Bromodomain-containing protein 4 (BRD4) is an epigenetic reader and oncology drug target that regulates gene transcription through binding to acetylated chromatin via bromodomains. Phosphorylation by casein kinase II (CK2) regulates BRD4 function, is necessary for active transcription and is involved in resistance to BRD4 drug inhibition in triple-negative breast cancer. Here, we provide the first biophysical analysis of BRD4 phospho-regulation. Using integrative structural biology, we show that phosphorylation by CK2 modulates the dimerization of human BRD4. We identify two conserved regions, a coiled-coil motif and the Basic-residue enriched Interaction Domain (BID), essential for the BRD4 structural rearrangement, which we term the phosphorylation-dependent dimerization domain (PDD). Finally, we demonstrate that bivalent inhibitors induce a conformational change within BRD4 dimers in vitro and in cancer cells. Our results enable the proposal of a model for BRD4 activation critical for the characterization of its protein-protein interaction network and for the development of more specific therapeutics.
BRD4 (BRomoDomain protein 4) is an epigenetic reader belonging to the BET (Bromodomain and Extra-Terminal domain) protein family, which also includes BRD2, BRD3, and BRDT (BRomoDomain protein Testes-specific)\(^2\). BRD4 has key functions in multiple processes including transcriptional regulation\(^1\), DNA damage response\(^6\), and virus maintenance and replication\(^5\). At the basis of most BRD4 functions is the ability to bind to acetylated chromatin through bromodomain 1 (BD1) and bromodomain 2 (BD2) that are located in tandem within the N-terminal part of the protein. These are highly conserved 110 amino acid (aa) domains composed of a bundle of four helices separated by two loops that form a hydrophobic pocket for interaction with mono- or di-acetylated peptides\(^4\). Despite the high sequence identity between the two bromodomains, they recognize different epigenetic marks: BD1 binds in vitro to mono- and multiply-acetylated H4 peptides, while BD2 exhibits promiscuous interaction with both acetylated H3 and H4 histone tails\(^4\) and it can also bind to acetylated transcription factors, such as TWIST1\(^1\). All members of the BET family also contain an extra-terminal domain (ET), which in BRD4 has been demonstrated to interact with multiple binding partners and influence gene transcription\(^6\). Finally, the long isoforms of BRD4 and BRDT display an intrinsically disordered region, shown to form in BRD4 phase-separated droplets at the chromatin to compartmentalize transcription\(^7\), and a conserved C-Terminal Motif that, together with BD2, contributes to activate transcription of targeted genes by recruiting the positive transcriptional elongation factor\(^6\). It has been recently reported that the BRD4 isoform C (aa 1–722), which lacks the long C-terminal intrinsically disordered region and has the last three residues ETA substituted by GPA, is also able to form liquid-like condensates at the nucleus, similarly to the long isoform A (aa 1–1362). The two isoforms seem to modulate the expression of a subset of genes in an opposite fashion\(^10\) and have been demonstrated to locate to distinct nuclear compartments\(^12\).

The discovery that the transcription of c-MYC and other oncogenic genes is regulated by BRD4\(^13\) and that selective inhibition of BET bromodomains with small molecules, JQ1 and I-BET, is effective against various hematological cancers\(^14\)–\(^18\), encouraged further development of BET inhibitors towards the clinic. BET inhibitors act by binding to the acetylated lysine binding pockets of BD1 and BD2 and disrupting interactions with chromatin and transcription factors, thus suppressing transcription of c-MYC and other proto-oncogenes. Although the majority of BET inhibitors bind to both BD1 and BD2, specific compounds targeting either BD1 or BD2 of BET proteins were recently developed\(^19\),\(^20\). Importantly, the efficacy of some of the BET inhibitors against hematological and solid tumors has been demonstrated in pre-clinical studies\(^21\), and they are also of interest in inflammatory and viral diseases. Although the inhibition of BRD4 is likely to be the main target of BET inhibitors, it has to be stressed that these small-molecules bind to all members of the BET protein family and that specific inhibitor of each member has been difficult to identify. Recently, several bivalent BET inhibitors (biBETs) were developed by three distinct groups, which are able to target two bromodomains (BD1 or BD2) simultaneously and show higher potencies and efficacies compared to monovalent counterparts\(^22\)–\(^25\).

Despite the broad therapeutic interest, the molecular details of BRD4 function and regulation are not fully understood. Phosphorylation of BRD4 by casein kinase 2 (CK2) is necessary for active gene transcription and controls the activity of BRD4 by positively regulating its binding to acetylated chromatin, as well as to human p53 and viral E2 transcription factors\(^26\),\(^27\). In addition, hyperphosphorylation of BRD4 has been identified as a resistance mechanism in triple-negative breast cancer against BET inhibition due to an increased p-BRD4-mediated recruitment of the Mediator complex, a multi-protein activator of RNA pol II\(^28\).

As in many targets of CK2, BRD4 harbors multiple highly conserved consensus sites for CK2 phosphorylation (S/TxxE/D, where x is any residue) that are located in two main clusters: one, named N-terminal phosphorylation sites (NPS) downstream of BD2, and another, C-terminal phosphorylation sites (CPS), after the ET domain. A proposed phospho-regulation mechanism involves a conformational switch driven by the NPS\(^29\). It was suggested that the unphosphorylated NPS interacts with BD2 to inhibit chromatin binding. Upon phosphorylation by CK2, NPS was proposed to bind to a lysine-rich region immediately downstream, called BD (Basic-residue enriched Interaction Domain), thereby releasing auto-inhibition of BD2 and allowing chromatin interaction. Although the “phospho-switch” is an elegant and simple model for the regulation of BRD4 activity, there are currently no structural or biophysical reports in its support.

Here, we provide insights into the phospho-regulation of human BRD4. Using an integrative structural biology approach, we demonstrate that BRD4 dimerizes upon phosphorylation of NPS by CK2. We identify BD and a conserved coiled-coil region downstream of the bromodomains as required for dimerization. Finally, we show the effects of biBETs on the BRD4 conformation in vitro and in cellular NanoBRET assays. Guided by our analyses, we propose a revised model for the regulation of BRD4 in which phosphorylation modulates the conformation and oligomeric state of the protein, thus creating a multi-valent platform for co-localization of transcriptional complexes. This work not only provides a key for the interpretation of phospho-regulated protein-protein interactions of BRD4, but it also gives mechanistic insight into the control of BRD4 activity while underlining the importance of biophysical and structural data on physiologically relevant constructs in the understanding of protein functional mechanisms.

Results

Dimerization of BRD4 is driven by phosphorylation and requires the BD region. To dissect the effects of phosphorylation on the structure of BRD4, we used three previously described constructs\(^29\): 1) BRD4\(^{1–530}\), encompassing BD1, BD2, and NPS; 2) BRD4\(^{1–579}\), which further includes BD; 3) BRD4\(^{1–722}\), which additionally spans the ET domain and the CPS region, and comprises the isoform C (Fig. 1a). Attempts to obtain protein samples of isoform A (aa 1–1362), failed due to low expression levels in insect cells and proteolytic instability.

Unphosphorylated proteins were produced by expression in E. coli, while phosphorylated samples were generated by expression in insect cells or by in vitro CK2-mediated phosphorylation. Purified proteins were obtained from both bacteria and insect cells (Fig. 1b) and multiply phosphorylated sites were observed by mass spectrometry from insect cells and in vitro phosphorylated samples (Supplementary Fig. 1 and 2). A single acetylation site was also identified in purified proteins produced from insect cells, but not in proteins from E. coli. Although BRD4 has been reported to be an atypical protein kinase with auto-phosphorylation activity\(^30\), we did not observe any phospho-adducts in the bacterial samples by mass spectrometry and we did not detect any auto-phosphorylation of BRD4 by ADP-β-s assay in the presence of ATP (Supplementary Fig. 3).

Analysis by analytical size-exclusion chromatography (SEC) revealed differences in elution profiles that were construct and phosphorylation dependent: while phosphorylated BRD4\(^{1–530}\) eluted later than the unphosphorylated form, indicating protein compaction, the peaks of both phosphorylated BRD4\(^{1–579}\) and
BRD4<sup>1-722</sup> shifted toward earlier elution volumes, suggesting a phosphorylation-dependent oligomerization or structural elongation (Fig. 1c). Interestingly, the addition of high salt abrogated the effects of phosphorylation on BRD4<sup>1-530</sup>, 1-579, 1-722. c Elution profiles of analytical size-exclusion chromatography (SEC) performed with 20 µM of the indicated constructs in the presence of 250 mM NaCl or 1 M NaCl. Std: Gel filtration standards (Bio-Rad) analysed in the corresponding low or high salt running buffer. d Elution profiles of the indicated constructs analysed by SEC-MALS. Samples are labeled as follows: black line indicates purified from bacteria, blue line indicates purified from insect cells, orange line indicates phosphorylated in vitro using CK2, dotted grey line in panels c, and d indicate protein molecular weight standards. The dotted line at each peak, colored as above, indicates the measured MW. The dotted horizontal grey lines in panel d represent the theoretical MW of the monomer or dimer of BRD4<sup>1-530</sup>, BRD4<sup>1-579</sup>, BRD4<sup>1-722</sup>, calculated from the primary sequence.
Table 1 Summary of the SEC-MALS and AUC sedimentation velocity and equilibrium experiments.

| BRD4 construct State | Theoretical mass (Da) | Mass SEC-MALS (Da) | Equilibrium mass AUC (Da) | Average sedimentation coefficient (S) | f/f₀ * | Mass estimate |
|----------------------|-----------------------|--------------------|--------------------------|--------------------------------------|--------|---------------|
| 1-530 unphos. (bacteria) | 60,512.7 | 81,330 | 62,883 | 2.58 | 2.35 | 64,200 |
| 1-579 CK2 phos. | 60,512.7 | 79,600 | 65,609 | 3.10 | 1.73 | 59,500 |
| 1-579 unphos. (bacteria) | 66,376.5 | 87,130 | 80,511 | 3.13 | 1.97 | 86,100 |
| 1-722 CK2 phos. | 64,335.5 | 129,500 | 91,765 | 4.42 | 2.40 | 152,000 |
| 1-722 unphos. (bacteria) | 82,415.3 | 147,200 | 145,923 | 5.65 | 1.58 | 123,000 |
| 7A CK2 phos. | 82,303.3 | 105,200 | - | - | - | - |
| 6A CK2 phos. | 82,319.3 | 134,600 | - | - | - | - |
| ΔS506–530 insect | 80,404.9 | 91,200 | - | 3.71 | 1.76 | 80000 |
| ΔS506–530 λ-phosphatase | 80,404.9 | 417,200 | - | 2.95 | 2.41 | 82700 |
| 1-722 λ-phosphatase | 88,427.3 | 88,157 | - | - | - | - |
| 1-722 + iBET | 82,415.3 | 84,003 | - | - | - | - |
| 1-722 + AZD5153 | 82,415.3 | 83,858 | - | - | - | - |
| 1-722 + 6 | 82,415.3 | 84,747 | - | - | - | - |
| 1-722 + 7 | 82,415.3 | 86,097 | - | - | - | - |
| 1-722 insect | 80,404.9 | 120,321 | - | - | - | - |
| 1-722 + iBET | 80,404.9 | 117,168 | - | - | - | - |
| 1-722 + AZD5153 | 80,404.9 | 117,258 | - | 3.53 | 1.72 | 78,200 |
| 1-722 + 6 | 80,404.9 | 112,824 | - | 3.45 | 2.19 | 142,000 |
| 1-722 + 7 | 80,404.9 | 117,168 | - | 3.71 | 1.76 | 80000 |

1/f₀: frictional coefficient.

The largest change in deuterium incorporation was found in 6 overlapping peptides in BRD41–579 and in BRD41–722 spanning the region between the NPS and BID (aa 506-527) (Fig. 2a, b and Supplementary Fig. 4). Notably, these peptides exhibit reduced HDX in the phosphorylated proteins compared to the unphosphorylated counterparts, but this only occurred in BRD41–579 and BRD41–722, the two constructs that dimerize upon phosphorylation. In contrast, there was no change in deuterium uptake in the corresponding residues of BRD41–530. The 506-527 region contains three heptad repeats, a characteristic of coiled-coil structures (Fig. 2c). Analysis of the BRD4 sequence using the LOGICOIL coiled-coil prediction algorithm, strongly predicts a coiled-coil structure involving residues 506-527 with an anti-parallel dimer configuration (Supplementary Fig. 5). Moreover, residues 506-527 are part of a region, named “motif B”, conserved among BET proteins (Fig. 2c) that has been proposed to mediate dimerization of BRD2 and other BET proteins, based on yeast 2-hybrid and co-immunoprecipitation data.

Minor reductions in HDX were also observed in three other regions of phosphorylated BRD41–722: aa 65–71 of the helix Z of BD1, aa 184–190 of the linker region immediately downstream of BD1, aa 362–386, which are part of the helix Z and the ZA-loop of BD2, and aa 621–644 and 657–675, which comprise most of the ET domain (Fig. 2a and Supplementary Fig. 6).

All of the HDX changes described above exhibit EX2 kinetics, which occur when the rate of protein refolding from a temporarily unfolded state is much faster than the rate of HDX, resulting in a gradual exchange of hydrogen. On the other hand, if the rate of protein refolding is slower than the rate of HDX, some residues will exchange before the protein returns to the folded state. In the HDX-MS analysis of all protein batches of BRD41–530 and BRD41–722, we identified peptides from the BD2 domain that displayed EX1 kinetics. This was not observed in the phosphorylated forms or in BRD41–579 (Supplementary Fig. 7). The presence of EX1 kinetics suggests that BD2 has a more plastic structure than BD1, and that BD2 may be stabilized in the context of other proteins such as iBET.

Motif B is involved in the phosho-driven structural change of BRD4. To gain insight into the structural rearrangements that occur upon phosphorylation, we compared the unphosphorylated and in vitro phosphorylated BRD4 constructs by Hydrogen-Deuterium eXchange Mass Spectrometry (HDX-MS). For all constructs, we identified a high number of unique peptides, common between the two phosphorylated states, which provided good coverage of BD1, BD2 (≥92%), and ET domains (74.7%) (Supplementary Fig. 4). Coverage of some parts of the BD1-BD2 linker and the BID region was not obtained, most probably due to the hydrophobic or highly positively charged primary sequences. Peptides derived from the NPS and CPS regions were excluded from the analysis, as these were differentially modified in the phosphorylated and unphosphorylated forms.
of the BRD4\textsuperscript{1–579} construct or by the phosphorylation-driven conformational rearrangement of BRD4\textsuperscript{1–530} and BRD4\textsuperscript{1–722}.

**Phosphorylation of BRD4 brings BD, BID, and ET regions in proximity.** To gain further insight into the architecture of the BRD4 dimer, we performed chemical crosslinking followed by MS (XL-MS) of the most relevant physiological form, BRD4\textsuperscript{1–722}. When preparing cross-linked samples, a distinct slower-migrating band with a MW consistent with a dimer (165 kDa) appeared in both unphosphorylated and CK2 in vitro phosphorylated BRD4\textsuperscript{1–722} samples at increasing concentrations of cross-linker (Supplementary Fig. 8). The accumulation of the dimeric band was clearly more marked in the phosphorylated sample. This is consistent with BRD4\textsuperscript{1–722} having a propensity to form a dimer via the coiled-coil motif B that is stabilized only upon phosphorylation, thus resulting in the dimer being the main species present in solution for phosphorylated BRD4\textsuperscript{1–722}. The unphosphorylated monomer and the phosphorylated dimeric species were purified by SEC prior to the analysis of the cross-linked peptides. In total, 69 putative intra-molecular cross-links and 104 inter-molecular cross-links were found, unique to the monomer or dimer, respectively (Fig. 3). These were mainly within or in the proximity of the BD1, BD2, BID, and ET regions, which suggests...
To test the role of motif B in the phospho-driven dimerization of BRD4Δ506-722, we then produced mutant constructs in which residues 506-530 of BRD4Δ506-722 were deleted and replaced with a 12-aa glycine-serine rich flexible linker (Fig. 4e). E. coli expression of BRD4Δ506-722(Δ506-530) was greatly reduced suggesting that deletion of the coiled-coil region impacted protein stability; however, insect cell expression enabled purification of the samples. We then generated the unphosphorylated protein by incubating the insect cell BRD4Δ506-722(Δ506-530) with λ-phosphatase. Mass spectrometry confirmed a substantial reduction in phosphorylation levels (Supplementary Fig. 9). Comparison of the oligomerization state of the phosphorylated and λ-phosphatase-treated BRD4Δ506-722(Δ506-530) by SEC-MALS and AUC revealed that both the samples were present in monomeric state in solution (Fig. 4f, Table 1). Interestingly, the analysis of the frictional and sedimentation coefficients indicates a large conformational difference between the two samples, suggesting a more compact shape of BRD4Δ506-722(Δ506-530) when phosphorylated (Table 1). This molecular rearrangement resembles the one observed for the BRD4Δ506-722 dimer and indicates that phosphorylation is triggering a molecular compaction regardless of the change in oligomerization.

In summary, the Δ506-530 mutant protein analysis confirms that motif B is required for BRD4 dimerization and that the formation of the coiled-coil interface is essential for the stability of the BRD4Δ506-722 dimer.

BRD4 dimerization is detected in HCT116 cells by NanoBRET.

The results of the in vitro biophysical analysis prompted us to test BRD4 dimerization in human cancer cells. We performed bioluminescence resonance energy transfer (NanoBRET) in HCT116 cells by transiently expressing two constructs of BRD4Δ506-722 fused to either a Nanoluminase tag (NanoLuc, donor) or a Halo-tag (acceptor) (Fig. 5a). A NanoBRET signal was observed in the presence of the BRD4Δ506-722 NanoBRET pair (Fig. 5b, c), in contrast to control (Halo-tag only and NanoLuc-BRD4Δ506-722) indicating that dimerization of BRD4Δ506-722 occurs in this cellular context. It is worth to note that a higher NanoBRET signal was observed when the NanoLuciferase and the Halo-tag were added at the N-ter and C-ter of the molecules, respectively, consistent with an antiparallel arrangement of the dimer. The specificity of BRD4Δ506-722 dimerization was confirmed in a saturation binding experiment with increasing concentrations of acceptor expression plasmid DNA (Fig. 5d), and in a competition experiment, in which untagged BRD4Δ506-722 expression plasmid DNA was titrated against a constant amount of BRD4Δ506-722 acceptor/donor pair plasmid DNA, leading to a reduction of the NanoBRET signal (Fig. 5e).

These results support the biophysical driven hypothesis that BRD4 dimersizes in cells.

Effects of the binding of bivalent BET inhibitors to BRD4 conformation.

We recently described a series of highly potent biBETs that simultaneously bind tandem bromodomains.23,33 (Supplementary Fig. 10). These compounds were found to efficiently displace both BRD4 isoform A (1–1362) (FL) and isoform C (1–722) from histone H3 in NanoBRET assays with a much lower IC50 than the monovalent I-BET (Fig. 6a). We asked whether modulation of BRD4 oligomeric state might be relevant in the mechanism by which biBETs achieve exceptional potency in cell assays. Using our NanoBRET assay, we observed a concentration-dependent increase in BRD4Δ506-722 BET signal after addition of bivalent compounds, which was not detected with the monovalent I-BET (Fig. 6b), reflecting either an increase in oligomerization or a conformational change that brings the
NanoBRET donor and acceptor into closer proximity. Interestingly, for all but one of the bivalent compounds, the EC\textsubscript{50} is >10-fold higher than the IC\textsubscript{50} required to inhibit BRD4\textsubscript{1–722} binding. AZD5153 is the exception, which may suggest a different mode of inhibition relative to the other bivalent compounds. In order to differentiate between an increased oligomerization or a conformational change induced by iBETs, we performed SEC-MALS experiments on BRD4\textsubscript{1–722} in the presence of compounds (Fig. 6c, d). We tested both the insect cell-expressed BRD4\textsubscript{1–722}, and its dephosphorylated monomeric form. Although SEC-MALS indicates a mixed population of dimer and monomer BRD4\textsubscript{1–722} before dephosphorylation, all biBETs shift the BRD4 peak to a faster migrating population without change in molecular weight, whereas I-BET failed to shift the peak. The same was observed for the dephosphorylated BRD4 monomer. Our results indicate that biBETs induce conformational compaction in BRD4 with no effect on oligomeric state. This is consistent with previously observed biBET induced compaction of BRD4\textsubscript{41–460} (BD1-BD2 tandem domain) in SAXS and AUC studies\textsuperscript{23}.

**Discussion**

In this study, we provide a comprehensive biophysical and structural analysis of a series of constructs of human BRD4, including BRD4\textsubscript{1–722}, which represents the isoform C. Using SEC-MALS and AUC, we observed that BRD4\textsubscript{1–579} and BRD4\textsubscript{1–722}, but not BRD4\textsubscript{1–530}, which lacks the BID domain,
dimerize after phosphorylation by CK2. Dimerization of BRD4\(^{1-722}\) was also observed in mammalian cancer cells using NanoBRET assays. Based on the increased protection identified by HDX-MS, and on our mutation studies, we demonstrated that BRD4\(^{1-722}\) dimerization requires a coiled-coil region (aa 506-530) within motif B, BID, and phosphorylation of NPS. We propose a model for the phosphorylation-driven conformational change of BRD4 (Fig. 6e). In this model, unphosphorylated BRD4 is a monomer; however, upon phosphorylation by CK2, BRD4 forms a stable homodimer through an interface comprising the phosphorylated NPS, coiled-coil motif B and BID. Guided by I) the overall topology of the dimer obtained by XL-MS, II) the coiled-coil oligomeric state prediction by LOGICOIL and III) the previous observation with isolated domains that phosphorylated NPS binds to BID\(^{29}\), we propose a head to tail conformation of the dimer. In this conformation, the negatively charged phosphorylated NPS of one monomer contacts the positively charged BID of the other monomer, thus stabilizing the coiled-coil interaction. Although our data are based on the analysis of the BRD4 isoform C, the same phospho-regulated dimerization can be envisioned also for the long isoform A, which shares the same residues, including the regions forming the dimer interface. The biophysical and structural analysis of phosphorylated BRD4\(^{1-722}\) also suggests a compact shape for the dimer in which the bromodomains and the ET domain are in proximity to each other. This configuration would therefore create a multi-valent platform that brings protein ligands of BD2 and ET to the chromatin. Additional structural studies employing cryo-EM analysis of BRD4 in complex with binding partners and chromatin will be necessary to confirm this hypothesis.

The conformational phospho-switch model of BRD4 proposed by Wu et al.\(^{1}\) is based on an autoinhibitory activity of NPS toward BD1 and BD2, released by its phosphorylation and intramolecular interaction with BID\(^{29}\). In our HDX-MS analysis, we did not observe an increase of HDX in BD1 or BD2 upon phosphorylation, as would be expected from the phospho-switch model due to release of autoinhibitory interactions. On the contrary, we observed a small HDX reduction in several peptides of BD1 and BD2, but only in BRD4\(^{1-722}\). Interestingly, the peptides of BD2 with reduced HDX are located in the ZA-loop and involve the WPF (W374 P375 F376) shelf important for creating the hydrophobic pocket hosting the acetylated lysines (Supplementary Fig. 6). The intra-molecular interactions, established upon BRD4\(^{1-722}\) phosphorylation and dimerization, may therefore stabilize BD2 for binding to chromatin, positive transcriptional elongation factor b or transcription factors. This hypothesis is further supported by the EX1 kinetics of HDX observed in BD2, which suggests an inherent plasticity of this specific bromodomain, with greater propensity to unfold in the unphosphorylated state.

Using phospho-deficient mutants in either NPS (7A mutant) or CPS (6A mutant), we identified NPS as a regulator of BRD4 dimerization. We did not observe any effect of phosphorylation of CPS on BRD4 structure in our biophysical analyses (Fig. 4), however this does not rule out a role for CPS in modulating interactions with other co-regulatory proteins. We have therefore identified a phosphorylation-dependent dimerization domain (PDD) in BRD4, spanning residues 484–579 and comprising NPS, coiled-coil motif B, and BID regions (Fig. 6e).

The coiled-coil interface of the BRD4 dimer is located within motif B, a region that was shown to be required for BD2-chromatin binding in mitosis and was proposed to be a universal dimerization motif in BET proteins\(^{32}\). Our structural, biophysical and cellular analysis on BRD4 not only supports this hypothesis but also shows that dimerization is modulated by CK2-mediated phosphorylation of NPS and suggests a dynamic equilibrium between monomer and dimer. The NPS region of BRD2 has an insertion of around 13 negatively charged residues relative to

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**Fig. 5 NanoBRET analysis of BRD4 dimerization in cells.**

(a) Schematic summary of the NanoBRET assays to test BRD4 homo-dimerization in HCT116 cells. The curved black arrow represents Bioluminescence Resonance Energy Transfer (BRET) between the Nanoluciferase donor (blue circle) and the Halo-tag acceptor ligand (orange circle). (b) Overview of the NanoBRET transfected constructs used in the NanoBRET experiments, with BRD4\(^{1-722}\) tagged at the N-terminus with Nanoluciferase, and at the N-terminus or at the C-terminus with Halo-tag. (c) NanoBRET signal observed using NanoLuc-BRD4\(^{1-722}\) and Halo-BRD4\(^{1-722}\) (dark grey bar) or NanoLuc-BRD4\(^{1-722}\) and BRD4\(^{1-722}\)-Halo (grey bar). The single measurements and the mean with SD are reported (n = 8 for samples and n = 4 for control independent experiments). (d) Titration NanoBRET experiments where increasing amounts of acceptor DNA (BRD4\(^{1-722}\)-Halo) were transfected with a fixed amount of donor DNA (Nanoluc-BRD4\(^{1-722}\)). The mean with the standard error of the mean (SEM) is reported (n = 4 independent experiments). (e) Competition NanoBRET experiments where increasing amount of untagged BRD4\(^{1-722}\) DNA were transfected with a fixed amount of donor and acceptor DNA pair (Nanoluc-BRD4\(^{1-722}\) and BRD4\(^{1-722}\)-Halo). The reduction of the NanoBRET signal at increasing values of untagged/Halo-tagged BRD4\(^{1-722}\) ratio indicates a specific competition of the untagged protein towards BRD4\(^{1-722}\)-Halo for binding to Nanoluc-BRD4\(^{1-722}\). The mean with SEM is reported (n = 4 independent experiments).
BRD4 and fewer CK2 consensus phosphorylation sites, perhaps suggesting that BRD2 dimerization may not require phosphorylation. Our AUC data also demonstrate that the isolated BRD4 bromodomains are monomeric, whereas BRD2 BD1 has been shown to form stable dimers, indicating differences in the nature or affinity of the dimer contacts in BRD4 and BRD2.

It is interesting to note that many reported BRD4 binding partners are oligomers: p53 (tetramer) binding to BD1, viral latency-associated nuclear antigens kLANA and mLANA (both the dimers and higher oligomers) binding to ET, histone H3 and H4 (two copies of which are included in the octameric nucleosome), binding to BD1 and BD2. Dimerization of BRD4, and the resulting spatial proximity of four bromodomains, two ET domains, and two BIDs, could therefore lead to the right architecture not only for binding to multiple acetylated histone tails of the same or adjacent nucleosome, but also for the efficient recruitment of the other interacting oligomers.

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The BRD4 isoform C forms liquid-like phase separations (LLPS) at the nucleus involved in active gene transcription similar to those previously reported for the long isoform A. LLPS and DNA binding was inhibited by phosphorylation via CK2 and LLPS were also inhibited by the addition of a bivalent inhibitor, but not by JQ1. Phosphorylation of BRD4 therefore promotes dimerization and inhibits interaction with DNA and formation of LLPS, while being necessary for active gene transcription. The apparently conflicting effects of phosphorylation in promoting gene transcription, while reducing LLPS in chromatin, may suggest that phosphorylated and unphosphorylated BRD4 form different molecular associations in LLPS. The transient polyvalent self-associations of unphosphorylated BRD4 LLPS contrast with the stable dimeric interaction of phosphorylated BRD4. The level of BRD4 phosphorylation may therefore modulate the structural and ligand binding properties of the BRD4 LLPS to decrease DNA binding and enhance interaction with transcriptional...
complexes, while maintaining interaction with acetylated histones so that when levels of BRD4 phosphorylation rise above a certain threshold transcription is triggered at gene loci. This suggests that phosphorylation and dephosphorylation of BRD4 mediates a dynamic interplay between DNA binding and dimerization to regulate transcription.

In the last few years, potent and selective inhibitors targeting the bromodomains of BET proteins have been developed. The biochemical and structural assays guiding drug design were mainly based on truncated constructs comprising BD1, BD2 or BD1-BD2. In BRD4, the finding that regions downstream of BD2 are involved in phospho-regulation of binding to chromatin and other interacting partners, and in the development of resistance against BETs, underlines the need to employ more physiologically relevant constructs in biochemical and biophysical studies to better resemble the native context.

Our NanoBRET experiments in HCT116 cells and SEC-MALS data indicated a conformational change of BRD4 upon addition of bivalent compounds, which target two bromodomains simultaneously (Fig. 6). We previously showed that biBETs afford an increased affinity in cell assays over corresponding monovalent inhibitors of up to four orders of magnitude and that biBETs are capable of engaging both bromodomains in BD1-BD2 constructs simultaneously. Here, we confirm that biBETs are able to induce a protein rearrangement of BRD4, independently of its oligomeric state.

Our results show that phospho-dependent BRD4 dimerization brings BD2 and ET domains into proximity, suggesting that a bivalent strategy, similar to that of biBETs, might be a viable approach to simultaneously target BD and ET domains, thus influencing coregulator interactions and perhaps resulting in inhibitors with different pharmacological and safety profiles. The NMR-derived structure of the ET domain was recently solved with a peptide ligand suggesting the ET domain as a target for small-molecules inhibitors. Bivalent strategies may be of wider value in targeting phase-separated condensates where the high concentrations of multi-domain proteins with flexible intrinsically disordered regions may provide enhanced avidity for binding.

In conclusion, our study provides an important contribution to the understanding of the molecular details of BRD4 function, and a refined model for BRD4 activation and inhibition that can be employed in drug discovery for the development of more specific, effective and safe therapies and suggests a general strategy for therapeutic targeting of epigenetics by exploiting avidity to achieve potency and selectivity in binding.

Materials and methods

Recombinant protein production. Variants of human BRD4 were generated by gene synthesis (GeneArt, Life Technologies) with an N-terminal 6xHis/6xHsHd (6xHis) tag or 6xHis/6xHis-Halo tag followed by tobacco etch virus (TEV) protease site. For bacterial expression, constructs were subsequently cloned into a plasmid vector and transformed into Escherichia coli (E. coli) BL21 Gold (DE3) strain (Novagen). Protein expression was induced at 0.6–0.8 OD$_{600}$ with 0.1 mM IPTG and sustained overnight at 18 °C. For insect cell expression, constructs were cloned into a baculovirus expression vector (pFastBac1 and bacmid DNA) and expressed in Sf21 cells (ThermoFisher) and protein expression was conducted at 27 °C for 48 h. Cells were lysed using a Standard Cells system cell disruptor in 50 mM Hepes pH 8.0, 300 mM NaCl, 5 mM Tris(2-carboxyethyl) phosphate hydrochloride (TCEP), 10 mM MgCl$_2$, 10% Glycerol, 1.5 mM DTT and 1 Complete EDTA-free protease inhibitors (Roche) and benzamide (5 mM, Sigma). For insect cell samples, 1x Hal1 phosphatase inhibitors (Thermo Fisher Scientific) and 1x Complete EDTA-free protease inhibitors (Roche), and benzamide (5 mM, Sigma). For insect cell samples, 1x Hal1 phosphatase inhibitors (Thermo Fisher Scientific) and 1x Complete EDTA-free protease inhibitors (Roche) were added. Cells were clarified by centrifugation at 43260 rcf for 45 min at 4 °C, the supernatant was loaded onto a HitTrap Heparin column (GE Healthcare) and eluted with a 0.3–1 M NaCl linear gradient. Fractions containing CK2α1–335 were diluted to a final NaCl concentration of 100 mM, loaded onto Resource Q (GE Healthcare) and eluted with a 500–5000 mM NaCl linear gradient. Protein fractions were further purified by SEC with a HiLoad 16/600 Superdex 75 column (GE Healthcare) in 25 mM Tris pH 8.5, 500 mM NaCl, 1 mM DTT.

In vitro phosphorylation of BRD4 by CK2. During purification, ion-exchange fractions containing bacterial BRD4 constructs were pulled together and diluted 1:2 into a final reaction including 50 mM Tris pH 7.5, 10 mM MgCl$_2$, 0.1 mM EDTA, 2 mM DTT, 500 µM ATP, 0.5x Complete EDTA-free protease inhibitors (Roche), 0.5X Hal1 phosphatase inhibitors (Thermo Fisher Scientific) and CK2α1-335 with a protein:kinase ratio of 15:1 (w/w). The reaction was incubated overnight at 4 °C, supplemented with 500 µM ATP and further incubated at 30 °C for 2 h. Phosphorylated BRD4 constructs were isolated by anion-exchange with a Resource Q column and further subjected to SEC using Superdex 200 10/300 GL column in storage buffer (10 mM Tris pH 8.6, 500 mM NaCl and 1 TCEP).

Intact mass spectrometry analysis. Samples were desalted and concentrated with 0.5 mL Millipore Amicon Ultra cut-off filters (UF505008, UFC503024) in a refrigerated centrifuge (4 °C). The mobile phase used for gradient elution consisted of (A) 0.1% formic acid (Fluka 5630-10XML-F) in water (JT Baker, 4128-02) and (B) 0.1% formic acid in Acetonitrile (JT Baker, TJ9017-2). The LC/MS system used a Shimadzu Prominence HPLC with a Agilent C8 column (Poroshell StableBond 300 C8, 2.1 × 75 mm, 5 µm) at 500 µl/min flow rate with a gradient consisting of 1 min at 20% B, then ramp to 95% B over 4 min, then hold for 1 min at 95% B before returning to 20% B. Mass spectra (LC/MS) were acquired on a Scieq 5600 TripleTOF+ mass spectrometer (Foster City, CA) using Analyst 1.6 software (Foster City, CA). Source temperature was 450 °C, spray voltage (ESVe) was 5500 V, curtain gas was 30, GS1 = 60, GS2 = 70, and data were acquired over 1000–4000 Da mass range. Protein peak reconstruction (charge state deconvolution) used the BioToolKit Microapp v2.2 in the Sciex PeakView 2.2 software.

ADPglc assay. Luminescent ADP detection assay was performed using the ADPglc kit (Promega). Eleven serial two-fold dilutions of BRD4–BD2 start from 85 µM were prepared in assay buffer (50 mM Tris pH 7.5, 10 mM MgCl$_2$, 0.1 mM EDTA, 2 mM TCEP). For the reaction, 2 µl of each BRD4 dilution was mixed with 0.6 µM CK2α1–335 and 0.2 mM Ultra Pure ATP (Promega) in a final volume of 5 µl and incubated at RT for 1 h. The ADPglc reagents were added as described in the ADPglc kit protocol. Luminescence was quantified using the Envision 2014 plate reader (Perkin Elmer) and analysed with Prism (GraphPad).

Analytical SEC. For each protein sample, 25 µl at 20 µM was injected into a Superdex200 50.10/300 GL column (GE Healthcare) equilibrated with 10 mM Tris pH 7.5, 250 mM NaCl, 1 mM TCEP. For high salt analysis, a buffer containing 10 mM Tris pH 7.5, 1 M NaCl, 1 mM TCEP was used.

Analytical ultracentrifugation. All analytical ultracentrifugation experiments were performed on either a Beckman XL-A or XL-I ultracentrifuge. Sedimentation equilibrium experiments were performed using 6-sector cells with 110 µl of sample in 10 mM Tris·HCl pH 7.5 with either 250 mM or 1 M NaCl. Samples of between 0.1 µM and 15 µM were centrifuged at speeds of 9,000, 13,000, and 20,000 rpm for 15-hours where equilibrium was attained and scanned using wavelengths of 230 nm and 280 nm. Data was selected based on appropriate absorbance and speed and analysed using HeteroAnalysis developed by James Cole and Jeffrey Lary, Version 1.1.37 using a single species model and floating the buoyant molecular weight. Sedimentation velocity was used to ascertain the sedimentation coefficients in both 250 mM NaCl and 1 M NaCl. Sedimentation velocity experiments were performed using a An50Ti rotor and standard 2-sector Epon centriplexes and quartz windows. Samples were diluted to loading in 1 M NaCl and referenced with the corresponding buffer. The ultracentrifuge was run at a speed of 45,000 rpm collecting scans at 280 nm until full sedimentation had been reached. Samples were analysed using the program Sedfit developed by Peter Schuck. Sedimentation coefficient distributions were corrected to standard conditions using the buffer density and viscosity correction. The partial specific volume was set to 0.73 cm$^3$/g.

SEC-MALS. In order to characterize BRD4 oligomeric state, size-exclusion chromatography coupled to multi-angle light scattering was used to ascertain the weight-average mass of particles eluting from a gel filtration column using a Wyatt
Hydroxy Succinimide (NHS) ester BS3 (H2/D2O) purchased from Creative Molecules (Canada) at a concentration of 0.058 mg/ml.

**Cross-linking mass spectrometry (XL-MS).** Purified solutions of both unphosphorylated and CK2 in vitro phosphorylated BRD4-722 were diluted to a concentration of 0.082 mg/ml in 20 mM HEPES pH 7.8, 250 mM NaCl, and 1 mM DTT and cross-linked using a homobifunctional, isotopically-coded N-...
experimental samples at a final concentration of 10 μM. Plates were read within 10 min using a PheraStar F5 multimode plate reader (BMG Labtech) equipped with a NanoBRET filter module (excitation 450 nm, emission 610 nm-LP). The results were reported as milliBRET units (acceptor emission value 450 nm) × 1000. Data were fitted with Prism (GraphPad), using the following equations: one-site-total non-linear equation for the titration experiment 
\[ Y = \frac{B_{\text{max}}}{1 + 10^{(\log_{10} c_{\text{IC}_{50}} - X)}} \times (K_{d} + X) + N \] , where \( B_{\text{max}} \) is the maximum binding constant, \( K_{d} \) is the equilibrium binding constant, \( N \) is the slope of non-specific binding; variable four-parameter curve fit for testing effects of the compounds on BRD4 dimerization or BRD4-H3 interaction 
\[ Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + (10^{(\log_{10} c_{\text{IC}_{50}} - X)}}] \times (K_{d} + X) + N \] , where \( c_{\text{IC}_{50}} \) is EC50, \( c_{\text{IC}_{50}} \) is IC50, and \( K_{d} \) is dissociation constant.

Statistics and reproducibility. The details about experimental design and statistics used in different data analyses performed in this study are given in the respective sections of results and methods. For the HDX-MS experiments using BRD4^1-330, BRD4^1-279, and BRD4^1-726, different biological samples were analyzed comprising protein from different purification batches.

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Author contributions
M.S.B.M., A.J.B. and F.M. designed the project. F.M., T.A.J., X.G., J.P.D., G.D. designed, conducted the experiments, and analysed the data. C.J.S., I.L.D. and J.M.S. contributed with the experiment design and data analysis. F.M. and M.S.B.M. wrote the manuscript with the help of all authors.

Competing interests
F.M., C.J.S., I.L.D., X.G., J.P.D. and M.S.B.M. are or have been employees of AstraZeneca and may have stock/stock options in AstraZeneca. T.A.J., G.D, J.M.S. and A.J.B declare no competing interests.

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