active and indistinguishable from SynGRs that bear pan-BET ligands. Surprisingly, the untethered BD1 ligand (GSK778) significantly enhanced the activity of all non-BD1 targeting SynGRs. Our findings reveal distinct and nonidentical roles of each bromodomain in stably engaging and selectively regulating gene expression at a targeted genomic locus. Moreover, the results demonstrate that SynGRs function akin to natural transcription factors, which preferentially engage BD2, rather than acetylated histone tails, which are thought to engage BD1 to recruit BET proteins to the sites of active gene transcription. As such, SynGRs function as bona fide synthetic transcription factors and not as "epi-mimics" or molecules that mimic the epigenetic chromatin marks.[22,59]

RESULTS

Synthesis of SynGRs. Here, we synthesized polyamide PA1, which targets the 5′AAGAAGAAG3′ sequence[60] via manual solid-phase synthesis using Boc β-alanine PAM resin,

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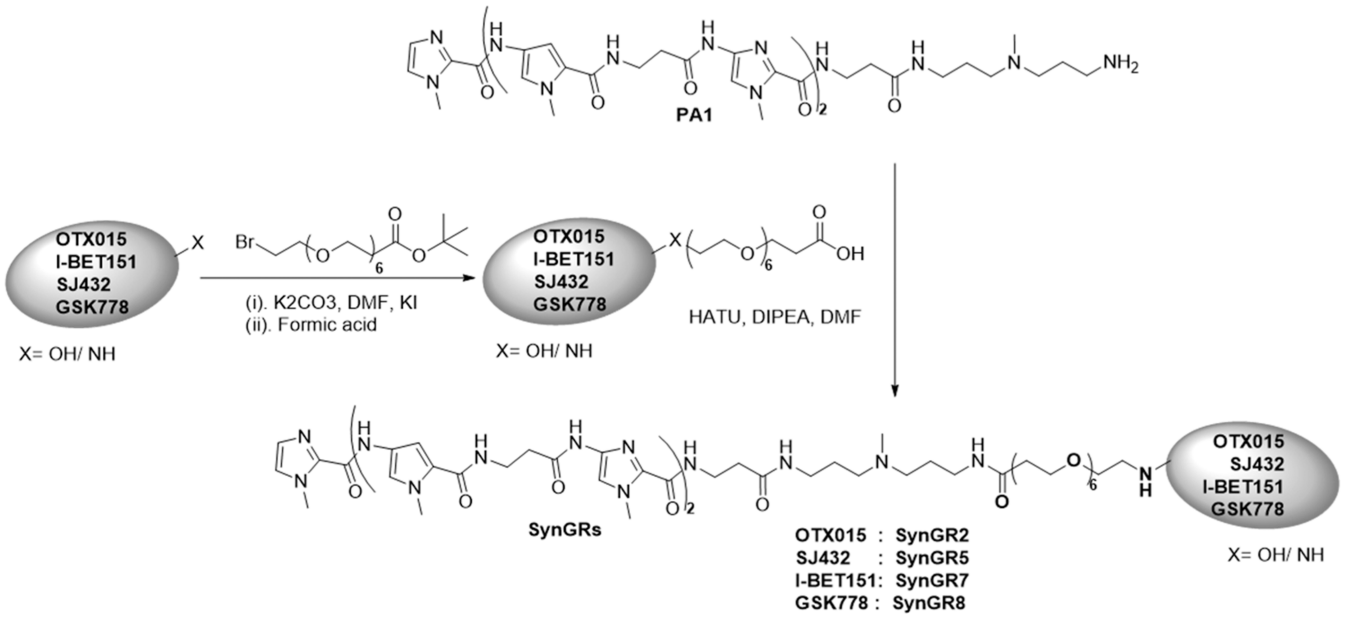
Scheme 2. Synthesis of SynGR2, SynGR5, SynGR7, and SynGR8



BocHN-Py−OH, BocHN-Im−OH, BocHN-β-alanine, and Im−OH building blocks and used this polyamide for the synthesis of all SynGRs by one of two methods. In the first method (Scheme 1), PEG-linked PA1 (PA1-PEG6-NH_2) was synthesized by nucleophilic substitution of Fmoc-PEG6-NHS ester with polyamide PA1 in the presence of DIPEA and DMF followed by 10% piperidine. PA1-PEG6-NH_2 was then coupled to the BET-binding ligands JQ1-COOH, I-BET762-COOH, I-BET726, and PLX51107 using standard coupling conditions (PyBOP, DIPEA in DMF) to produce SynGR1, SynGR3, SynGR4, and SynGR6, respectively (see the Supporting Information for details). This new method is synthetically more facile and led to negligible, if any, side products compared to our previous method.[19] For BET ligands that do not have an available carboxylic acid moiety, a different method was developed (Scheme 2), where the suitable bromo-PEG6-tBu ester was reacted with BET-binding ligands OTX015, I-BET151, SJ432, and GSK778 by using K_2CO_3 as a base. The hydrolysis of the resulting tBu-PEG6-linked ligand was carried out using formic acid, followed by conjugation to PA1 using standard coupling conditions (PyBOP and DIPEA), yielding SynGR2, SynGR5, SynGR7, and SynGR8, respectively (see the Supporting Information for details). All SynGRs were purified by Prep HPLC, and pure fractions were characterized by liquid chromatography−mass spectrometry (LC−MS) and lyophilized to offer white fluffy compounds (Supporting Figures S1−S22).

Binding of SynGRs to DNA. Linear polyamide PA1 binds to the minor groove of dsDNA with 1:1 stoichiometry in reverse orientation.[5,11,60] Surface plasmon resonance (SPR) was used to determine the binding affinities of PA1 and SynGRs to three different sequences of biotinylated hairpin DNA (hDNA) that display the PA1 target sequence ($^{5'}$AAGAAGAAG$^{3'}$; hDNA1), a single mutation (5′-AGGAA-GAAG-3′; hDNA2), or two mutations (5′-AGGAGGAAG-3′; hDNA3; Figures 2 and S23 and Supporting Table 1). The SPR sensorgrams of PA1 and SynGR binding to hDNA1 from low (3.25 nM) to high (100 nM) concentrations permit affinity measurements. Kinetic fits were used to determine K_D values because sensorgrams did not always reach a steady-state level, especially at lower concentrations.[61]

PA1 has a very strong binding affinity for its target sequence (K_D = 0.36 nM), while a single mutation within the binding site reduces the binding affinity by 26-fold, and two mutations abolish measurable binding (Supporting Table 1). This trend holds for all of the SynGRs as well, where conjugation to BET ligands modestly impacts binding to the target site. This observation is consistent with previous studies where tethering ligands to polyamides reduced affinity with minimal consequences on sequence specificity.[12,18,62] Interestingly, in the context of SynGRs, the ability of PA1 to discriminate between its target sequence and near-cognate sites appears to increase upon conjugation to BET ligands, as the binding affinities to hDNA2 are reduced by 1.5- to 6-fold greater than the sequence selectivity displayed by PA1. The basis for this nonlinear sensitivity to binding site perturbation can have complex kinetic and mechanistic underpinnings. Nevertheless, the finding highlights the importance of balancing affinity and specificity, as molecules with very high affinities more readily overcome the penalty in binding to near-cognate target sites.[60]

Recruitment of BRD4 and Activation of *FXN* Transcription by SynGRs. Having confirmed their sequence selectivity and DNA-binding properties, the biological function of SynGRs was tested by monitoring their ability to restore *FXN* transcription in Friedreich's ataxia patient-derived cells (GM15850; Figures 3A and S24). SynGR1, SynGR2, and SynGR3 are structurally similar and have similar effects on *FXN* expression, except for SynGR2, which peaks at a slightly lower concentration (0.3 vs 1 μM) despite having comparable affinity for BRD4 and an ∼2-fold lower affinity for the target DNA. Maximal activation at lower concentrations is also displayed by SynGR4 and SynGR5, which are structurally related but behave differently in terms of *FXN* expression and bromodomain selectivity. SynGR4, which is dual bromodomain-targeting with a 7-fold lower affinity for DNA than SynGR1, is the most potent of all tested SynGRs at 0.1 μM, but its activity drops off at higher concentrations. Meanwhile, BD2-selective SynGR5 shows comparable activity to the dual

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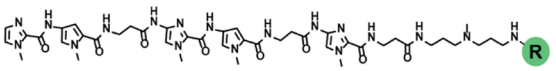


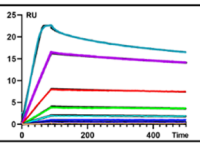
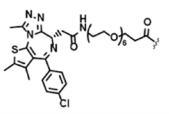
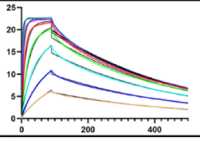
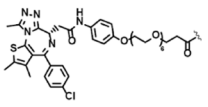
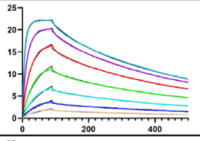
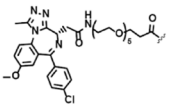
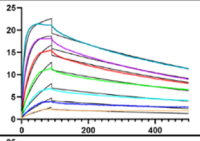
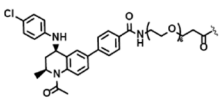
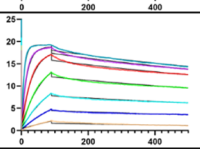
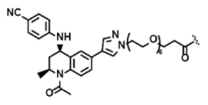
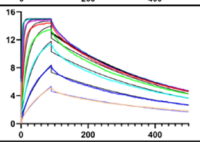
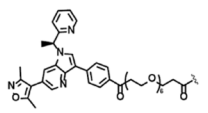
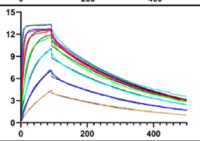
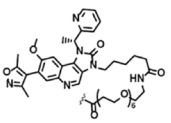
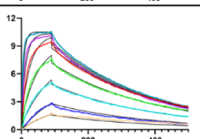
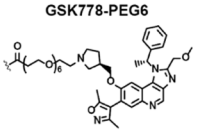
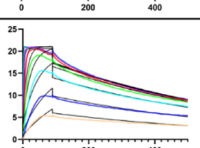
SynGR	R	SPR Sensorgrams	hDNA1 K_D (nM)	BRD4: K_D (nM)	
				BD1	BD2
PA1	H		0.36±0.01	nb	nb
SynGR1	JQ1-PEG6 		1.24±0.01	49	90 [ref. 26]
SynGR2	OTX015-PEG6 		2.35±0.01	92-112 (IC_{50}) [ref. 49]	
SynGR3	I-BET762-PEG6 		1.68±0.01	95	65 [ref. 50]
SynGR4	I-BET726-PEG6 		8.73±0.01	3.16 (IC_{50})	0.39 (IC_{50}) [ref. 51]
SynGR5	SJ432-PEG6 		3.64±0.01	301	2 [ref. 40]
SynGR6	PLX51107-PEG6 		1.16±0.01	1.7	6.1 [ref. 52]
SynGR7	I-BET151-PEG6 		3.57±0.01	19	172 [ref. 36]
SynGR8	GSK778-PEG6 		3.76±0.01	19	2480 [ref. 36]

Figure 2. Binding of PA1 and SynGRs to DNA and bromodomains in vitro. Structures of PA1-based SynGRs and representative SPR sensorgrams for PA1 alone and SynGR1 to SynGR8 were measured in the presence of hairpin DNA (hDNA1) with the PA1 target sequence. For each compound (PA1 or SynGR), the sensorgrams represent 3, 6.25, 12.5, 25, 50, 100, and 200 nM (bottom to top) compound. Solid black lines are best-fit values for the global kinetic fitting of the results with a single-site function. BRD4 K_D values of parent BET ligands are taken from the literature; for OTX015 and I-BET726, binding affinities to individual bromodomains are not available.

bromodomain-targeting SynGRs1−4. SynGR6, SynGR7, and SynGR8 are also structurally related to one another; however, their BD1 selectivity increases from SynGR6 (~3-fold selective) to SynGR8 (~130-fold selective). Puzzlingly, *FXN* gene expression decreases with increasing BD1 selectivity in this set of three SynGRs. SynGR8, which is the most selective for BD1, barely activates *FXN* even at the highest tested concentration, while SynGR7, which is moderately selective (~9-fold), promotes *FXN* expression only at higher concentrations (1 μM). In contrast, the dual bromodomain-targeting SynGR6, which binds BRD4 more than an order of magnitude better than the first set of pan-BET SynGRs, displays activity at the *FXN* locus almost equal to that of SynGR1.

The data suggest that increased selectivity of SynGRs for BD1 correlates negatively with activity (Figure 3B), while the affinity for BD2 correlates positively with activity (Figure 3C). Rather than a linear correlation, it also appears that beyond a threshold dissociation constant of approximately 100 nM, further increasing affinity for BD2 does not result in increased activity in cells (Supporting Figures S25 and S26). This

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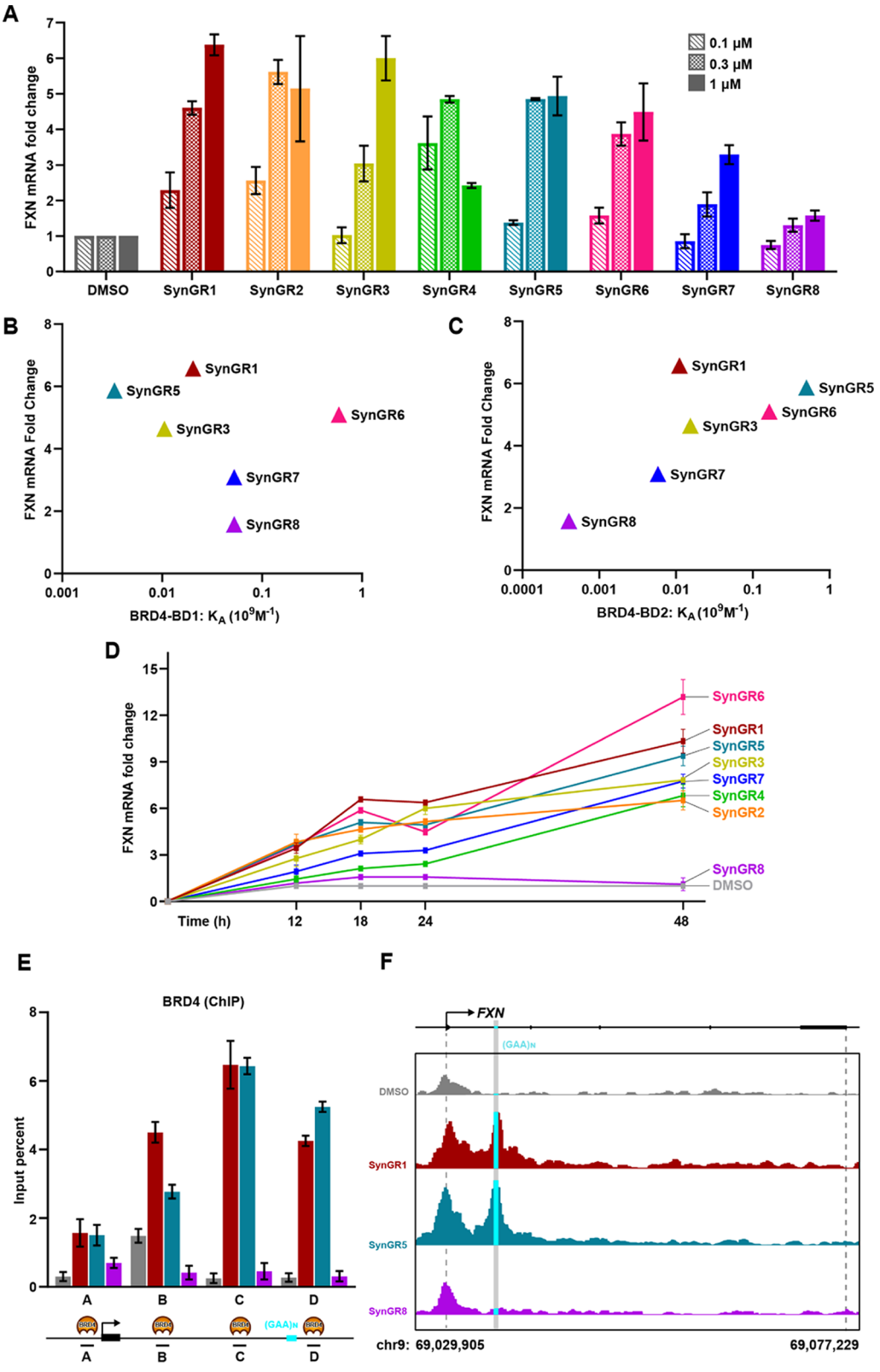

Figure 3. Licensing of *FXN* transcription by SynGRs in FA cells. (A) Relative expression of *FXN* mRNA in GM15850 cells following treatment for 24 h with 0.1, 0.3, and 1 μM of the indicated SynGR, normalized to 0.1% DMSO solvent control. Error bars are the SD of three replicates. (B, C) Correlation of SynGR activity with binding affinities of unconjugated BET ligands to individual bromodomains BD1 (B) and BD2 (C) with activity at the *FXN* locus, following treatment with 1 μM SynGR for 18 h. (D) Relative expression of *FXN* mRNA in GM15850 cells following treatment at increasing time intervals up to 48 h with 1 μM of the indicated SynGR, normalized to 0.1% DMSO solvent control. Error bars are the SD of three replicates. (E) ChIP-qPCR for BRD4, normalized to percent input, at amplicons across the *FXN* gene in GM15850 cells following treatment for 24 h with 1 μM SynGR1, SynGR5, or SynGR8, or 0.1% DMSO. Error bars are the SD of two replicates. (F) Next-generation sequencing of genomic DNA enriched from GM15850 cells treated for 24 h with 1 μM SynGR1, SynGR5, or SynGR8, or 0.1% DMSO.

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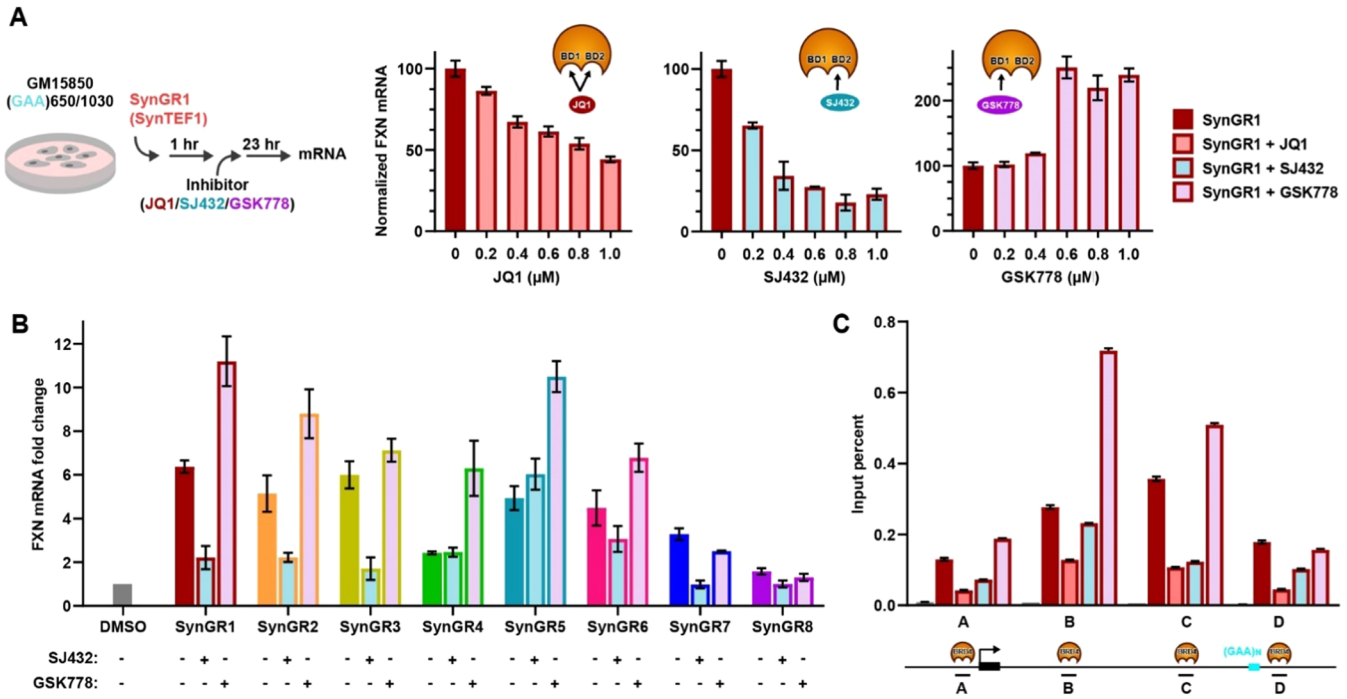

Figure 4. Cotreatment of SynGRs with BET-binding ligands. (A) Relative expression of *FXN* mRNA, normalized to SynGR1 treatment alone, after the treatment of GM15850 cells with SynGR1 (1 μM) for 1 h, followed by BET-binding ligands JQ1, SJ432, or GSK778 at increasing concentrations for 23 h. Error bars are the SD of three replicates. (B) Relative expression of *FXN* mRNA, normalized to 0.1% DMSO control, after the treatment of GM15850 cells with each SynGR (1 μM) alone for 24 h (white bars) or each SynGR for 1 h followed by BD2-binding ligand SJ432 (0.5 μM, cyan bars) or BD1-binding ligand GSK778 (0.5 μM, purple bars) for 23 h. Error bars are the SD of three replicates. (C) ChIP-qPCR for BRD4, normalized to percent input, at amplicons across the *FXN* gene in GM15850 cells following treatment for 24 h with 1 μM SynGR1 (white bars) or for 1 h with 1 μM SynGR1 followed by 23 h with 0.5 μM JQ1, SJ432, or GSK778 (colored bars). Error bars are the SD of two replicates.

observation prompted us to investigate whether kinetics could also play a role in determining SynGR activity. We thus tested these SynGRs at different time intervals, up to 48 h (Figure 3D), and found that only SynGR6 exhibits a striking increase in activity at later time points. Meanwhile, the BD1-selective SynGR8 does not activate the *FXN* gene, even upon prolonged treatment.

To verify that conjugation to the polyamide does not materially affect the ability of GSK778 to bind BET bromodomains, we used SPR to measure the binding affinity (K_D) of SynGR8 to recombinant BD1 and BD2 domains from BRD2/BET (Supporting Table 2). As positive and negative controls, we also tested the binding of both domains to SynGR1 and parent polyamide PA1 (Supporting Table 2). As expected, PA1 did not display any binding to either domain, while the affinities of the two SynGRs for BD1 and BD2 were comparable to those of their unconjugated BET ligands (GSK778 and JQ1). Importantly, the selectivity for BD1 over BD2 reported for GSK778 is maintained by SynGR8. Moreover, an ~20-fold increase in affinity for BD2 displayed by PA1-tethered JQ1 is consistent with the ability of SynGR1 to license *FXN* expression at the levels evoked by higher affinity BD2 ligands in SynGR5 and SynGR6. Taken together, these results indicate that the engagement of the BD2 domain is critical for licensing *FXN* transcription in patient-derived cells.

Our previous results demonstrated that *FXN* expression is restored by recruiting BET proteins to rescue stalled RNA polymerase II in the disease-causing GAA triplet repeat expansions (Figure 1A). The discordant functional outcomes of targeting BD1 versus BD2 were unexpected because SynTEF1 (SynGR1) was designed to recruit BET proteins in a domain-agnostic manner. Despite the growing appreciation for nonidentical biological roles of the two domains, current mechanistic models would predict that the recruitment of BET proteins to the target *FXN* locus via either domain should be equally effective.[19] To determine if this were the case, we performed chromatin immunoprecipitation (ChIP) experiments to monitor BRD4 enrichment upon treatment with SynGR1 (pan-BET), SynGR5 (BD2-selective), or SynGR8 (BD1-selective) (Figure 3E,F). Upon incubation with 1 μM SynGR for 24 h, the cells were treated with 37% v/v formaldehyde to induce covalent cross-links between biomolecules. BRD4 cross-linking to four distinct sites across the *FXN* locus was quantified by PCR amplification using primer pairs that amplified genomic DNA at the promoter (A), promoter-proximal intronic region (B), or (C, D amplicons) flanking the GAA repeats that are targeted by the SynGRs (Figure 3E). Consistent with expectations, the quantitative ChIP-qPCR data show that SynGR1 and SynGR5 effectively recruit BRD4 to the targeted region of *FXN* (Figure 3E). In stark contrast, SynGR8, despite its high affinity for the BD1 domain in vitro, failed to stably recruit BRD4. While the inability to recruit BRD4/BET is consistent with the lack of transcription activity displayed by SynGR8 in cells, the result suggests that the stable engagement of BET at repressive chromatin may be differently mediated in FA patient-derived lymphoblasts. The inability to directly test SynGR binding to genomic loci by ChIP methodology[6,63−66] prevents us from determining if the tethered BET ligand cooperatively enables

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SynGR association with repressive GAA repeat expansions. To further ensure that SynGR8 does not enrich BET proteins at genomic sites other than those queried by the four bespoke *FXN* amplicons, we performed unbiased next-generation sequencing (ChIP-seq) of the BRD4 enriched genomic DNA from cells treated with the three SynGRs. These higher resolution profiles unambiguously demonstrate that SynGR8 does not recruit BRD4 to the GAA repeats within the *FXN* locus (Figure 3F). The near-identical BRD4 profiles of SynGR5 and SynGR1 buttress the view that BD2 binding is necessary and sufficient for stably engaging BRD4 at the targeted genomic site despite the underlying repressive chromatin environment.

Cotreatment of SynGRs with Domain-Specific Binding Ligands. These opposite effects observed for ligands that target one or the other of the two tandem BET bromodomains prompted us to further explore the underlying mechanism and dependence on BD1 versus BD2 engagement by prototype pan-BET SynGR1. Beginning with the administration of SynGR1 to GM15850 cells for an hour, we examined the effect of competitive inhibition by subsequent treatment with the cognate pan-BET ligand (+)-JQ1 (Figure 4A). With increasing JQ1 concentration, the expression levels of *FXN* decreased up to 50%. As JQ1 targets both bromodomains of BET proteins, this result indicates that blocking the binding of SynGR1 to either (or both) BD1 or BD2 reduces its ability to recruit BET proteins to the expanded GAA triplet repeats within the *FXN* gene in patient-derived cells.

To distinguish the effects of binding to each bromodomain, we treated SynGR1-incubated cells with domain-selective binding ligand SJ432 (BD2) or GSK778 (BD1). Cotreatment with SJ432 largely mirrored the cotreatment with JQ1, though with a somewhat stronger effect at high concentrations, decreasing *FXN* expression by up to 75% (Figure 4A). On the other hand, contrary to all expectations, GSK778 further stimulated the activity of SynGR1 at high concentrations, almost doubling *FXN* gene expression compared to treatment with SynGR1 alone. In control experiments, treatment with these ligands alone or with unlinked PA1 did not promote *FXN* expression above the baseline (Supporting Figure 4).

To investigate these effects further, we tested the effect of domain-selective inhibitors on the activity of the entire set of SynGRs. Cotreatment with SJ432 generally reduced the *FXN* transcription activity of the nonselective pan-BET SynGRs (Figure 4B). This result reinforces the importance of BD2 even for SynGR8 and SynGR7, which selectively engage the BD1 module of BET proteins but have a residual affinity for BD2. Remarkably, for those SynGRs that have particularly high affinity for BD2, *FXN* expression following cotreatment with SJ432 was essentially unchanged (SynGRs4 and 5) or only modestly decreased (SynGR6) relative to those SynGRs alone, suggesting that free SJ432 is not effective at competitively displacing BET proteins from the genomic locus once it is recruited to hundreds of tandemly organized GAA repeats and potentially engaged in higher-order transcriptional complexes. Conversely, cotreatment with GSK778 enhanced the activity of the BD2-selective and pan-BET SynGRs while negligibly reducing the activity of the BD1-selective SynGRs (Figure 4B). This effect highlights the importance of BD2 in the BET engagement by active SynGRs, as discussed below. In support of the competitive displacement of SynGR-bound BRD4/BET, ChIP-qPCR analysis shows that the addition of JQ1 or SJ432 reduces the recruitment of BRD4 at the *FXN* locus, whereas the addition of GSK778 enhances BRD4 recruitment (Figure 4C).

DISCUSSION

Mechanistic models of human gene regulation guided our design of SynTEF1, the prototype SynGR that recruits BET proteins to the expanded GAA repeats in the diseased *FXN* locus (Figure 1A). We reasoned that SynTEF1/SynGR1 utilized conjugated JQ1 to bind either one or both tandem bromodomains of BET proteins. As more effective and domain-selective BET ligands were developed, we incorporated them here in our modular SynGR design. These new chemical probes enabled us to investigate the contribution of increased affinity and domain selectivity toward restoring *FXN* expression in patient-derived cells. The results unequivocally demonstrate that SynGRs function by engaging the BD2 domain of the BET proteins rather than BD1.

BD1 and BD2 were initially considered functionally redundant due to their high sequence and structural similarities and comparable affinities for ligands such as JQ1. With this framework, SynGRs bearing high-affinity synthetic BET ligands that bind either domain would have been expected to recruit BET proteins to targeted genomic loci. However, more recent genetic, structural, and pharmacological studies have revealed nuanced differences between the two tandem bromodomains of BET proteins.[36,39,67] The fact that BD1-selective SynGRs do not recruit BRD4 further reveals an unexpected functional distinction in the mode by which each domain "reads" acetyl-lysine marks on its interacting partners. In particular, ligands that selectively bind BD1 elicit broad transcriptome-wide perturbations, more widely evict BRD4 from chromatin, and effectively block cell proliferation.[36] As such, it is inferred that diacetylated lysine residues on the unstructured N-terminal "tails" of histones that project out of the nucleosome preferentially associate with BD1 (Figure 5A). Conversely, BD2 appears more permissive and is targeted by multiple client proteins, including transcription factors such as MYC, NFkB, and Twist.[68−70] These distinctions are not absolute: GATA1, a transcription factor that governs erythropoiesis and red blood cell maturation, targets BD1,[71] while acetylated histone tails are widely documented to bind BD2 as well.[32] Nevertheless, BD2-selective pharmacological agents have a narrower and more targeted disruptive impact on gene expression, blunting specific cellular/physiological responses rather than bluntly blocking cellular proliferation. These observations are consistent with a transcription factor-directed engagement of BD2 to regulate selective gene networks (Figure 5B).

Given this context, the ability of BD2-binding SynGRs to recruit BRD4 to targeted genomic loci when contrasted with the inability of BD1-binding SynGRs to do so indicates that SynGR functions as a synthetic transcription factor rather than mimicking the acetylated histone tail, a so-called "epi-mimic" (Figure 5C). This is not a semantic distinction, as it explains the mechanistic basis for the single-gene selective activity of SynGRs at the targeted GAA repeats of the *FXN* locus, whereas an "epi-mimic" would have elicited broader perturbations in gene expression. More importantly, only through domain-selective SynGRs were we able to reveal distinct functional roles of the two tandem bromodomains despite their similar affinities for diacetylated histone tails. Notably, BD1 is exquisitely dependent on local context to stably associate with the genome, whereas BD2 is context-agnostic and can stably associate with the genome through

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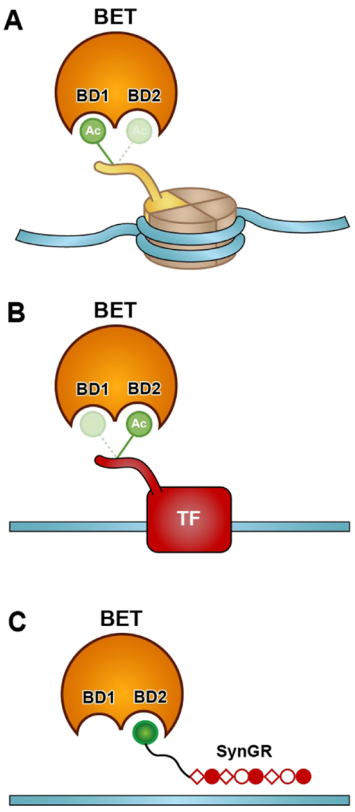

Figure 5. Models of the BET protein interactions with acetylated client proteins. (A) Interaction between acetylated chromatin and BD1 of a BET protein. (B) Interaction between the acetylated transcription factor and BD2 of a BET protein. (C) BET protein recruitment by SynGRs occurs via the binding of BD2, indicating that SynGRs behave as synthetic transcription factors, not mimics of epigenetic marks.

binding to a tethered small molecule ligand of modest affinity (~0.1 μM). This insight enables a more sophisticated design of next-generation SynGRs for other genome-targeted operations. We intend to exploit this insight in the design of future SynGRs targeted to other genomic loci.

The cotreatment of cells with SynGRs and untethered BET ligands also brought to light at least two unexpected physiological and physical phenomena. The first was the surprising result that GSK778, a BD1-selective ligand, substantively increased the activity of SynGRs when it was expected to decrease activity, similar to the effects of JQ1 and SJ432 (Figure 4A,B). One explanation for this result is that by binding to and occupying BD1, GSK778 redirects pan-BET SynGRs to the BD2 domain, leading to a more effective recruitment and stable association of BRD4. However, such redirection to BD2 would not explain the increased expression by SynGR5, which is highly BD2-selective. It is possible that the binding of GSK778 to BD1 may contribute allosterically to BD2 binding or coactivator recruitment. On the other hand, based on previous reports that free BD1 ligands can drive the global release of chromatin-bound BRD4,[36] it is possible that GSK778 treatment increases the available pool of nuclear BET proteins for recruitment by BD2-targeting SynGRs. The cotreatment data further suggests that under physiological conditions, access to BET proteins is limiting, and active recruitment by transcription factors overcomes this rate-limiting step in gene expression. The second intriguing result is that SynGRs conjugated to ligands that bind BD2 with high affinity (2−23 nM) are remarkably resistant to competitive inhibition by free SJ432, the BD2-selective ligand. This observation stands in contrast to the substantial loss of activity displayed by all pan-BET SynGRs and even BD1-selective SynGRs upon cotreatment with free/untethered SJ432. The results suggest a DNA-templated assemblage of higher-order BET-Pol II complexes that are not readily disassembled. Whether such assemblages occur and perhaps adopt emergent properties within biomolecular condensates (as BRD4 is known to do) remains to be determined.

In summary, this study provides new design principles for the creation of sophisticated and precision-tailored SynGRs with potential therapeutic applications. Moreover, our chemical approach enabled the dissection of molecular recognition features that govern rate-limiting steps in gene regulation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c06297.

Experimental characterization data, synthetic schemes, and experimental procedures of all compounds; MALDI analysis spectra of PA1; LC−MS analysis spectra of PA1-PEG6-NH_2; LC−MS analysis spectrum of SynGRs; HPLC analysis spectrum of SynGRs; binding affinities of SynGRs; binding affinities and sensorgrams of PA1, SynGR1, and SynGR8 for BRD2-BD1 and BRD2-BD2; and methods (PDF)

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Notes
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■ REFERENCES

(1) Dervan, P. B. A Personal Perspective on Chemical Biology: Before the Beginning. *Isr. J. Chem.* **2019**, *59* (1−2), 71−83.
(2) Ptashne, M.; Gann, A. *Genes & Signals*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor: NY, 2002; Vol. *402*.
(3) Henley, M. J.; Koehler, A. N. Advances in targeting 'undruggable'transcription factors with small molecules. *Nat. Rev. Drug Discovery* **2021**, *20* (9), 669−688.
(4) White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B. Recognition of the four Watson-Crick base pairs in the DNA minor groove by synthetic ligands. *Nature* **1998**, *391* (6666), 468−471.
(5) Carlson, C. D.; Warren, C. L.; Hauschild, K. E.; Ozers, M. S.; Qadir, N.; Bhimsaria, D.; Lee, Y.; Cerrina, F.; Ansari, A. Z. Specificity landscapes of DNA binding molecules elucidate biological function. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107* (10), 4544−4549.
(6) Erwin, G. S.; Grieshop, M. P.; Bhimsaria, D.; Do, T. J.; Rodríguez-Martínez, J. A.; Mehta, C.; Khanna, K.; Swanson, S. A.; Stewart, R.; Thomson, J. A.; Ramanathan, P.; Ansari, A. Z. Synthetic genome readers target clustered binding sites across diverse chromatin states. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113* (47), E7418−E7427.
(7) Edayathumangalam, R. S.; Weyermann, P.; Gottesfeld, J. M.; Dervan, P. B.; Luger, K. Molecular recognition of the nucleosomal "supergroove. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (18), 6864−6869.
(8) Gottesfeld, J. M.; Belitsky, J. M.; Melander, C.; Dervan, P. B.; Luger, K. Blocking transcription through a nucleosome with synthetic DNA ligands. *J. Mol. Biol.* **2002**, *321* (2), 249−263.
(9) Suto, R. K.; Edayathumangalam, R. S.; White, C. L.; Melander, C.; Gottesfeld, J. M.; Dervan, P. B.; Luger, K. Crystal structures of nucleosome core particles in complex with minor groove DNA-binding ligands. *J. Mol. Biol.* **2003**, *326* (2), 371−380.
(10) Puckett, J. W.; Muzikar, K. A.; Tietjen, J.; Warren, C. L.; Ansari, A. Z.; Dervan, P. B. Quantitative microarray profiling of DNA-binding molecules. *J. Am. Chem. Soc.* **2007**, *129* (40), 12310−12319.
(11) Puckett, J. W.; Muzikar, K. A.; Tietjen, J.; Warren, C. L.; Ansari, A. Z.; Dervan, P. B. Quantitative microarray profiling of DNA-binding molecules. *J. Am. Chem. Soc.* **2007**, *129* (40), 12310−12319.
(12) Warren, C. L.; Kratochvil, N. C.; Hauschild, K. E.; Foister, S.; Brezinski, M. L.; Dervan, P. B.; Phillips, G. N., Jr.; Ansari, A. Z. Defining the sequence-recognition profile of DNA-binding molecules. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (4), 867−872.
(13) Meier, J. L.; Yu, A. S.; Korf, I.; Segal, D. J.; Dervan, P. B. Guiding the design of synthetic DNA-binding molecules with massively parallel sequencing. *J. Am. Chem. Soc.* **2012**, *134* (42), 17814−17822.
(14) Mapp, A. K.; Ansari, A. Z.; Ptashne, M.; Dervan, P. B. Activation of gene expression by small molecule transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (8), 3930−3935.
(15) Ansari, A. Z.; Mapp, A. K.; Nguyen, D. H.; Dervan, P. B.; Ptashne, M. Towards a minimal motif for artificial transcriptional activators. *Chem. Biol.* **2001**, *8* (6), 583−592.
(16) Arora, P. S.; Ansari, A. Z.; Best, T. P.; Ptashne, M.; Dervan, P. B. Design of artificial transcriptional activators with rigid poly-L-proline linkers. *J. Am. Chem. Soc.* **2002**, *124* (44), 13067−13071.
(17) Ansari, A. Z.; Mapp, A. K. Modular design of artificial transcription factors. *Curr. Opin. Chem. Biol.* **2002**, *6* (6), 765−772.
(18) Arndt, H. D.; Hauschild, K. E.; Sullivan, D. P.; Lake, K.; Dervan, P. B.; Ansari, A. Z. Toward artificial developmental regulators. *J. Am. Chem. Soc.* **2003**, *125* (44), 13322−13323.
(19) Erwin, G. S.; Grieshop, M. P.; Ali, A.; Qi, J.; Lawlor, M.; Kumar, D.; Ahmad, I.; McNally, A.; Teider, N.; Worringer, K.; Sivasankaran, R.; Syed, D. N.; Eguchi, A.; Ashraf, M.; Jeffery, J.; Xu, M.; Park, P. M. C.; Mukhtar, H.; Srivastava, A. K.; Faruq, M.; Bradner, J. E.; Ansari, A. Z. Synthetic transcription elongation factors license transcription across repressive chromatin. *Science* **2017**, *358* (6370), 1617−1622.
(20) Mapp, A. K.; Ansari, A. Z. A TAD further: exogenous control of gene activation. *ACS Chem. Biol.* **2007**, *2* (1), 62−75.
(21) Xiao, X.; Yu, P.; Lim, H. S.; Sikder, D.; Kodadek, T. Design and synthesis of a cell-permeable synthetic transcription factor mimic. *J. Comb. Chem.* **2007**, *9* (4), 592−600.
(22) Taniguchi, J.; Feng, Y.; Pandian, G. N.; Hashiya, F.; Hidaka, T.; Hashiya, K.; Park, S.; Bando, T.; Ito, S.; Sugiyama, H. Biomimetic Artificial Epigenetic Code for Targeted Acetylation of Histones. *J. Am. Chem. Soc.* **2018**, *140* (23), 7108−7115.
(23) Vaijayanthi, T.; Pandian, G. N.; Sugiyama, H. Pyrrole−Imidazole Polyamides−A Frontrunner in Nucleic Acid-Based Small Molecule Drugs. *Adv. Ther.* **2023**, *6*, No. 2300022, DOI: 10.1002/adtp.202300022.
(24) Stanton, B. Z.; Chory, E. J.; Crabtree, G. R. Chemically induced proximity in biology and medicine. *Science* **2018**, *359* (6380), No. eaao5902.
(25) Gerry, C. J.; Schreiber, S. L. Unifying principles of bifunctional, proximity-inducing small molecules. *Nat. Chem. Biol.* **2020**, *16* (4), 369−378.
(26) Filippakopoulos, P.; Qi, J.; Picaud, S.; Shen, Y.; Smith, W. B.; Fedorov, O.; Morse, E. M.; Keates, T.; Hickman, T. T.; Felletar, I.; Philpott, M.; Munro, S.; McKeown, M. R.; Wang, Y.; Christie, A. L.; West, N.; Cameron, M. J.; Schwartz, B.; Heightman, T. D.; La Thangue, N.; French, C. A.; Wiest, O.; Kung, A. L.; Knapp, S.; Bradner, J. E. Selective inhibition of BET bromodomains. *Nature* **2010**, *468* (7327), 1067−1073.
(27) Shi, J.; Vakoc, C. R. The mechanisms behind the therapeutic activity of BET bromodomain inhibition. *Mol. Cell* **2014**, *54* (5), 728−736.
(28) Schwalm, M. P.; Knapp, S. BET bromodomain inhibitors. *Curr. Opin. Chem. Biol.* **2022**, *68*, No. 102148.

(29) Vollmuth, F.; Blankenfeldt, W.; Geyer, M. Structures of the dual bromodomains of the P-TEFb-activating protein Brd4 at atomic resolution. *J. Biol. Chem.* **2009**, *284* (52), 36547−36556.
(30) Jung, M.; Philpott, M.; Müller, S.; Schulze, J.; Badock, V.; Eberspächer, U.; Moosmayer, D.; Bader, B.; Schmees, N.; Fernández-Montalván, A.; Haendler, B. Affinity map of bromodomain protein 4 (BRD4) interactions with the histone H4 tail and the small molecule inhibitor JQ1. *J. Biol. Chem.* **2014**, *289* (13), 9304−9319.
(31) Zaware, N.; Zhou, M. M. Bromodomain biology and drug discovery. *Nat. Struct. Mol. Biol.* **2019**, *26* (10), 870−879.
(32) Filippakopoulos, P.; Picaud, S.; Mangos, M.; Keates, T.; Lambert, J. P.; Barsyte-Lovejoy, D.; Felletar, I.; Volkmer, R.; Müller, S.; Pawson, T.; Gingras, A. C.; Arrowsmith, C. H.; Knapp, S. Histone recognition and large-scale structural analysis of the human bromodomain family. *Cell* **2012**, *149* (1), 214−231.
(33) Pérez-Salvia, M.; Esteller, M. Bromodomain inhibitors and cancer therapy: From structures to applications. *Epigenetics* **2017**, *12* (5), 323−339.
(34) Chen, J.; Tang, P.; Wang, Y.; Wang, J.; Yang, C.; Li, Y.; Yang, G.; Wu, F.; Zhang, J.; Ouyang, L. Targeting Bromodomain-Selective Inhibitors of BET Proteins in Drug Discovery and Development. *J. Med. Chem.* **2022**, *65* (7), 5184−5211.
(35) Liu, Z.; Wang, P.; Chen, H.; et al. Drug Discovery Targeting Bromodomain-Containing Protein 4. *J. Med. Chem.* **2017**, *60*, 4533−4558, DOI: 10.1021/acs.jmedchem.6b01761.
(36) Gilan, O.; Rioja, I.; Knezevic, K.; Bell, M. J.; Yeung, M. M.; Harker, N. R.; Lam, E. Y. N.; Chung, C. W.; Bamborough, P.; Petretich, M.; Urh, M.; Atkinson, S. J.; Bassil, A. K.; Roberts, E. J.; Vassiliadis, D.; Burr, M. L.; Preston, A. G. S.; Wellaway, C.; Werner, T.; Gray, J. R.; Michon, A. M.; Gobbetti, T.; Kumar, V.; Soden, P. E.; Haynes, A.; Vappiani, J.; Tough, D. F.; Taylor, S.; Dawson, S. J.; Bantscheff, M.; Lindon, M.; Drewes, G.; Demont, E. H.; Daniels, D. L.; Grandi, P.; Prinjha, R. K.; Dawson, M. A. Selective targeting of BD1 and BD2 of the BET proteins in cancer and immunoinflammation. *Science* **2020**, *368* (6489), 387−394.
(37) Picaud, S.; Wells, C.; Felletar, I.; Brotherton, D.; Martin, S.; Savitsky, P.; Diez-Dacal, B.; Philpott, M.; Bountra, C.; Lingard, H.; Fedorov, O.; Müller, S.; Brennan, P. E.; Knapp, S.; Filippakopoulos, P. RVX-208, an inhibitor of BET transcriptional regulators with selectivity for the second bromodomain. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110* (49), 19754−19759.
(38) Jiang, F.; Hu, Q.; Zhang, Z.; Li, H.; Li, H.; Zhang, D.; Li, H.; Ma, Y.; Xu, J.; Chen, H.; Cui, Y.; Zhi, Y.; Zhang, Y.; Xu, J.; Zhu, J.; Lu, T.; Chen, Y. Discovery of Benzo[cd]indol-2(1H)-ones and Pyrrolo-[4,3,2-de]quinolin-2(1H)-ones as Bromodomain and Extra-Terminal Domain (BET) Inhibitors with Selectivity for the First Bromodomain with Potential High Efficiency against Acute Gouty Arthritis. *J. Med. Chem.* **2019**, *62* (24), 11080−11107.
(39) Rianjongdee, F.; Atkinson, S. J.; Chung, C. W.; Grandi, P.; Gray, J. R. J.; Kaushansky, L. J.; Medeiros, P.; Messenger, C.; Phillipou, A.; Preston, A.; Prinjha, R. K.; Rioja, I.; Satz, A. L.; Taylor, S.; Wall, I. D.; Watson, R. J.; Yao, G.; Demont, E. H. Discovery of a Highly Selective BET BD2 Inhibitor from a DNA-Encoded Library Technology Screening Hit. *J. Med. Chem.* **2021**, *64* (15), 10806−10833.
(40) Slavish, P. J.; Chi, L.; Yun, M. K.; Tsurkan, L.; Martinez, N. E.; Jonchere, B.; Chai, S. C.; Connelly, M.; Waddell, M. B.; Das, S.; Neale, G.; Li, Z.; Shadrick, W. R.; Olsen, R. R.; Freeman, K. W.; Low, J. A.; Price, J. E.; Young, B. M.; Bharatham, N.; Boyd, V. A.; Yang, J.; Lee, R. E.; Morfouace, M.; Roussel, M. F.; Chen, T.; Savic, D.; Guy, R. K.; White, S. W.; Shelat, A. A.; Potter, P. M. Bromodomain-Selective BET Inhibitors Are Potent Antitumor Agents against MYC-Driven Pediatric Cancer. *Cancer Res.* **2020**, *80* (17), 3507−3518.
(41) Zhao, Y.; Yang, C.-Y.; Wang, S. The making of I-BET762, a BET bromodomain inhibitor now in clinical development. *J. Med. Chem.* **2013**, *56*, 7498−7500.
(42) Rogers, D.; Hahn, M. Extended-connectivity fingerprints. *J. Chem. Inf. Model.* **2010**, *50* (5), 742−754.
(43) Liu, Z.; Wang, P.; Chen, H.; Wold, E. A.; Tian, B.; Brasier, A. R.; Zhou, J. Drug Discovery Targeting Bromodomain-Containing Protein 4. *J. Med. Chem.* **2017**, *60* (11), 4533−4558.
(44) Chen, H.; Liu, Z.; Zheng, L.; Wang, R.; Shi, L. BET inhibitors: an updated patent review (2018−2021). *Expert Opin. Ther. Pat.* **2022**, *32* (9), 953−968.
(45) Liu, C. S.; Rioja, I.; Bakr, A.; Veldwijk, M. R.; Sperk, E.; Herskind, C.; Weichenhan, D.; Prinjha, R. K.; Plass, C.; Schmezer, P.; Popanda, O. Selective inhibitors of bromodomain BD1 and BD2 of BET proteins modulate radiation-induced profibrotic fibroblast responses. *Int. J. Cancer* **2022**, *151* (2), 275−286.
(46) Megiorni, F.; Camero, S.; Pontecorvi, P.; Camicia, L.; Marampon, F.; Ceccarelli, S.; Anastasiadou, E.; Bernabò, N.; Perniola, G.; Pizzuti, A.; Panici, P. B.; Tombolini, V.; Marchese, C. OTX015 Epi-Drug Exerts Antitumor Effects in Ovarian Cancer Cells by Blocking GNL3-Mediated Radioresistance Mechanisms: Cellular, Molecular and Computational Evidence. *Cancers* **2021**, *13* (7), No. 1519, DOI: 10.3390/cancers13071519.
(47) Nicodeme, E.; Jeffrey, K. L.; Schaefer, U.; Beinke, S.; Dewell, S.; Chung, C. W.; Chandwani, R.; Marazzi, I.; Wilson, P.; Coste, H.; White, J.; Kirilovsky, J.; Rice, C. M.; Lora, J. M.; Prinjha, R. K.; Lee, K.; Tarakhovsky, A. Suppression of inflammation by a synthetic histone mimic. *Nature* **2010**, *468* (7327), 1119−1123.
(48) Asangani, I. A.; Dommeti, V. L.; Wang, X.; Malik, R.; Cieslik, M.; Yang, R.; Escara-Wilke, J.; Wilder-Romans, K.; Dhanireddy, S.; Engelke, C.; Iyer, M. K.; Jing, X.; Wu, Y. M.; Cao, X.; Qin, Z. S.; Wang, S.; Feng, F. Y.; Chinnaiyan, A. M. Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. *Nature* **2014**, *510* (7504), 278−282.
(49) Noel, J. K.; Iwata, K.; Ooike, S.; Sugahara, K.; Nakamura, H.; Daibata, M. Abstract C244: Development of the BET bromodomain inhibitor OTX015. *Mol. Cancer Ther.* **2013**, *12*, No. C244, DOI: 10.1158/1535-7163.TARG-13-C244.
(50) Baud, M. G. J.; Lin-Shiao, E.; Zengerle, M.; Tallant, C.; Ciulli, A. New Synthetic Routes to Triazolo-benzodiazepine Analogues: Expanding the Scope of the Bump-and-Hole Approach for Selective Bromo and Extra-Terminal (BET) Bromodomain Inhibition. *J. Med. Chem.* **2016**, *59* (4), 1492−1500.
(51) Gosmini, R.; Nguyen, V. L.; Toum, J.; Simon, C.; Brusq, J. M.; Krysa, G.; Mirguet, O.; Riou-Eymard, A. M.; Boursier, E. V.; Trottet, L.; Bamborough, P.; Clark, H.; Chung, C. W.; Cutler, L.; Demont, E. H.; Kaur, R.; Lewis, A. J.; Schilling, M. B.; Soden, P. E.; Taylor, S.; Walker, A. L.; Walker, M. D.; Prinjha, R. K.; Nicodème, E. The discovery of I-BET726 (GSK1324726A), a potent tetrahydroquinoline ApoA1 up-regulator and selective BET bromodomain inhibitor. *J. Med. Chem.* **2014**, *57* (19), 8111−8131.
(52) Ozer, H. G.; El-Gamal, D.; Powell, B.; Hing, Z. A.; Blachly, J. S.; Harrington, B.; Mitchell, S.; Grieselhuber, N. R.; Williams, K.; Lai, T. H.; Alinari, L.; Baiocchi, R. A.; Brinton, L.; Baskin, E.; Cannon, M.; Beaver, L.; Goettl, V. M.; Lucas, D. M.; Woyach, J. A.; Sampath, D.; Lehman, A. M.; Yu, L.; Zhang, J.; Ma, Y.; Zhang, Y.; Spevak, W.; Shi, S.; Severson, P.; Shellooe, R.; Carias, H.; Tsang, G.; Dong, K.; Ewing, T.; Marimuthu, A.; Tantoy, C.; Walters, J.; Sanftner, L.; Rezaei, H.; Nespi, M.; Matusow, B.; Habets, G.; Ibrahim, P.; Zhang, C.; Mathé, E. A.; Bollag, G.; Byrd, J. C.; Lapalombella, R. BRD4 Profiling Identifies Critical Chronic Lymphocytic Leukemia Oncogenic Circuits and Reveals Sensitivity to PLX51107, a Novel Structurally Distinct BET Inhibitor. *Cancer Discovery* **2018**, *8* (4), 458−477.
(53) Seal, J.; Lamotte, Y.; Donche, F.; Bouillot, A.; Mirguet, O.; Gellibert, F.; Nicodeme, E.; Krysa, G.; Kirilovsky, J.; Beinke, S.; McCleary, S.; Rioja, I.; Bamborough, P.; Chung, C. W.; Gordon, L.; Lewis, T.; Walker, A. L.; Cutler, L.; Lugo, D.; Wilson, D. M.; Witherington, J.; Lee, K.; Prinjha, R. K. Identification of a novel series of BET family bromodomain inhibitors: binding mode and profile of I-BET151 (GSK1210151A). *Bioorg. Med. Chem. Lett.* **2012**, *22* (8), 2968−2972.
(54) Chaidos, A.; Caputo, V.; Gouvedenou, K.; Liu, B.; Marigo, I.; Chaudhry, M. S.; Rotolo, A.; Tough, D. F.; Smithers, N. N.; Bassil, A. K.; Chapman, T. D.; Harker, N. R.; Barbash, O.; Tummino, P.; Al-

Mahdi, N.; Haynes, A. C.; Cutler, L.; Le, B.; Rahemtulla, A.; Roberts, I.; Kleijnen, M.; Witherington, J. J.; Parr, N. J.; Prinjha, R. K.; Karadimitris, A. Potent antimyeloma activity of the novel bromodomain inhibitors I-BET151 and I-BET762. *Blood* **2014**, *123* (5), 697−705.
(55) Winter, G. E.; Buckley, D. L.; Paulk, J.; Roberts, J. M.; Souza, A.; Dhe-Paganon, S.; Bradner, J. E. DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for in vivo target protein degradation. *Science* **2015**, *348* (6241), 1376−1381.
(56) Nowak, R. P.; DeAngelo, S. L.; Buckley, D.; He, Z.; Donovan, K. A.; An, J.; Safaee, N.; Jedrychowski, M. P.; Ponthier, C. M.; Ishoey, M.; Zhang, T.; Mancias, J. D.; Gray, N. S.; Bradner, J. E.; Fischer, E. S. Plasticity in binding confers selectivity in ligand-induced protein degradation. *Nat. Chem. Biol.* **2018**, *14* (7), 706−714.
(57) Lu, J.; Qian, Y.; Altieri, M.; Dong, H.; Wang, J.; Raina, K.; Hines, J.; Winkler, J. D.; Crew, A. P.; Coleman, K.; Crews, C. M. Hijacking the E3 Ubiquitin Ligase Cereblon to Efficiently Target BRD4. *Chem. Biol.* **2015**, *22* (6), 755−763.
(58) Chan, K. H.; Zengerle, M.; Testa, A.; Ciulli, A. Impact of Target Warhead and Linkage Vector on Inducing Protein Degradation: Comparison of Bromodomain and Extra-Terminal (BET) Degraders Derived from Triazolodiazepine (JQ1) and Tetrahydroquinoline (I-BET726) BET Inhibitor Scaffolds. *J. Med. Chem.* **2018**, *61* (2), 504−513.
(59) Yu, Z.; Pandian, G. N.; Hidaka, T.; Sugiyama, H. Therapeutic gene regulation using pyrrole-imidazole polyamides. *Adv. Drug Delivery Rev.* **2019**, *147*, 66−85.
(60) Burnett, R.; Melander, C.; Puckett, J. W.; Son, L. S.; Wells, R. D.; Dervan, P. B.; Gottesfeld, J. M. DNA sequence-specific polyamides alleviate transcription inhibition associated with long GAA.TTC repeats in Friedreich's ataxia. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (31), 11497−11502.
(61) Guo, P.; Farahat, A. A.; Paul, A.; Kumar, A.; Boykin, D. W.; Wilson, W. D. Extending the σ-Hole Motif for Sequence-Specific Recognition of the DNA Minor Groove. *Biochemistry* **2020**, *59* (18), 1756−1768.
(62) Hauschild, K. E.; Metzler, R. E.; Arndt, H. D.; Moretti, R.; Raffaelle, M.; Dervan, P. B.; Ansari, A. Z. Temperature-sensitive protein-DNA dimerizers. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (14), 5008−5013.
(63) Erwin, G. S.; Bhimsaria, D.; Eguchi, A.; Ansari, A. Z. Mapping polyamide-DNA interactions in human cells reveals a new design strategy for effective targeting of genomic sites. *Angew. Chem., Int. Ed.* **2014**, *53* (38), 10124−10128.
(64) Finn, P. B.; Bhimsaria, D.; Ali, A.; Eguchi, A.; Ansari, A. Z.; Dervan, P. B. Single position substitution of hairpin pyrrole-imidazole polyamides imparts distinct DNA-binding profiles across the human genome. *PLoS One* **2020**, *15* (12), No. e0243905.
(65) Kurmis, A. A.; Dervan, P. B. Sequence specific suppression of androgen receptor−DNA binding in vivo by a Py-Im polyamide. *Nucleic Acids Res.* **2019**, *47* (8), 3828−3835.
(66) Nickols, N. G.; Dervan, P. B. Suppression of androgen receptor-mediated gene expression by a sequence-specific DNA-binding polyamide. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (25), 10418−10423.
(67) Faivre, E. J.; McDaniel, K. F.; Albert, D. H.; Mantena, S. R.; Plotnik, J. P.; Wilcox, D.; Zhang, L.; Bui, M. H.; Sheppard, G. S.; Wang, L.; Sehgal, V.; Lin, X.; Huang, X.; Lu, X.; Uziel, T.; Hessler, P.; Lam, L. T.; Bellin, R. J.; Mehta, G.; Fidanze, S.; Pratt, J. K.; Liu, D.; Hasvold, L. A.; Sun, C.; Panchal, S. C.; Nicolette, J. J.; Fossey, S. L.; Park, C. H.; Longenecker, K.; Bigelow, L.; Torrent, M.; Rosenberg, S. H.; Kati, W. M.; Shen, Y. Selective inhibition of the BD2 bromodomain of BET proteins in prostate cancer. *Nature* **2020**, *578* (7794), 306−310.
(68) Zou, Z.; Huang, B.; Wu, X.; Zhang, H.; Qi, J.; Bradner, J.; Nair, S.; Chen, L. F. Brd4 maintains constitutively active NF-κB in cancer cells by binding to acetylated RelA. *Oncogene* **2014**, *33* (18), 2395−2404.
(69) Gamsjaeger, R.; Webb, S. R.; Lamonica, J. M.; Billin, A.; Blobel, G. A.; Mackay, J. P. Structural basis and specificity of acetylated transcription factor GATA1 recognition by BET family bromodomain protein Brd3. *Mol. Cell. Biol.* **2011**, *31* (13), 2632−2640.
(70) Shi, J.; Wang, Y.; Zeng, L.; Wu, Y.; Deng, J.; Zhang, Q.; Lin, Y.; Li, J.; Kang, T.; Tao, M.; Rusinova, E.; Zhang, G.; Wang, C.; Zhu, H.; Yao, J.; Zeng, Y. X.; Evers, B. M.; Zhou, M. M.; Zhou, B. P. Disrupting the interaction of BRD4 with diacetylated Twist suppresses tumorigenesis in basal-like breast cancer. *Cancer Cell* **2014**, *25* (2), 210−225.
(71) Lamonica, J. M.; Deng, W.; Kadauke, S.; Campbell, A. E.; Gamsjaeger, R.; Wang, H.; Cheng, Y.; Billin, A. N.; Hardison, R. C.; Mackay, J. P.; Blobel, G. A. Bromodomain protein Brd3 associates with acetylated GATA1 to promote its chromatin occupancy at erythroid target genes. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108* (22), E159−68.

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