Transcriptional addiction in cancer cells is mediated by YAP/TAZ through BRD4

Francesca Zanconato*[1], Giusy Battilana*[1], Mattia Forcato[2], Luca Azzolin[1], Andrea Manfrin[1], Letizia Filippi[1], Erika Quaranta[1], Daniele Di Biagio[1], Gianluca Sigismondo[3], Vincenza Guzzardo[4], Pascale Lejeune[5], Bernard Haendler[5], Jeroen Krijgsveld[3], Matteo Fassan[4], Silvio Bicciato[2], Michelangelo Cordenonsi[1§♯] and Stefano Piccolo[1§♯]

[1] Department of Molecular Medicine, University of Padua School of Medicine, Viale Colombo 3, 35126 Padua, Italy

[2] Center for Genome Research, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via G. Campi 287, 41100 Modena, Italy

[3] German Cancer Research Center (DKFZ) and Heidelberg University, Im Neuenheimer Feld 581, 69120 Heidelberg, Germany

[4] Department of Medicine (DIMED), Surgical Pathology and Cytopathology Unit, Via Giustiniani 2, 35126 Padua, Italy

[5] Bayer AG, Drug Discovery, Müllerstr. 178, 13353 Berlin, Germany

§ To whom correspondence should be addressed

*These authors contributed equally to the work

♯Co-last authors

Mail to:
Stefano Piccolo
Department of Molecular Medicine, Univ. of Padua School of Medicine,
viale Colombo 3, 35100 Padua, Italy
TEL 0039049 8276098
FAX 0039049 8276079
piccolo@bio.unipd.it

ABSTRACT

Cancer cells rely on dysregulated gene expression. This establishes specific transcriptional addictions that may be therapeutically exploited. Yet, the mechanisms ultimately responsible for these addictions are poorly understood. Here we show that the transcription factors YAP and TAZ mediate transcriptional dependencies of cancer cells. YAP/TAZ physically engage the general coactivator BRD4, dictating the genome-wide association of BRD4 to chromatin. YAP/TAZ flag a large set of enhancers with super-enhancer-like functional properties. YAP/TAZ-bound enhancers mediate recruitment of BRD4 and Pol II at YAP/TAZ-regulated promoters, boosting expression of a host of growth-regulating genes. Treatment with small molecule inhibitors of BRD4 phenocopies the effects of YAP/TAZ inactivation and causes an epigenetic reprogramming of cancer cells that leads to regression of pre-existing early neoplastic lesions and reverts drug resistance. This work sheds light on essential mediators, mechanisms and genome-wide regulatory elements responsible for transcriptional addiction in cancer and lays the groundwork for a rational use of BET inhibitors according to YAP/TAZ biology.

An emerging paradigm in cancer biology relates to the concept of "transcriptional addiction": it posits that, to support their uncontrolled proliferation or other needs, tumor cells set high demands on transcriptional regulators, including chromatin regulators and even the basal transcriptional machinery (*1, 2*). The molecular mechanisms underlying the transcriptional dependency of cancer cells are poorly understood. Yet, it is an appealing concept, as general chromatin regulators/transcriptional cofactors are amenable to inhibition with small-molecules (*2*); thus, identifying the most sensitive nodes of these regulations offers the potential of defining new targets and therapeutics with selective antitumor effects. The emblematic example is the antitumor activity of BET inhibitors in various xenograft model systems and clinical trials (*3-6*). BET inhibitors oppose the activity of BET (Bromodomain and Extraterminal)-coactivators (that is, BRD4 and its related factors BRD2 and BRD3) (*6*). Although BET proteins have been proposed to serve as general regulators of RNA polymerase II (Pol II)-dependent transcription, genome-wide studies have instead shown that BET inhibitors display selective effects on gene expression (*6, 7*). In particular, BET inhibitors have been reported to have disproportional effect on a set of highly expressed genes associated with large enhancer regions operationally defined by exceptionally high levels of BRD4 and other chromatin marks (i.e., super-enhancers) (*6, 7*). The molecular basis of the transcriptional addiction associated to super-enhancers in cancer cells, as well as the determinants of the selectivity of BET inhibitors remain undefined (*8*). It is unclear whether specific transcription factors (TFs) are responsible for engaging BRD4 at these sites, and also to what extent the effect of BET inhibitors in cancer can be ascribed to a very restricted number of super-enhancers and their controlled genes (*6*).

The transcription coactivators YAP/TAZ are ideal candidates to mediate cancer-specific transcriptional addictions. In fact, YAP/TAZ are genetically dispensable for homeostasis in many adult tissues; in contrast, once activated by injury or oncogene-induced responses, YAP/TAZ play fundamental roles in

tissue regeneration and cancer in those same tissues (*9*). In line, YAP/TAZ activation is a hallmark of many human malignancies and, in mouse models, YAP and/or TAZ hyperactivation is instrumental to install a robust gene-expression program driving cell proliferation and cell fate plasticity (*9-11*). Once YAP/TAZ enter the nucleus, they control gene expression by binding mainly to distal enhancers that become physically juxtaposed to target promoters through chromatin looping (*12*). Yet, large gaps of mechanistic understanding remain on how YAP/TAZ regulate gene expression, which are their co-factors on chromatin, and to what extent the tumor reliance on YAP/TAZ may in fact coincide with tumor transcriptional dependencies. Filling these gaps is relevant to develop effective and specific antitumor approaches.

With this background in mind, we started this investigation by carrying out ChIP-MS for endogenous YAP/TAZ, a procedure that allows studying the composition of the native protein complexes entertained by YAP/TAZ, and in particular nuclear interactions. As in conventional ChIP protocols, we used formaldehyde crosslinking and performed immunoprecipitations with anti-YAP or anti-TAZ antibodies from sonicated lysates of MDA-MB-231 cells. Proteins captured in YAP/TAZ complexes were identified by MS (*13*). We detected some well-known nuclear partners of YAP/TAZ, including TEAD (the main YAP/TAZ DNA interacting partner) and Activator Protein 1 family members (*12*) and several subunits of the Swi/Snf complex (*16*). YAP/TAZ protein complexes were also enriched in chromatin readers/modifiers, such as BRD4, histone acetyltransferases (p300, p400) and the histone methyltransferase KMT2D/MLL2 (Table 1). The roles of p300, SWI/SNF and the H3K4 methyltransferase complexes in the context of YAP-dependent transcription have been previously described (*14-16*). The association with BRD4 attracted our attention, as this hinted to a connection between YAP/TAZ regulated gene expression and the transcriptional addiction of cancer cells.

In order to validate the interactions detected by Chip-MS, we performed co-immunoprecipitation of endogenous proteins with anti YAP/TAZ and anti-BRD4 antibodies from nuclear lysates of MDA-MB-231 cells. As shown in Fig. 1A, Western blot revealed the presence of BRD4 and TEAD1 in YAP and TAZ immunocomplexes, and of YAP, TAZ and TEAD1 in BRD4 immunocomplexes. We also transfected epitope-tagged BRD4 in HEK293T cells and performed proximity ligation assays (PLA), showing that exogenous BRD4 interacts with endogenous YAP, TAZ and TEAD1 in the nucleus (Fig. 1B). Overall, data indicate that YAP, TAZ, TEAD1 and BRD4 are part of the same nuclear multiprotein complex.

To study the connection between YAP/TAZ and transcriptional addiction in cancer, we used MDA-MB-231 cells, a well-established model of triple-negative breast cancer (TNBC), a tumor type that does not only feature YAP/TAZ prominently in its biology (*9, 12, 17*), but also requires high-levels of uninterrupted transcription of large set of genes to sustain its particularly aggressive nature (*18, 19*). Are YAP/TAZ causal to these dependencies? We first addressed this question by comparing the transcriptional profiles (obtained by RNA-seq) of control and YAP/TAZ-depleted cells, to identify genes activated by YAP/TAZ in MDA-MB-231 cells (figs. S1A-B). Notably, YAP/TAZ target genes were significantly more expressed than all the other genes (fig. 1C). Moreover, the transcriptional profile of control cells revealed that genes whose biological function is associated with cell proliferation (~1500 genes, as determined by Gene Ontology (GO) annotation) were transcribed at higher levels compared to the bulk of expressed genes (fig. 1D); silencing YAP/TAZ with siRNAs led to a global downregulation of such "growth program" (fig.1D), in line with the previous report that MDA-MB-231 cells depleted of YAP/TAZ undergo growth arrest (*12*). Indeed, 37% of the growth genes actively transcribed in these cells are YAP/TAZ targets (541/1449); their transcripts were particularly abundant, displaying higher level of expression than non-YAP/TAZ targets associated to

the same biological function (fig. S1C). Thus, the activation of essential growth genes in MDA-MB-231 cells relies on YAP/TAZ.

To assess if the interaction with BRD4 is causal for the activation of YAP/TAZ transcriptional targets, we performed RNA-seq in cells treated with JQ1, the most established BET inhibitor; JQ1 occupies the bromodomain pockets of BET proteins in a manner that is competitive with the binding to acetylated histone tails, causing their displacement from chromatin (*3*). Most YAP/TAZ regulated genes (68%) displayed exquisite sensitivity to JQ1 (fig. 1E). Indeed, treatment with JQ1 selectively decreased the transcript abundance of YAP/TAZ target genes, compared to all other active genes (figs. 1F and S1D). Moreover, the genes most effectively downregulated by JQ1 were in fact YAP/TAZ targets (fig. S1E). The bias of JQ1 towards inhibition of YAP/TAZ transcriptional targets was also evident when restricting the analysis to genes regulating cell proliferation (figs. S1F-G): BET-inhibition affected the expression of 604 genes associated to GO terms linked to cell proliferation, and 428 of these (71%) were YAP/TAZ-targets. Actually, the list of JQ1-sensitive YAP/TAZ-targets included essential factors involved in replication licencing, DNA synthesis and repair (for example, CDC6, GINS1, MCM3, TOP2A, RAD18 and many others); transcriptional regulators of the cell cycle (E2F2, E2F3, MYBL1 and others); cyclins and their activators (CCNA2, CCNE1, CDC25A); and factors required for mitosis (KIF23; CENPF; CENPV; CDCA5: CDCA8 and others). Thus, sensitivity of a broad number of growth-regulating genes to BET inhibition relies on YAP/TAZ. Effects similar to those of JQ1 were obtained with another BET inhibitor (OTX015, figs. 1H-I and S1G and S1K) and by knocking down BRD2/3/4 with two independent combinations of siRNAs (figs. 1E-F and S1H). Moreover, depletion of the sole BRD4 was sufficient, at least in part, to downregulate YAP/TAZ target genes (fig. S1I). We also found that endogenous YAP/TAZ remained nuclear upon treatment with BET inhibitors (fig. S1J), excluding the possibility that the compounds would indirectly cause YAP/TAZ cytoplasmic

relocalization. Consistent with the notion that YAP/TAZ act upstream of BRD4, exogenous YAP could not rescue the expression of YAP/TAZ target genes in BET-inhibited cells (figs. 1G and S1K). YAP/TAZ transcriptional control has been connected to CDK9-induced elongation of nascent transcripts by Pol II (*20*). Here we find that, in stark contrast with BET inhibitors, inhibition of transcriptional CDKs with flavopiridol or THZ1 failed to display any bias towards inhibition of YAP/TAZ transcriptional targets (figs. 1H-I and S1L). Collectively, the data indicate that the physical association between YAP/TAZ and BRD4 is functionally relevant; BRD4 is a required cofactor for YAP/TAZ, conferring to YAP/TAZ target genes a specifically high dependency on BRD4 and vulnerability to BET inhibitors.

Next, we evaluated the effect of BRD4 silencing on YAP-induced cell transformation. For this, we performed a colony formation assay in soft agar with mammary epithelial cells (MCF10A), which are per se unable to seed colonies, but acquire this capacity upon overexpression of YAP5SA (a constitutively-active version of YAP). MCF10A+YAP5SA were transduced with lentiviral vectors encoding doxycycline-inducible shRNA targeting BRD4 (two independent interfering sequences). Downregulation of BRD4 reduced the number of colonies initiated by YAP-overexpressing cells (figs. 2A and S2A). In line, addition of JQ1 to culture medium potently impaired colony formation (fig. 2B). To assess whether BET inhibition could block colony growth, and not just colony initiation, we started treatment with JQ1 1 or 2 weeks after seeding MCF10A+YAP5SA cells in soft agar, i.e., when colony outgrowth had already started. In this set-up, BET inhibitors prevented any further expansion of colonies (fig. 2C). Similar results were also obtained in MDA-MB-231 cells, whose colony forming capacity depends on endogenous YAP/TAZ (figs. S2B-C).

Extending the translational significance of these findings, we assayed whether inhibition of BET proteins could blunt the growth of, if not cure, YAP/TAZ-addicted mammary tumors in vivo. We modelled this by using mice bearing activation of the Wnt signalling cascade in the mammary gland, by crossing conditional *Apc* alleles with the MMTV-Cre driver. Indeed, constitutive activation of the Wnt cascade has been shown to induce TNBC-like tumors in mice (*21*), a finding that parallels the high frequency of APC epigenetic silencing in human TNBC (*22*). YAP/TAZ are potently activated by aberrant Wnt signalling (*23*) and, consistently, *MMTV-Cre;Apc*$^{fl/fl}$ mice exhibited massive YAP stabilization (fig. 2E). By 8 weeks of age, *MMTV-Cre;Apc*$^{fl/fl}$ mice displayed massive overgrowth of the mammary epithelium, with panductal and panlobular atypical hyperplasia and fibrosis (fig. 2D and S2D). By staining with luminal and basal keratines (K8 and K14, respectively), we detected expansion of the luminal layer and large discontinuities in the basal/myoepithelial layer (figs. 2E, 2G and S2E), collectively configuring a preneoplastic/early neoplastic scenario. Strikingly, all these lesions did not develop in *MMTV-Cre;Apc*$^{fl/fl}$ mice also bearing *Yap* and *Taz* conditional alleles (figs. 2D-E), indicating that YAP/TAZ are required for epithelial overgrowth and development of these mammary lesions. Since our in vitro data indicated that YAP/TAZ establish transcriptional dependencies that induce oncogenic growth in a manner dependent on BRD4, we next tested whether the lesions in *MMTV-Cre;Apc*$^{fl/fl}$ mice could be treated by administration of BET inhibitors. For this, we treated 8-week-old female mice (i.e., with an overt mammary gland phenotype) with a potent BET inhibitor (BAY-1238097 (*24*), 75 mg/kg/week) for 6 weeks. Strikingly, at the end of treatment, lesions had greatly regressed due to cell death and epithelial remodelling with few remaining signs of mammary hyperplasia or fibrosis to an extent that the main mammary ducts returned to a normal appearance, as defined by histology and K8 and K14 staining (figs. 2F-G and S2F). As a control, treatment of *Apc*$^{fl/fl}$ siblings (i.e., lacking Cre expression) was overtly well tolerated, and inconsequential for mammary

gland homeostasis (figs. S2G-H). Thus, BET inhibition defines a vulnerability for YAP/TAZ-driven tumors.

Beyond controlling tumour initiation and growth, YAP/TAZ endow cancer cells with the capacity to acquire resistance to chemotherapeutics and molecularly targeted drugs (*9*). Melanoma cells bearing BRAF activating mutations are a point in case. It has been recently shown that resistance to BRAF inhibitors (such as PLX4032/vemurafenib) is rapidly installed in a YAP/TAZ dependent manner in melanoma cells (*25, 26*). Consistently, YAP/TAZ activity is overtly induced in the resistant melanoma cell lines M229-R5, when compared to sensitive M229 cells (fig. 2H). YAP/TAZ knockout in resistant cells is sufficient to diminish their viability, and re-sensitize them to vemurafenib (fig. 2J). In light of the data presented above, we hypothesized that JQ1 could be used to inhibit YAP/TAZ in PLX4032-resistant melanoma cells, as such phenocopying the effect of YAP/TAZ inhibition at restoring sensitivity to BRAF inhibitors. Supporting this hypothesis, JQ1 inhibited the activity of the TEAD luciferase reporter in a dose-dependent manner in PLX4032-resistant M229-R5 cells (fig. 2H). To assess the functional implication of this finding, we performed viability assays by exposing M229-R5 cells to the BRAF inhibitor with or without JQ1. The combined treatment with JQ1 sensitized resistant cells to low doses of PLX4032, and impaired tumour cell viability to an extent that neither PLX4032 nor JQ1 could achieve when used individually (fig. 2I). The effect of JQ1 was phenocopied by combination of PLX4032 with two independent mixes of BRD siRNAs (fig. 2J). Similar results were obtained with two additional BRAF-mutant, PLX4032-resistant cell lines (figs. S2I-J). These experiments indicate the requirement of BET proteins in maintaining YAP/TAZ-induced resistance to vemurafenib in BRAF mutant melanoma cells, and suggest that BET inhibitors might indeed prove useful to revert YAP/TAZ-dependent drug resistance in melanoma cells.

To expand on the generality of the YAP/TAZ-BRD4 connection, we then asked whether YAP/TAZ transcriptional activity is especially sensitive to BET inhibitors also in different cancer cell lines. For this, we used publicly available transcriptomic data of cancer cells treated with JQ1 or OTX015, and performed GSEA using signatures of YAP/TAZ transcriptional activation. As shown in supplementary figure 3, YAP/TAZ-activated genes are over-represented among genes downregulated by BET inhibitors in different TNBC, prostate cancer, lung adenocarcinoma and melanoma cell types.

Data presented so far indicate that the transcriptional and biological functions of YAP/TAZ and BETs are intertwined, to the extent that YAP/TAZ targets are the most sensitive BRD4 targets and pharmacological inhibition of BRD4 interferes with YAP/TAZ activity. Still, what underlies the disproportionate sensitivity of YAP/TAZ targets to BET inhibitors? To gain mechanistic insights into this connection, we performed ChIP-seq experiments to compare BRD4 and YAP/TAZ binding to chromatin. YAP/TAZ bind almost exclusively to enhancers (*12, 15, 20*), whereas BRD4 binds both active enhancers and active promoters (figs. S4A-B; see Materials and Methods for the definition of enhancers and promoters). We started our analysis from enhancer elements, and found that BRD4 coverage was higher on enhancers containing YAP/TAZ binding sites when compared to active enhancer not occupied by YAP/TAZ (fig. 3A). We reasoned that differential BRD4 loading might correspond to differential responsiveness to JQ1; to verify this assumption, we performed BRD4 ChIP-seq in cells treated with JQ1. We found that JQ1 induced a preferential loss of BRD4 from YAP/TAZ-occupied enhancers, compared to active enhancers without YAP/TAZ binding sites (fig. 3A and S4C). Thus, the presence of YAP/TAZ peaks defines enhancers enriched of BRD4 and highly sensitive to BET inhibitors on the genome-wide scale.

We then assessed whether the presence of YAP/TAZ was required for the engagement of BRD4 to chromatin, by performing BRD4 ChIP-seq in MDA-MB-231 cells depleted of YAP/TAZ. As shown by the average BRD4 binding profile and some representative enhancers in figs. 3B-C, BRD4 recruitment to YAP/TAZ-containing enhancers was heavily reduced upon YAP/TAZ depletion, to an extent similar to JQ1 (see also figs. S4C-E). Thus, YAP/TAZ are required to keep BRD4 anchored to a specific group of enhancers. Do these elements correspond to super-enhancers? In fact, 80% of super-enhancers in MDA-MB-231 cells do contain YAP/TAZ peaks; yet, the vast majority (85%) of YAP/TAZ-occupied enhancers are by definition typical enhancers (figs. S4F-G). However, we observed that genes connected to YAP/TAZ-bound typical enhancers displayed sensitivity to JQ1 strikingly similar to the much more restricted number of genes associated with super-enhancers (fig. S4H).

We then surmised that the disproportionate sensitivity of YAP/TAZ targets to inhibition by JQ1 should be ultimately explained at the level of YAP/TAZ-regulated promoters. To study this, we focused on YAP/TAZ regulated genes whose promoters either contain YAP/TAZ binding sites or are connected to YAP/TAZ-bound enhancers through chromatin looping (*12*) (see Table 2). We found that: i) the TSS of these genes exhibited higher BRD4 occupancy when compared to the TSS of genes not activated by YAP/TAZ (fig. 3D); ii) YAP/TAZ were required for BRD4 accrual on the promoters of their targets, while marginally affecting the promoters of non-YAP/TAZ targets (figs. 3E-G; see also figs. 3H-I for representative individual gene tracks, and fig. S4I for validation by qPCR); iii) JQ1 caused preferential loss of BRD4 from YAP/TAZ-regulated promoters (figs. 3E-G), matching the effects of JQ1 on gene expression. Importantly, as exemplified in fig. 3H, JQ1 had only minor effects on BRD4 coverage on the promoters of genes not activated by YAP/TAZ, where it was in fact insufficient to induce a general downregulation of transcription (figs. 1F and S1D). Thus, BRD4 levels at promoters closely reflect the dynamic of YAP/TAZ-mediated engagement of BRD4 at distant enhancers. We thus envision a model

whereby YAP/TAZ bound to enhancers promote BRD4 overload on their target promoters, establishing higher expression levels of essential genes, and – concomitantly – their vulnerability to BET inhibitors.

We next investigated the mechanisms by which BRD4 accrual regulates activity of YAP/TAZ-target promoters. The role of BRD4 for transcriptional activation is best understood in terms of promotion of transcriptional elongation through recruitment of P-TEFb (*6*). If so, we should expect that, upon YAP/TAZ depletion or JQ1 treatment, Pol II should remain paused, if not accumulate, on the promoters of YAP/TAZ targets. To verify this hypothesis, we performed ChIP-seq experiments for RNA Pol II. In contrast to expectations, Pol II loading was selectively decreased on the promoters of YAP/TAZ targets in YAP/TAZ-depleted cells (figs. 4A-B, S5A-C). This implies that YAP/TAZ promote the recruitment of RNA-Pol II. In line, Pol II coverage on the TSSs of YAP/TAZ target genes was on average higher compared to all other expressed genes (fig. 4A), proportional to BRD4 binding (fig. 4C), and selectively reduced by JQ1 treatment (fig. 4D). Representative gene tracks showing comparable effects of JQ1 and YAP/TAZ depletion on Pol II loading on the TSS of YAP/TAZ target genes (but no substantial effects on not-YAP/TAZ targets) are presented in figs. 4E and S5D. Similar results were obtained after siRNA-mediated depletion of BRD2/3/4, as revealed by ChIP-qPCR at sampled promoters (fig 4F). This suggests that YAP/TAZ recruit Pol II by inducing BRD4 accumulation at TSS.

Since we found that transcriptional addiction, as defined by JQ1 sensitivity, is associated to differential Pol II recruitment, then BRD4 must entail additional mechanisms to regulate YAP/TAZ-dependent gene expression, other than favouring elongation. BRD4 has been recently reported to display an intrinsic acetyltransferase activity, leading to acetylation of K122 in the globular domain of H3 (*27*). Intriguingly, H3K122 acetylation is associated with Pol II loading on promoters and transcriptional

activation (*28*). We thus measured the levels of H3K122ac by ChIP-seq in control, YAP/TAZ-depleted or JQ1-treated cells. Strikingly, H3K122ac levels were significantly higher on the promoters of YAP/TAZ target genes (fig. 4G), in line with the higher coverage of BRD4 (fig. S5E) and Pol II, and with transcriptional activation. This differential enrichment of H3K122ac was dependent on YAP/TAZ, as robust loss in H3K122 acetylation was observed in YAP/TAZ targets upon YAP/TAZ depletion (figs. 4H-J and S5F, and H3K122ac profile at individual loci in fig. 4K). Paralleling BRD4 occupancy, acetylation of H3K122 on the promoters of YAP/TAZ targets was especially sensitive to JQ1-treatment (figs. 4H-J and S5F). We thus propose that YAP/TAZ promote transcriptional activation of their target genes by favouring BRD4 overload on their promoters, in turn increasing the acetylation of H3K122 and subsequent Pol II recruitment.

The work here presented holds a number of implications. We have advanced on the molecular mechanisms underlying transcriptional addiction in TNBC and other tumor types, identifying YAP/TAZ as essential players in this phenomenon. Indeed, we have demonstrated that cancer transcriptional addiction and cancer reliance on YAP/TAZ represent two sides of the same coin, at least in the model system here investigated. The underlying molecular event is the physical and functional association between YAP/TAZ and BRD4: YAP/TAZ-bound enhancers recruit BRD4, leading to BRD4 accrual on their target promoters. The YAP/TAZ/BRD4 complex confers a transcriptional advantage to a broad number of YAP/TAZ target genes; this edge can be targeted by BET inhibitors with remarkable antitumor responses. Drugging YAP/TAZ is clearly a very challenging yet exciting goal for cancer research (*11*), given the widespread and pervasive functions of YAP/TAZ in cancer cells, contrasting their dispensability for healthy tissues. BET inhibitor may start to fulfil this unmet need.

These findings offer a solution to the conundrum of how a general transcriptional regulator such as BRD4 can regulate transcription in a gene-specific manner in tumor cells. Although other transcription factors have been demonstrated to bind BRD4, in no case these could explain genome-wide recruitment of BRD4 at enhancers and promoters of entire groups of genes essential for cancer biology, including genes controlling S-phase entry, DNA synthesis and repair, and cell cycle control (*6*). The fact that BET inhibitors and YAP/TAZ inactivation cause remarkably similar impoverishment of BRD4 on chromatin suggests that the associations of BRD4 with acetylated histones and YAP/TAZ are cooperative. This is reminiscent of previously described bromodomain-dependent and -independent functions of BRD4 (*6, 19*), and suggests that further investigations of the BET-YAP/TAZ interaction may be fruitful toward the design of new therapeutics. The intimate connection between YAP/TAZ and BET proteins also offers unexpected perspectives for the use of BET inhibitors. Although these are promising anticancer drugs, drug resistance and identification of the most sensible patient populations or choice of effective combinations with other drugs, all remain as open issues (*4*). Our data would suggest that the response to BET inhibitors may be in fact correlated to the biology of YAP/TAZ; as proof of principle, YAP/TAZ-induced resistance to vemurafenib can be reverted by BET inhibitors. Alternatively, patients stratified according to YAP/TAZ classifiers may display differential sensitivity to these drugs.

More broadly, the present results advance on the molecular definition of enhancer elements that are responsible for transcriptional dysregulations in cancer. Major emphasis in this respect has recently been placed on super-enhancers, although the molecular identity of the key TFs underlying the properties of these regulatory elements remains mysterious (*8*). We found that super-enhancers largely consist of YAP/TAZ-occupied enhancers; more importantly, super-enhancers, at least those active in TNBC cells, may simply represent the more noticeable "tip of the iceberg" of a larger set of YAP/TAZ-

bound enhancers that nonetheless display super-enhancer-like properties, as defined by strong enrichment of BRD4, higher expression level of regulated target genes and higher than average sensitivity to BET inhibitors.

Finally, YAP/TAZ are critical for inducing cell-fate plasticity in normal and tumor cells alike (*9*). For example, they reprogram normal/differentiated mammary cells into mammary stem cells (*10*); or more differentiated tumor cells into cancer stem cells (*29*). The nature of the epigenetic barriers controlling these transitions remains unknown, but it is tempting to speculate that BRD4 availability, and potentially other factors assembled by YAP/TAZ on chromatin, may link YAP/TAZ function to permissive vs. restrictive chromatin states, as such guiding cell reprogramming or barring it. Thus the YAP/TAZ-BRD4 connection may hold relevance in contexts other than cancer in which YAP/TAZ play essential roles, such as heart repair and tissue regeneration.

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Table 1. ChIP-MS.

Protein IDs	Gene names	Protein names	Unique peptides			LFQ value		
			IP: YAP	IP: TAZ	IP: IgG	IP: YAP	IP: TAZ	IP: IgG
Q9GZV5	WWTR1; TAZ	WW domain-containing transcription regulator protein 1	7	13	0	9.00E+07	3.00E+09	0
P46937	YAP1	Yes-associated protein 1	3	3	0	2.00E+10	8.00E+09	4.00E+06
P28347	TEAD1;TEAD4	TEF-1, TEF3	2	1	0	1.00E+07	8.00E+07	0
P05412	JUN	Transcription factor AP-1	2	2	0	7.00E+06	5.00E+06	0
Q53GM9	FOSL1	Fos-related antigen 1	1	1	0	2.00E+06	3.00E+06	0
F8VZ70	SMARCD1; SMARCD3	SWI/SNF complex subunit	1	1	0	2.00E+06	3.00E+06	0
O60264	SMARCA5	SWI/SNF complex subunit	2	3	0	4.00E+06	3.00E+06	0
P51532	SMARCA4	Transcription activator BRG1	5	2	0	7.00E+06	2.00E+07	0
Q05BW5	SMARCC1	SWI/SNF complex subunit	3	1	0	3.00E+07	2.00E+07	0
Q59FG6	KMT2D	Histone-lysine N-methyltransferase 2D	1	1	0	1.00E+06	9.00E+06	0
Q96L91	EP400	E1A-binding protein p400	1	2	0	1.00E+06	2.00E+06	0
Q9Y265	RUVBL1	RuvB-like 1	3	2	0	1.00E+07	8.00E+06	0
Q09472	EP300	Histone acetyltransferase p300	2	1	0	6.00E+06	3.00E+06	0
O00422	SAP18	Histone deacetylase complex subunit SAP18	3	2	0	5.00E+07	1.00E+07	0
O60885	BRD4	Bromodomain-containing protein 4	10	2	0	8.00E+07	1.00E+07	0

Main Figure Legends

Figure 1. BRD4 is a required cofactor for YAP/TAZ transcriptional activity.

A) Interaction of endogenous YAP/TAZ, TEAD1 and BRD4 in MDA-MB-231 cells.

B) *In situ* PLA detection of interactions between endogenous YAP, TAZ or TEAD1 and exogenous FLAG- or HA-BRD4 in HEK293T cells. The detected dimers are represented by fluorescent dots (red). Nuclei are counterstained with DAPI (blue). No dots could be detected in the nuclei of cells transfected with empty vector, confirming the specificity of interactions.

C) Box plot of expression values of genes not activated by YAP/TAZ (not YT targets) vs. activated by YAP/TAZ in MDA-MB-231 cells. Expression values were determined by RNA-seq and are presented as RPKM. Values within the 10^{th} and 90^{th} percentile are plotted. **** $p<10^{-10}$ (one-tailed Mann-Whitney U test)

D) Box plot of expression values of genes classified according to GO annotation as genes involved in cell proliferation vs. genes associated to all other functions. **** $p<10^{-10}$ (one-tailed Mann-Whitney U test); $^{++++}$ $p<10^{-10}$ (one-tailed Wilcoxon matched-pairs signed rank test)

E) The fraction of genes activated by YAP/TAZ which are inhibited by JQ1 or BRD2/3/4 siRNAs is larger than the fraction of all expressed genes downregulated by the same treatments.

F) Box plots showing fold change in gene expression upon treatment with JQ1 (1μM, 24h) or transfection with BRD2/3/4 siRNAs (siBRD mix A or B, 72h). The y axis shows the fold change in transcript levels versus DMSO-treated cells (left) or cells transfected with control siRNA (siCO, right). **** $p<10^{-10}$ (one-tailed Mann-Whitney U test)

G) RT-qPCR showing that sustained expression of wild-type YAP does not rescue the expression of YAP/TAZ target genes in MDA-MB-231 cells depleted of BET proteins or treated with BET inhibitors (1μM, 24h). Exogenous YAP, instead, can rescue the expression of the same genes after YAP/TAZ depletion. Data are presented as individual data points + average (bar).

H) Average expression level of YAP/TAZ activated genes in MDA-MB-231 cells, measured by RNA-seq. Cells were treated with DMSO (vehicle), BET inhibitors (JQ1, OTX015), CDKs inhibitors (flavopiridol, THZ1) or RG-108 (a DNA methyltransferase inhibitor, here used as negative control to assess the effect of a compound targeting an epigenetic function not related to transcription). Data are presented as mean of z-scores ± SEM.

I) Odds ratio plot: genes activated by YAP/TAZ are more likely to be inhibited by BET inhibitors than not-YAP/TAZ target genes. CDK inhibitors and RG-108 do not display such property (see Methods).

Figure 2. BET inhibitors impair YAP/TAZ biological functions.

A) Quantification of colonies formed by YAP5SA-overexpressing MCF10A cells in soft agar. Data are presented as mean + SD of 3 biological replicates.

B) Quantification of colonies formed by YAP5SA-overexpressing MCF10A cells in soft agar, upon treatment with 0,1μM or 1μM JQ1 for the entire experiment. Data are presented as in A.

C) Inhibition of the growth of colonies initiated by YAP5SA-overexpressing MCF10A cells in soft agar upon addition of JQ1 (1μM) to culture medium 8 or 15 days after seeding (treatment with JQ1 at day1 is presented as reference for maximal inhibition). Data are presented as in A.

D) Representative hematoxylin and eosin (H&E) staining of sections of mammary glands from *MMTV-Cre;Apc*$^{+/+}$, *MMTV-Cre;Apc*$^{fl/fl}$, or *MMTV-Cre;Apc*$^{fl/fl}$*;Yap*$^{fl/fl}$*;Taz*$^{fl/fl}$ mice. Scale bar is 0.1 mm.

E) Representative immunofluorescence (IF) pictures of mammary glands from the indicated mice, showing YAP accumulation and K14 discontinuities in *MMTV-Cre;Apc*$^{fl/fl}$. Ducts of *MMTV-Cre;Apc*$^{fl/fl}$*;Yap*$^{fl/fl}$*;Taz*$^{fl/fl}$ mice display a normal morphology. Scale bar is 25 μm.

F) Representative H&E staining of sections of mammary glands from *MMTV-Cre;Apc*$^{fl/fl}$ mice, treated with vehicle (n=3) or BAY-BET inhibitor (n=3) for 6 weeks. All scale bars are 0.1mm.

G) Representative IF pictures of mammary glands from *MMTV-Cre;Apc*$^{fl/fl}$ mice, treated with vehicle or BAY-BET inhibitor for 6 weeks, showing that treatment with BET inhibitor restores normal distribution of K8 and K14 in the mammary ducts. Scale bars are 25 μm.

H) TEAD luciferase reporter assay (8xGTIIC-lux) in M229 and M229-R5 melanoma cells. JQ1 doses ranged from 1nM to 1μM. Data are normalized to parental M229 cells and are presented as mean + SD of two biological replicates.

I) Viability curves of M229 and M229-R5 cells, treated with increasing doses of PLX4032 (1nM to 10μM) with or without 1μM JQ1. The green lines shows the effect of 1μM JQ1 alone. Data are mean + SD of 8 technical replicates.

J) Cell viability assay of M229-R5 cells, transfected with the indicated siRNAs and treated with DMSO or 3μM PLX4032. Data are mean + SD of 8 technical replicates.

Figure 3. YAP/TAZ are required for BRD4 recruitment to chromatin.

A) Box plot showing the distribution of BRD4 ChIP-seq signal (expressed as normalized read density, RPKM) comparing active enhancers with or without YAP/TAZ peaks in MDA-MB-231 cells treated with DMSO or JQ1 (1μM, 6h), or transfected with YAP/TAZ siRNAs (48h). **** $p<10^{-10}$ (one-tailed Mann-Whitney U test); $^{++++}$ $p<10^{-10}$ (one-tailed Wilcoxon matched-pairs signed rank test)

B) Average signal of BRD4 ChIP-seq reads in enhancers with YAP/TAZ peaks in a window of ±1 kb centered on the summit of YAP/TAZ peaks.

C) Genome browser view of YAP, BRD4 and H3K4me1 binding profiles at representative active enhancers in MDA-MB-231 cells. Both JQ1 and YAP/TAZ siRNA induce a strong decrease in BRD4 binding.

D) Box plot of BRD4 ChIP-seq signal (RPKM) comparing promoters of genes not activated by YAP/TAZ or of YAP/TAZ target genes (YT targets) in MDA-MB-231 cells (treated with DMSO). **** $p<10^{-10}$ (one-tailed Mann-Whitney U test)

E) Box plots showing the change in BRD4 genomic occupancy in JQ1-treated (left) or YAP/TAZ-depleted cells (right) vs. control cells (DMSO), comparing promoters of genes not activated by YAP/TAZ with promoters of YAP/TAZ target genes. The y axes reports the fold change calculated as $RPKM_{(JQ1 \text{ or } siYT)}$ divided by $RPKM_{(DMSO)}$. **** $p<10^{-10}$ (one-tailed Mann-Whitney U test)

F) Heatmap showing BRD4 binding on the promoters of YAP/TAZ targets in MDA-MB-231 cells, in a window of ±1.5kb centered on the transcription start site (TSS).

G) Average signal of BRD4 ChIP-seq reads on the promoters of YAP/TAZ target genes in MDA-MB-231 cells, in a window of ±1.5 kb centered on TSS.

H) YAP, BRD4 and H3K4me3 binding profiles at representative promoters of YAP/TAZ target genes or not-YAP/TAZ targets. Arrows indicate BRD4 enrichment at the TSS. JQ1 and siYAP/TAZ induce a strong decrease in BRD4 binding on YAP/TAZ targets, whereas there is no overt variation of BRD4 binding on the TSS of not YAP/TAZ targets.

I) YAP, BRD4 and H3K4me1/H3K4me3 binding profiles on a distal enhancer and on CDCA5 promoter. JQ1 (1μM, 6h) and siYAP/TAZ (48h) induce a strong decrease in BRD4 binding both on the enhancer, containing YAP/TAZ peak, and on TSS of CDCA5.

Figure 4. YAP/TAZ and BRD4 regulate Pol II loading and H3K122 acetylation on TSSs.

A) Box plot showing the distribution of RNA-Pol II ChIP-seq signal (expressed as normalized read density, RPKM) comparing promoters of genes not activated by YAP/TAZ or of YAP/TAZ target genes in control (DMSO) or YAP/TAZ depleted cells. **** $p<10^{-10}$ (one-tailed Mann-Whitney U test)

B) Heatmap showing RNA-Pol II loading on the promoters of YAP/TAZ targets in MDA-MB-231 cells, in a window of ±1.5kb centered on the transcription start site (TSS).

C) Linear correlation between BRD4 and RNA-Pol II occupancy (both expressed in RPKM) on the TSS of YAP/TAZ target genes. R^2 is the coefficients of determination of the correlation ($p<0.0001$).

D) Box plots showing the change in RNA-Pol II promoter occupancy in JQ1-treated cells vs. control cells (DMSO), comparing promoters of genes not activated by YAP/TAZ with promoters of YAP/TAZ target genes. **** $p<10^{-10}$ (one-tailed Mann-Whitney U test)

E) Genome browser view of RNA-Pol II binding profiles at representative promoters of YAP/TAZ target genes or not-YAP/TAZ targets. Pol II binding is reduced upon JQ1 treatment or YAP/TAZ depletion on the TSS of YAP/TAZ targets.

F) ChIP-qPCR verifying RNA-Pol II binding to promoters of established YAP/TAZ targets upon depletion of BET proteins. GAPDH promoter represents a non-YAP/TAZ target. ChIP with pre-immune IgG displayed background signal (which was comparable in all samples). DNA enrichment was calculated as fraction of input and is presented as % of RNA-Pol II binding in control cells (siCO).

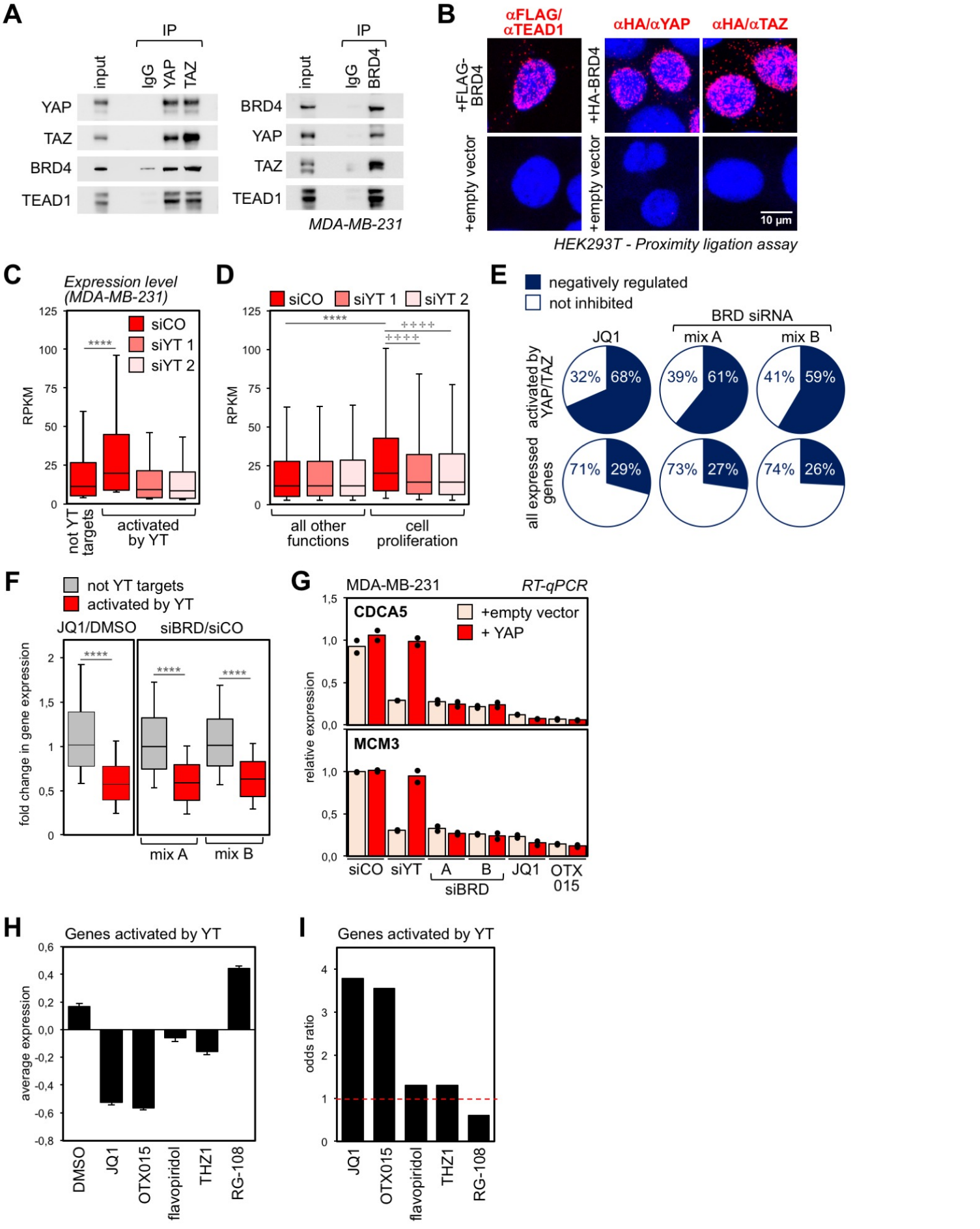
G) Box plot of H3K122ac ChIP-seq signal (RPKM) comparing inactive promoters with the TSS of genes not activated by YAP/TAZ or of YAP/TAZ target genes in control cells (treated with DMSO). **** $p<10^{-10}$ (one-tailed Mann-Whitney U test)

H) Box plots showing the change in H3K122ac promoter levels in YAP/TAZ-depleted (left) or JQ1-treated cells (right) vs. control cells (DMSO), comparing genes not activated by YAP/TAZ with YAP/TAZ target genes. **** $p<10^{-10}$ (one-tailed Mann-Whitney U test)

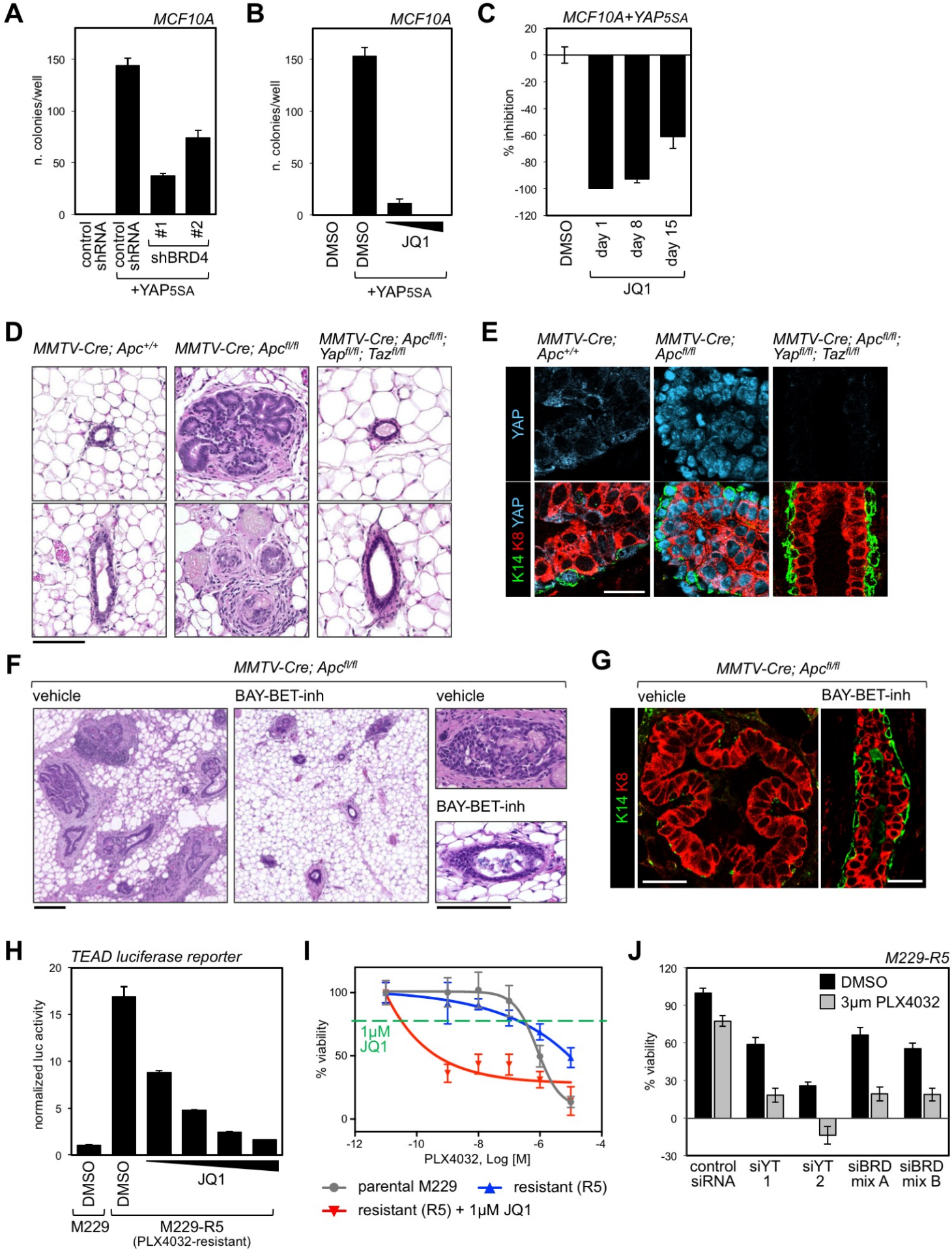
I) Heatmap showing acetylation of H3K122 on the promoters of YAP/TAZ targets in MDA-MB-231 cells, in a window of ±1.5kb centered on the transcription start site (TSS).

J) Average ChIP-seq profile of H3K122ac on the promoters of YAP/TAZ target genes in MDA-MB-231 cells, in a window of ±1.5 kb centered on TSS.

K) Genome browser view of H3K122ac levels at representative promoters of YAP/TAZ target genes or not-YAP/TAZ targets.



Piccolo, Zanconato, et al. - MAIN FIGURE 1



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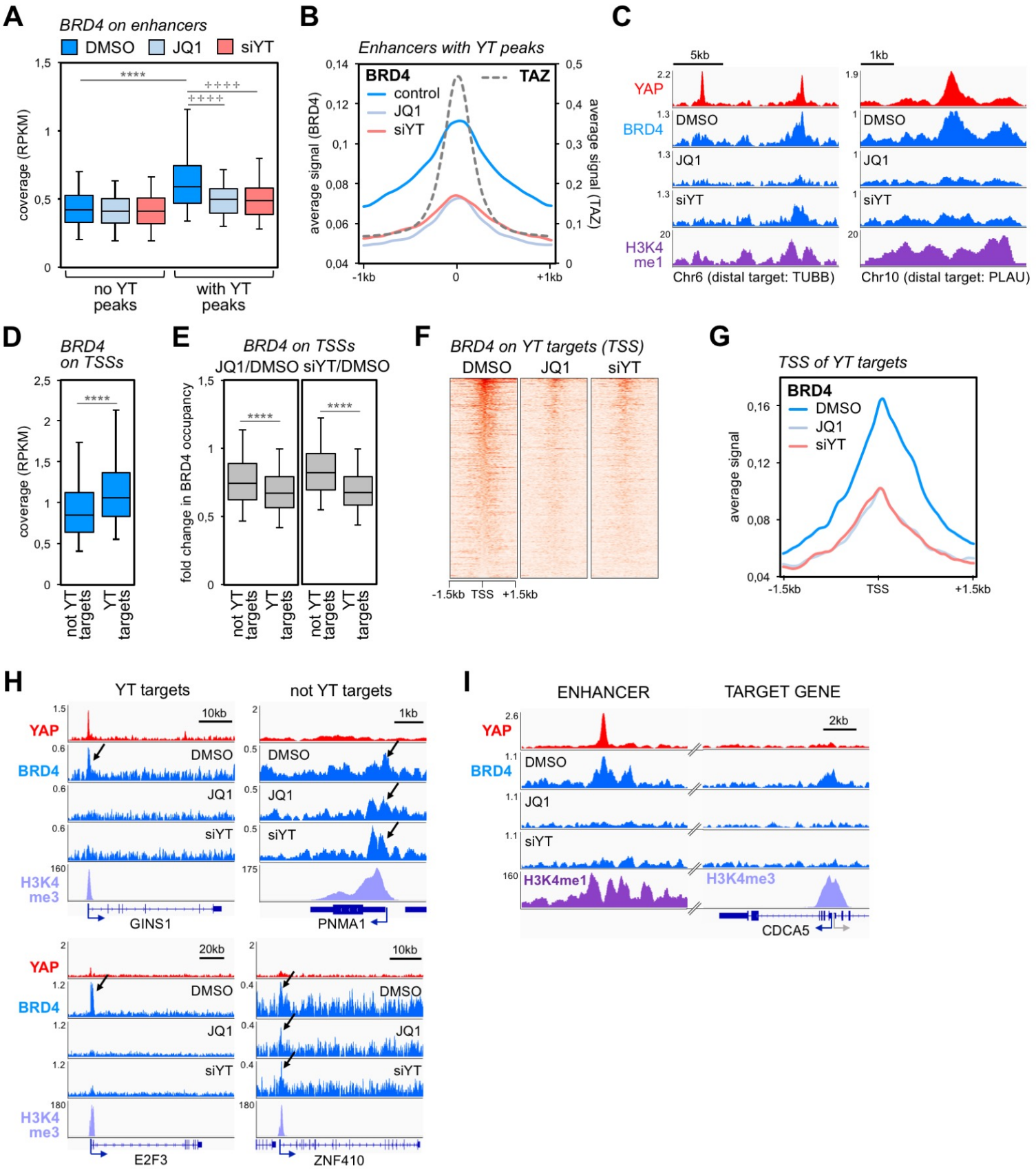

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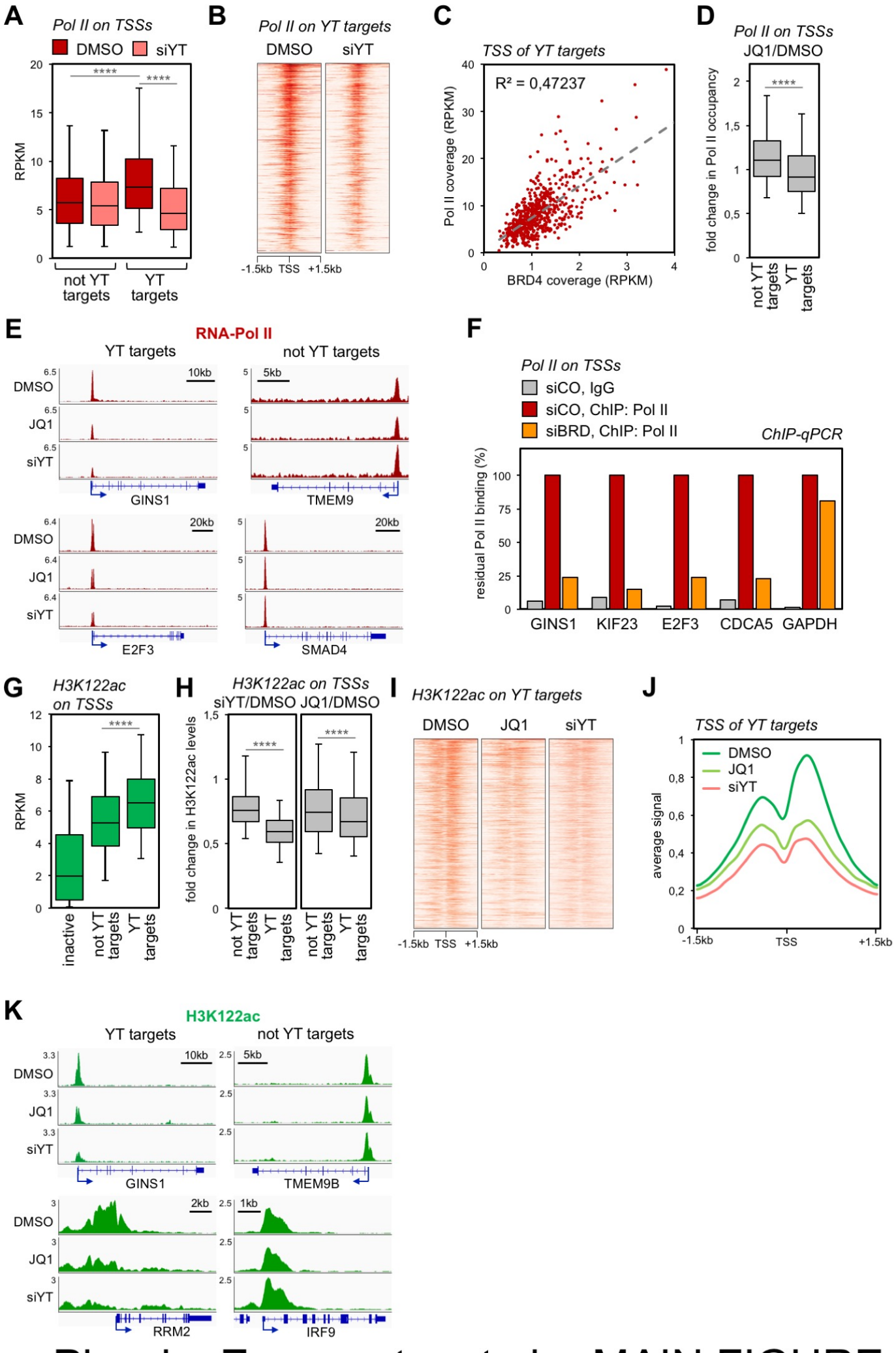

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