

ORIGINAL ARTICLE

Molecular networks of hepatoblastoma predisposition and oncogenesis in Beckwith-Wiedemann syndrome

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Abstract

Beckwith-Wiedemann Syndrome (BWS) is the most common human overgrowth disorder caused by structural and epigenetic changes to chromosome 11p15. Patients with BWS are predisposed to developing hepatoblastoma (HB). To better understand the mechanism of HB oncogenesis in this cancer predisposition background, we performed the first multi-dimensional study of HB samples collected from patients diagnosed with BWS. This multi-omic investigation of seven BWS HB and five matched nontumor BWS liver samples from 7 unique patients included examination of whole exome sequences, messenger RNA/microRNA expression, and methylation levels to elucidate the genomic, transcriptomic, and epigenomic landscape of BWS-associated HB. We compared the transcriptional profiles of the BWS samples, both HB and nontumor, to that of control livers. Genes differentially expressed across BWS tissues were identified as BWS HB predisposition factors; this gene group included cell cycle regulators, chromatin organizers, and WNT, mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)/AKT members. We also compared transcriptional changes associated with non-syndromic HB carrying BWS-like 11p15 alterations compared to those without, as well as to BWS HB. Through this analysis, we identified factors specific to 11p15-altered HB oncogenesis, termed the BWS oncogenesis network. We propose that 11p15 alterations drive HB oncogenesis by initially dysregulating cell-cycle regulators and chromatin organizers, including *histone deacetylase 1 (HDAC1)*, *ATP-dependent helicase X*, and *F-Box and WD repeat domain containing 7*. Furthermore, we found oncogenic factors such as dickkopf WNT signaling pathway inhibitor 1 and 4, *WNT16*, *forkhead box O3 (FOXO3)*, and *MAPK10* are differentially expressed in 11p15-altered HB in both the BWS and non-syndromic backgrounds. These genes warrant further investigation as diagnostic or therapeutic targets.

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INTRODUCTION

Hepatoblastoma (HB) is a rare embryonal tumor comprising 1% of pediatric cancer cases^[1]; it is also the most common malignant liver tumor diagnosed in children under 5 years of age.^[2] The most studied driver gene of HB is the WNT pathway member β -catenin (*CTNNB1*); over 60% of HB carry mutations in *CTNNB1*, specifically in the exon 3 region.^[3] These sequence alterations stabilize the β -catenin protein in the nucleus, where it acts as a transcription factor; its targets include the proto-oncogene *MYC*, which has been implicated in liver neoplasm malignancy.^[4]

Another locus that contributes to HB development is human chromosome 11p15. Nearly one-third of all HB samples investigated for structural genomic alterations demonstrated loss of heterozygosity (LOH) at or including 11p15.^[5–12] Often, these 11p15 LOH are categorized as copy-neutral and result from the duplication of DNA content from one parental allele with loss of commensurate DNA content from the other allele, or uniparental disomy (UPD).^[5,6,8,10–12] In several studies, parental DNA was available and contained informative variants, allowing researchers to conclude that the maternal allele was lost and the paternal allele was duplicated, thereby resulting in paternal UPD of 11p15 (pUPD11).^[5,10] One study also noted an increased risk of HB relapse in patients whose tumors carry this 11p15 LOH.^[7]

Genetic and epigenetic alterations affecting the 11p15 region also cause one of the most common HB predisposition syndromes, Beckwith-Wiedemann Syndrome (BWS; OMIM 130650). BWS can also be classified as a genomic imprinting disorder, which is caused by disruption of monoallelic parent-specific gene expression. Within the 11p15 region, there are two independent domains subject to this parent-specific gene expression: *H19/IGF2:IG-DMR* is the imprinting control region (ICR) for Imprinting Center 1 (IC1), and *KNCQ1OT1:TSS-DMR* is the ICR for Imprinting Center 2 (IC2) (Figure S1). Within IC1, gain of methylation (GOM) at the ICR causes an increase of the paternally expressed *insulin-like growth factor 2 (IGF2)* and its regulatory microRNA (miRNA) *miR-483* as well as a decrease of the maternally expressed noncoding RNA *H19* and its regulatory miRNA *miR-675* (Figure S1A). Within IC2, loss of methylation (LOM) at the ICR causes a decrease in the maternally expressed *potassium voltage-gated channel subfamily Q member 1 (KCNQ1)* and *cyclin-dependent kinase inhibitor 1C (CDKN1C)* as well as an increase in the noncoding antisense RNA *KCNQ1OT1* (Figure S1B).

Combinations of these alterations in the 11p15 region cause a range of fetal and neonatal overgrowth characteristics including hepatomegaly and development of embryonal tumors.^[13] BWS occurs in about 1 of 10,000 live births.^[13] The relative risk of HB development in the BWS population was estimated at 2280-fold compared with age-matched controls.^[14] Of the 29 HB tumors

reported in previously published BWS cohort studies, 20 occurred in patients with pUPD11, five in patients with IC2 LOM, one in a patient with IC1 GOM, and three in patients with other subtypes or who were not molecularly diagnosed.^[15–21] Due to this increased cancer risk, all patients with BWS are screened by liver ultrasound and serum α -fetoprotein (AFP) every 3 months from BWS diagnosis until four years of age.^[22]

BWS HB neoplasms may be different from those that arise sporadically in the nonsyndromic population. A meta-analysis of BWS HB cohorts observed that no individuals developed HB after 30 months of age.^[23] The estimated median age of HB diagnosis is 6 months of age in the BWS population compared with 16 months in the nonsyndromic population.^[23,24] Additionally, the genetics driving BWS HB may differ from that of nonsyndromic HB. A previous study of two HB investigated the prevalence of *CTNNB1* nuclear localization in BWS HB; *CTNNB1* was observed in the nucleus of both BWS tumors, but not normal liver tissue.^[25] Additionally, a *CTNNB1* exon 3 mutation common to sporadic HB was only identified in one of the tumors.^[25] These limited data in BWS HB suggest differential HB oncogenic drivers in this patient population.

Aside from this one study focused on two BWS HB tumors,^[25] studies have not investigated the molecular nature of HB in this syndromic background. A recent paper by Nagae et al. noted that their HB cohort included five tumors derived from patients with BWS but did not analyze these samples independently.^[26] Other cohorts reporting on the molecular nature of HB do not note whether any samples were collected from patients with BWS.^[8,27–31] In this study, we analyzed HB and matched nontumor liver samples from patients with congenital BWS. We use a multi-omics approach to better understand the molecular contributors to BWS HB predisposition. In addition, we compared our BWS tumor data with that of a nonsyndromic HB cohort to identify BWS HB oncogenesis factors. We believe these genes represent potential targets for future therapeutic development and may inform future personalized cancer screening approaches in cancer predisposition syndromes like BWS.

METHODS

Patients and samples

Samples and clinical information were collected through the BWS Registry, which is a previously established institutional review board protocol (IRB 13–010658) at the Children's Hospital of Philadelphia (CHOP). Briefly, consent was obtained from all patients and/or legal guardians to collect longitudinal clinical information and samples that became available through clinical care.

During surgical procedures for HB management, liver samples were collected from CHOP patients,

then snap-frozen in liquid nitrogen and stored at -80°C . Control liver samples were collected by CHOP Pathology and Laboratory Medicine and were derived from autopsy specimens with appropriate consents provided. Basic and limited clinical information about these samples was provided through an honest-broker based on their protocol. These samples were selected from individuals who did not have clinical or molecular BWS, nor was the cause of death the result of liver-associated complications. We analyzed all BWS HB tumor samples available to us through this methodology, which was seven primary HBs collected from 7 patients affected by molecularly confirmed BWS. Five had matched nontumor liver samples available (Table 1).

Whole genome sequencing and methylation array

Genomic DNA was isolated using the AllPrep DNA/RNA Micro Kit (QIAGEN). Whole genome sequencing (WES) libraries were prepared by the CHOP Center for Applied Genomics (CAG) using the Twist Bioscience Human Core Exome kit with 50 ng of input DNA on controls 1, 2, and 3, as well as all BWS HB and matched nontumor samples presented in this study. DNA from controls 2, 4, and 5 was of suitable quality and concentration for methylation analysis alongside all BWS HB and matched nontumor samples presented in this study (Table 1). The DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research) for methylation analysis. The CAG performed Infinium MethylationEPIC array (Illumina) runs.

RNA sequencing

Total RNA was isolated from frozen tumor samples using the AllPrep DNA/RNA Micro Kit (QIAGEN). Large and small RNA fractions were isolated using the Monarch RNA Cleanup kit (NEB). Standard and small RNA-sequencing (RNA-seq) was performed by GENEWIZ using the Tru-Seq RNA Library Prep kit (Illumina) including polyA selection and Tru-Seq Small RNA Library Prep kit (Illumina), respectively. Large RNA was available from all BWS HB samples as well as BWS nontumor samples 3, 5, and 6. Small RNA at concentrations and qualities suitable for sequencing could only be isolated from BWS HB samples and controls 2 and 3; other controls and BWS nontumor samples are not included in this analysis (Table 1).

Additional details of library preparation and analysis are provided in the Supporting Data and have been deposited in the Database of Genotypes and Phenotypes (dbGAP) of the National Center for Biotechnology Information (United States National Library of Medicine, Bethesda, MD) under accession number phs002614.

v1.p1. Nonsyndromic data were retrieved from Gene Expression Omnibus GSE132219 accession.

RESULTS

Overview of the BWS HB cohort

Although HB affects more male than female children,^[32] our cohort represents 43% males and 57% females (Table 1). Hepatomegaly, or enlargement of the liver, is a suggestive feature of BWS^[13] and was previously observed in one-third of patients with BWS and HB.^[24] In our cohort, 3 of 7 (43%) patients with BWS and HB were also noted to have hepatomegaly (Table 1). The median age of HB diagnosis for these children was 4 months of age (Table 1), in line with the previously reported early development of HB in the BWS population compared with that of the general population.^[23] All patients had their HB detected through increased AFP and/or a liver mass detected as part of the routine BWS tumor surveillance program.^[22] Most of the tumors presented with a mixed fetal and embryonal histologic subtype (Table 1). In the present study, all patients who were affected by BWS HB were alive at last follow-up, with survival measured between 20 months to 15 years off therapy (Table 1).

Imprinting of 11p15 in BWS HB

All patients were diagnosed with BWS due to IC2 LOM or pUPD11 in blood before HB development (Table 1). Initially, we wanted to confirm these BWS subtypes within the liver samples. We determined 11p15 methylation status based on the results of the methylation array and determined whether UPD was likely present in the sample cell population based on whether single nucleotide polymorphisms (SNPs) were observed in an even proportion of WES reads.

Surprisingly, several liver samples indicated a different BWS subtype compared with that initially identified in blood. For patients 2 and 6, IC2 LOM was indicated in blood, whereas HB presented as IC1 GOM and IC2 LOM without UPD (Table 1 and Figure S2A,B). For patient 3, pUPD11 was observed in blood, but the HB and nontumor samples presented with only IC1 GOM (Table 1 and Figure S2A). Interestingly, we confirmed that IC1 is likely affected by UPD, but not IC2 (Figure S2B), suggesting a shorter extent of UPD across 11p15 in liver samples compared with blood. Finally, for patients 4 and 7, pUPD11 was observed in blood; however, the respective methylation and SNP results in HB and nontumor conflictingly indicated that only IC1 or IC2 aberrant methylation was present along with UPD across the entirety of 11p15 (Table 1 and Figure S2A,B). As such, it is likely that these samples carry pUPD11 but may also have had subsequent methylation

TABLE 1 Clinical features of HB cases

	Sex	BWS subtype in blood	BWS subtype in tumor	BWS subtype in NT	Hepato-megaly	Age at tumor diagnosis	Months off therapy	Tumor histology	Genomic analysis ^b	Transcriptome analysis
Patient 1 (HB1)	M	pUPD11	pUPD11	N/A	No	7 months	168	Fetal	E, M	mRNA, miRNA
Patient 2 (HB2/NT2)	M	IC2 LOM	Aberrant IC1/IC2 methylation	IC2 LOM	No	1 month	67	Embryonal with scattered mesenchymal foci	HB: E, M NT: E, M	HB: mRNA, miRNA NT: N/A
Patient 3 (HB3/NT3)	F	pUPD11	IC1 GOM (by pUPD)	IC1 GOM (by pUPD)	Yes	1 month	44	Mixed fetal and embryonal	HB: E, M NT: E, M	HB: mRNA, miRNA NT: mRNA
Patient 4 (HB4)	F	pUPD11	pUPD11 with normal IC2	N/A	No	4 months	182	Mixed fetal and embryonal	E, M	mRNA, miRNA
Patient 5 (HB5/NT5)	F	pUPD11	pUPD11	pUPD11	No	4 months	27	Mixed fetal and embryonal	HB: E, M NT: E, M	HB: mRNA NT: mRNA
Patient 6 (HB6/NT6)	F	IC2 LOM	Aberrant IC1/IC2 methylation	No 11p15 change	Yes	4 months	20	Mixed fetal and embryonal	HB: E, M NT: E, M	HB: mRNA NT: mRNA
Patient 7 (HB7/NT7)	M	pUPD11	pUPD11 with normal IC1	pUPD11	Yes	13 months	21	Mixed fetal and embryonal	HB: E, M NT: E, M	HB: mRNA NT: N/A
Control 1	F	N/A	N/A	N/A	No	27 weeks ^a	N/A	N/A	N/A	mRNA
Control 2	F	N/A	N/A	N/A	No	20 days ^a	N/A	N/A	E, M	mRNA, miRNA
Control 3	M	N/A	N/A	N/A	No	10 days ^a	N/A	N/A	E	mRNA, miRNA
Control 4	M	N/A	N/A	N/A	No	3 months ^a	N/A	N/A	E, M	mRNA
Control 5	F	N/A	N/A	N/A	No	4 weeks ^a	N/A	N/A	E, M	N/A

Abbreviations: F, female; LOM, loss of methylation; M, male; miRNA, microRNA; N/A not applicable; pUPD11, paternal UPD (uniparental disomy) of 11p15.

^aAge at which control tissue was collected.

^b"E" denotes whole exome sequencing; "M" denotes methylation array.

changes in a population of cells that transformed. Of note, the confidence interval for methylation at IC2 was wider than that of IC1, which suggests that application of the methylation array at this locus may be less reliable (Figure S2A). In summary, we classified HB1, HB4, HB5, and HB7 as pUPD11; HB3 as IC1 GOM; and HB2 and HB6 as aberrant IC1/IC2 methylation (Table 1 and Figure S2A,B).

We also confirmed that the changes to the 11p15 ICRs influenced their imprinted genes as expected through RNA-seq analysis. Within IC1, *H19* and *miR-675* were both down-regulated in BWS samples compared with controls, as expected, in samples involving IC1 GOM (Figures S1A,B and S2C). *IGF2* was up-regulated in the BWS nontumor group, but *IGF2* and *miR-483* were expressed at a range closer to that of the controls in BWS HB, possibly as result of transcriptome changes associated with the oncogenic process (Figure S2C). Within IC2, *CDKN1C* was down-regulated in BWS HB and nontumor groups, as expected, in samples involving IC2 LOM (Figures S1A,C and S2C). The *KCNQ1* and *KCNQ1OT1* expression patterns were similar to those of *H19* and *IGF2*, respectively (Figure S2C).

Genomic changes associated with BWS HB

Aside from the previously discussed *CTNNB1* mutations and 11p15 alterations, several other somatic

structural and smaller-scale genomic alterations have been observed recurrently in HB. These include gains of chromosomal content from 1q, 2q, 6, 8, 12, 17, and 20; losses of 4q have also been noted.^[3,6,12,29] To determine whether these changes work cooperatively with 11p15 in BWS HB development, we examined the WES data for copy number alterations (CNAs). We found that HB6 carried a gain of 1q21-q44 encompassing nearly the entire arm (Figure 1A). Five other samples presented with 1p36.3 gains or losses, leading to its classification as a recurrent region of alteration (Figure 1A). A recurrent CNA was also identified at 2q35 in five BWS HB samples (Figure 1A). We noted additional recurrent gains and losses at 10q11.2, 12q21.31-q23.1, and across chromosomes 15, 16, and 17 (Figure 1A).

In line with the previously reported HB CNA rate,^[29] we identified just over 200 CNAs identified in tumors without a matched nontumor (HB1 and HB4) and just over 100 CNAs identified in the five HB normalized against their nontumor counterparts (Figure 1A). This result suggests that the BWS HB samples did not have a greater level of genome instability than previously studied sporadic HB by a CNA metric. To confirm, we calculated the tumor mutational burden (TMB). Overall, pediatric cancers are estimated to carry fewer than 25 nonsilent mutations per tumor, with HB carrying the lowest average number of mutations.^[34,35] As evidenced in our CNA analysis, samples without matched nontumor samples have an overestimated mutational

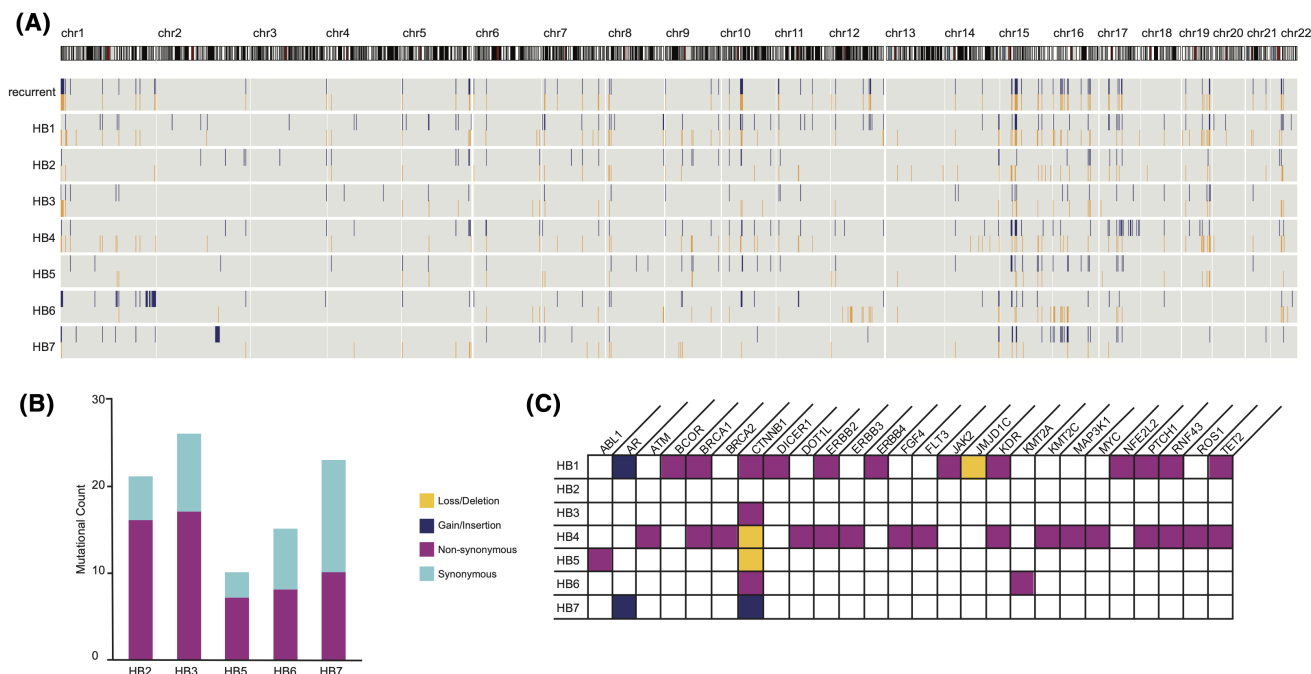


FIGURE 1 Genomic features of Beckwith-Wiedemann Syndrome (BWS) hepatoblastoma (HB) indicate genome stability and involvement of few cancer drivers. (A) Copy number alterations (CNAs) identified in BWS HB samples with recurrent alterations identified in at least two samples. Gains are shown in blue; losses are shown in orange. (B) Tumor mutational burden (TMB) in HB samples with a matched nontumor (NT). Synonymous mutations are shown in light blue; non-synonymous mutations are shown in green. (C) Mutations that map to known cancer-driving genes. Insertions are shown in blue, deletions in orange, and non-synonymous substitutions in green

load. For that reason, HB1 and HB4 were not included in this aspect of the analysis. For the five samples with a matched nontumor sample, we found an average of 19 ± 6.4 coding mutations overall, with an average of 11.6 ± 4.6 non-synonymous mutations (Figure 1B and Table S1).

While the CNA and TMB estimates indicate that BWS HB is similarly stable to nonsyndromic HB, we wanted to better understand how these mutations might contribute to oncogenesis. During the clinical evaluation of tumors, the Children's Hospital of Philadelphia uses a gene panel to assess mutations in 238 genes known to contribute to solid tumor development (listed in the Supporting Information). We used these genes to interrogate our WES-identified CNAs and SNPs to determine whether these common cancer drivers were altered in our BWS HB cohort. We observed a limited number of mutations overlapping with these known cancer-causing genes in our BWS HB, with the greatest number of mutations identified in HB1 and HB4 likely due to their lack of matched nontumor (Figure 1C). *CTNNB1* exon 3 was the only region of recurrent alteration identified in 6 of 7 (86%) samples (Figure 1C): Three of 7 (43%) were non-synonymous substitutions; 1 of 7 (14%) was an in-frame insertion; and 2 of 7 (29%) were in-frame deletions.

BWS HB predisposition transcriptome network

We next investigated transcriptome changes associated with BWS HB. Specifically, we wanted to identify the factors that work alongside *CTNNB1* in 11p15-dysregulated HB. To achieve this, we performed Gene Set Enrichment Analysis (GSEA) in a pairwise manner. While few pathways reached statistical significance, several demonstrated stronger expression correlations based on groups (i.e., KEGG_CELL_CYCLE, SMAD_PROTEIN_SIGNAL_TRANSDUCTION, JNK_CASCADE, and MAPK_CASCADE in BWS HB compared with BWS nontumor as well as JNK_CASCADE and JAK_STAT_SIGNALING in BWS nontumor compared with controls) (Figure 2A and Table S2). As BWS is an imprinting disorder caused by aberrant DNA methylation and chromatin organization, we also tested gene sets related to these terms. Of these, CHROMATIN_REMODELING, HISTONE_METHYLATION, as well as genomic imprinting factors were enriched in BWS tissue (Figure 2A and Table S2).

To visualize differentially expressed genes from these signaling pathways, we developed a heatmap of the 640 genes with the strongest correlation (Figure 2B). Notably, a subset of 139 genes was similarly expressed between BWS HB and BWS nontumor, but up-regulated or down-regulated comparing BWS and controls (Figure 2B). We highlight these genes

in brackets in Figure 2B and propose these genes as BWS HB predisposition factors, as their dysregulation is not restricted to the cancerous tissue.

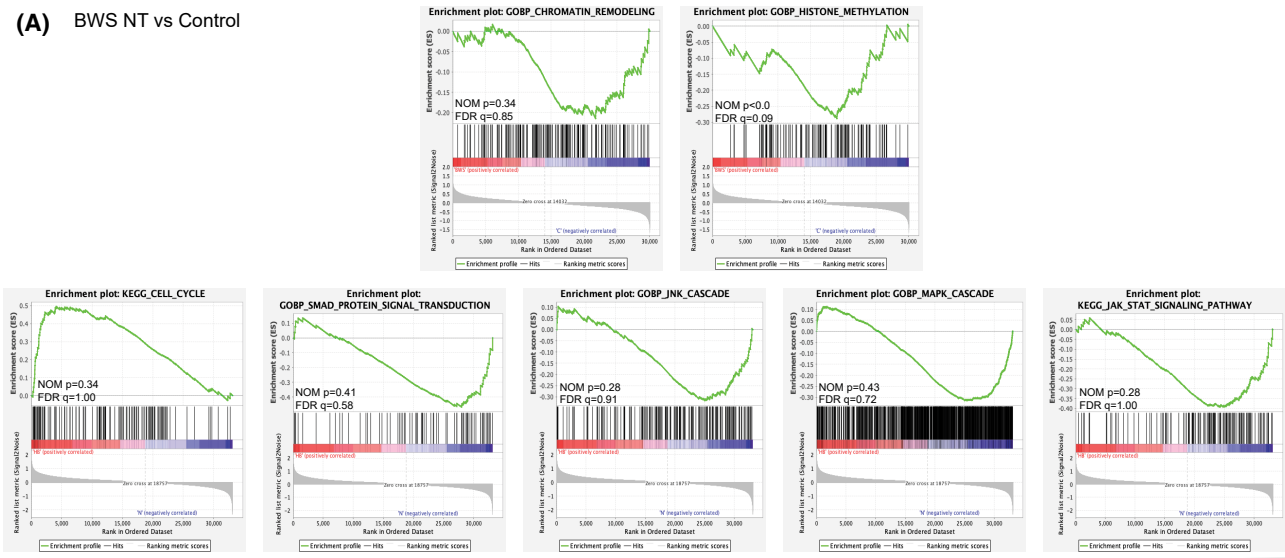
To understand how these 139 genes work together to influence BWS HB predisposition, we generated a protein–protein interaction (PPI) network (Figure 3A, Figure S3A). The top 10% of interactions were made by the following 10 hub genes: up-regulated *MYC*, *tumor growth factor beta R2 (TGFB2)*, *histone deacetylase 1 (HDAC1)*, mitogen-activated protein kinase (*MAPK8*), and *interleukin-2 (IL-2)*, and down-regulated *HRAS*, endothelial growth factor, *IL-6*, *SHC adaptor protein 1*, and *WNT1* (Figure 3A,B). There were an additional 18 BWS HB predisposition network genes significantly differentially expressed between the BWS HB/nontumor versus control samples (Figure S3B). Of these, eight function in chromatin organization and/or cell-cycle regulation (growth factor independent 1B transcriptional repressor (*GFI1B*), *RNA polymerase-associated protein CTR9 homolog*, *ubiquitin specific peptidase 8*, *ATP-dependent helicase X (ATR)*, *lysine methyltransferase 2E (KMT2E)*, *RTF1*, *F-Box and WD repeat domain containing 7 (FBXW7)*, and AT-Rich interaction domain 4A (*ARID4A*)) and three are associated with MAPK and/or PI3K/AKT signaling (erythropoietin, coiled-coil domain containing 88A, and histone deacetylase 3) (Figure S3). In fact, of the GSEA pathways initially enriched in our analysis, chromatin organization and cell-cycle regulation were the two most represented processes in this network (Figure 3C and Figure S3A). Overall, 77% of the predisposition factors belong to these pathways and/or insulin response, WNT, MAPK, and PI3K/AKT signaling pathways, even though the overall WNT and PI3K/AKT signaling pathways were not statistically significant (Figure 3C and Figure S3A).

BWS HB oncogenesis transcriptome network

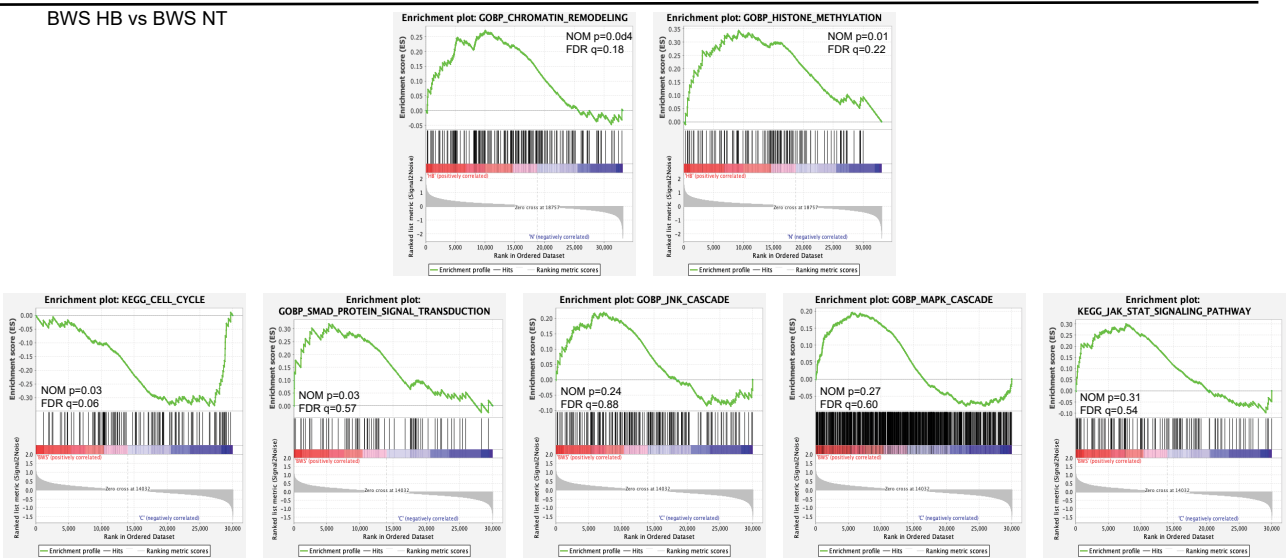
Previously Carrillo-Reixach et al. presented a multi-omic analysis of sporadic HB.^[28] We wanted to use these data to explore how BWS HB development might be similar or distinct from that of nonsyndromic HB. First, we needed to confirm that none of the samples were derived from patients with an 11p15 alteration indicative of congenital BWS. Comparing nontumor samples from this independent cohort with the controls generated in our cohort, we observed IC1 GOM in NT12, NT2, NT33, and NT7, but none of the samples exhibited robust IC2 LOM (Figure S4A). Although it is possible that these samples were derived from individuals with BWS, it is unlikely that all 4 of these patients had congenital BWS before HB development based on established (epi)genotype–phenotype correlations.

Previously, Carrillo-Reixach et al. evaluated their cohort for 11p15 LOH; 41% of the HB exhibited this

(A) BWS NT vs Control



BWS HB vs BWS NT



(B)

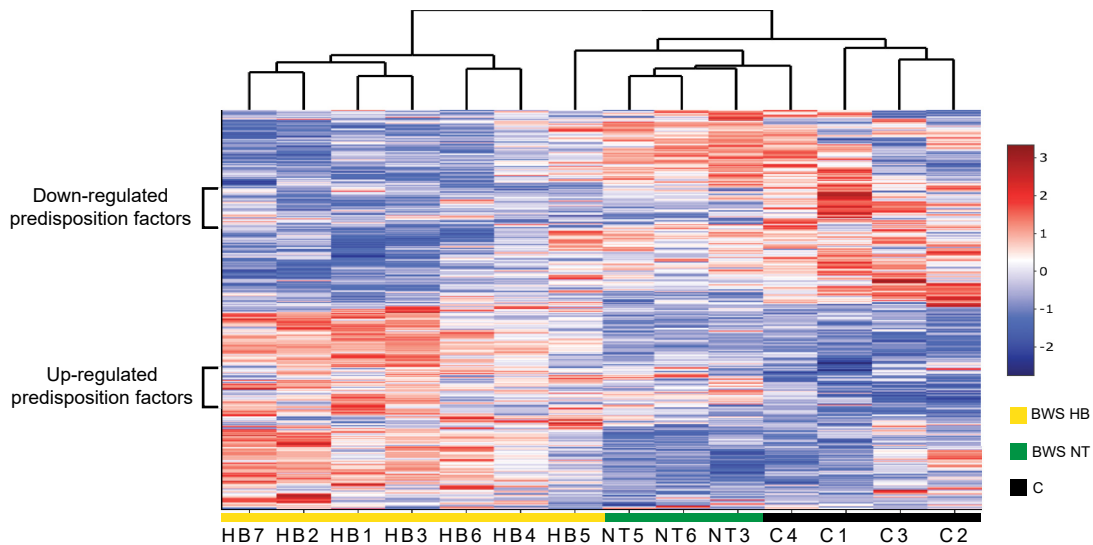


FIGURE 2 Transcriptome features of BWS liver and HB. (A,C) Preranked Gene Set Enrichment Analysis (GSEA) plots of enriched signaling pathways comparing expression of BWS HB with BWS NT liver (A) or of BWS NT with controls (C). False discovery rate (FDR) q value and nominal (NOM) p values are displayed. (B) Heatmap of genes differentially expressed between BWS HB, BWS NT, and controls identified through GSEA. Genes with similar expression levels between all BWS samples compared with controls are highlighted by the brackets on the left

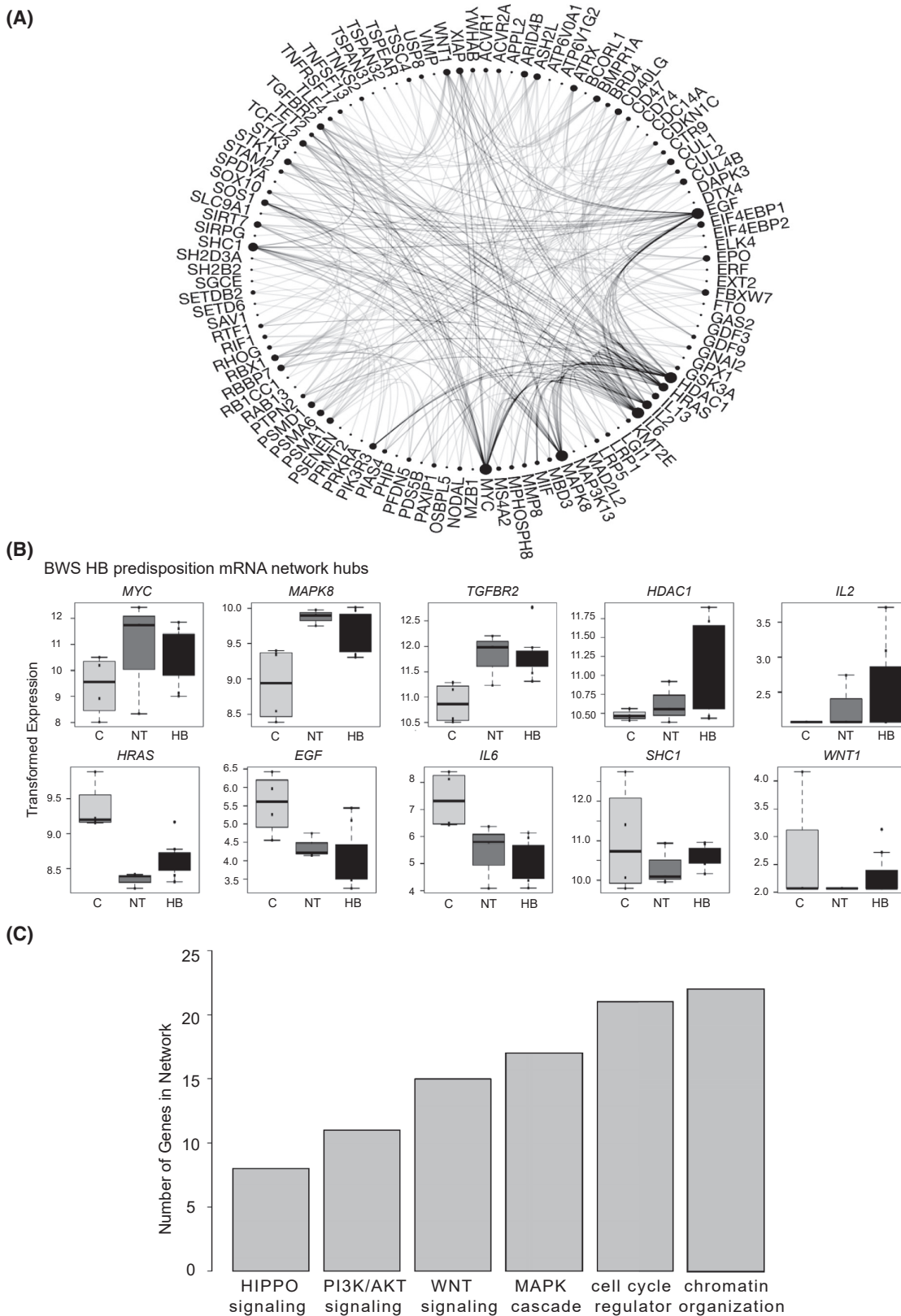


FIGURE 3 Features of expression factors comprising the BWS HB predisposition network. (A) Protein–protein interaction (PPI) of the 139 genes with similar expression between BWS HB and NT with differential expression in controls. Hub genes are indicated by the proportionally large number of interactions. (B) Expression of hub genes in control (C), BWS NT, and BWS HB samples. Variance stabilizing–transformed read counts detected by RNA-sequencing (RNA-seq) are shown. (C) Pathways represented in the predisposition PPI network based on the number of genes attributed to the biological process

structural alteration including tumors T12, T2, T33, and T7.^[28] We confirmed aberrant methylation in these samples as well as seven additional samples; Nineteen of 28 (68%) of the nonsyndromic tumors presented with BWS-like 11p15 alterations (Figure S4B). The samples with normal 11p15 methylation and no pUPD11 were T4, T5, T6, T9, T14, T15, T16, T17, and T18 (Figure S4B). To directly compare the BWS HB samples with these nonsyndromic HB, we performed a batch correction as described in the Supporting Information. After correction, we performed GSEA to compare HB-carrying 11p15 alterations, including those derived from patients with BWS, to HB with normal 11p15 methylation and structure. Here, KEGG_CELL_CYCLE, CHROMATIN_REMODELING, RAS_SIGNAL_TRANSDUCTION, MAPK_CASCADE, JNK_CASCADE, and JAK_STAT_SIGNALING were significantly differentially expressed between the groups (Figure 4A and Table S2).

Again, we visualized the top genes with the strongest expression correlation. These 303 genes cluster the HB without 11p15 alterations together on the right, while most of the nonsyndromic HB carrying 11p15 changes cluster with the BWS HB on the left (Figure 4B). The BWS HB formed three pairs that were not based on 11p15 status (i.e., whether aberrant methylation or pUPD11) (Figure 4B, Figure S2A,B, and Table 1). As the BWS HB did not form a single, distinct cluster, they are likely transcriptionally similar to those arising in a nonsyndromic background with spontaneous 11p15 changes (Figure 4B).

Of the 303 differentially expressed genes, 54 genes overlap with the analysis presented in Figure 2A (Figure 5A). To better understand the relationship between these genes identified by multiple comparisons, we generated a BWS HB oncogenesis PPI network (Figure 5B and Figure S5A). Supporting the similarity between BWS and nonsyndromic HB in the broader expression profile (Figure 4B), we identified the following hub genes: 11p15-down-regulated *janus kinase 2 (JAK2)*, *forkhead box O3 (FOXO3)*, and proto-oncogene *KIT*, 11p15-up-regulated proto-oncogene *SRC*, and similarly expressed among all HB is cyclin dependent kinase inhibitor 2A (*CDKN2A*) (Figure 5B,C). There were an additional 10 genes significantly differentially expressed between HB stratified by 11p15 status (Figure S5B). While several cell-cycle regulator and chromatin organization factors are represented in this network, a larger number of genes represent WNT, MAPK, PI3K/AKT, and JAK–signal transducer and activator of transcription (STAT) signaling factors (Figure 5C and Figure S5A).

Although these results suggest how BWS HB oncogenesis might be distinct from HB without 11p15 imprint disruption, we also wanted to determine how similar the BWS HB transcriptome might be compared with other HB. To this end, we tested whether gene signatures proposed to stratify HB in previous publications were

enriched in BWS HB (Figure S6A–E). Aside from the cell population and epigenetic machinery stratification proposed by Rivas et al.,^[36] the signatures were enriched in BWS HB samples (Figure S6A–E and Table S2).

miRNA regulation of BWS HB networks

Previously, miRNA dysregulation has been shown to contribute to HB oncogenesis, such as through WNT signaling and MYC regulation.^[37] As with the messenger RNA analysis, we wanted to determine whether previously proposed HB miRNA signatures were conserved in BWS HB. None were well-correlated with BWS HB miRNA expression patterns (Figure S6F–H and Table S2). To elucidate miRNAs that might play a more prominent role in BWS HB, we identified those miRNAs predicted to bind BWS HB predisposition and oncogenesis factors.

For the BWS HB predisposition and oncogenesis networks, 89 and 169 predicted that regulatory miRNAs were differentially expressed, respectively. Several miRNAs were predicted to co-regulate hub and significant factors to form three predisposition networks and four oncogenesis networks (Figure S7A,B). Of these miRNAs, 19 stem-loop structures (*miR-22*, *miR-30b*, *miR-33b*, *miR-92a-1*, *miR-100*, *miR-139*, *miR-203a*, *miR-205*, *miR-449b*, *miR-450a-2*, *miR-490*, *miR-579*, *miR-676*, *miR-3622a*, and *miR-4728*) were ranked with high annotation confidence by miRBase. *miR-30b-3p* and *miR-3622a-5p* were predicted components of both the predisposition and oncogenic networks. *miR-30b-3p* was up-regulated and may contribute to the down-regulation of 14 BWS HB predisposition factors as well as BWS HB oncogenesis hub genes *JAK2* and *FOXO3* (Figure S7A–C). *miR-3622a-5p* was down-regulated, which may contribute to the up-regulation of hub genes *MAPK8*, *HDAC1*, and *CDKN2A* (Figure S7A–C). Also of note, *miR-139* and *miR-490* were predicted to co-regulate the previously mentioned dickkopf WNT signaling pathway inhibitor 1 and 4 (*DKK1/4*), *WNT16*, and *SRC* members of the BWS HB oncogenesis network (Figure S7B and Figure 5B).

Epigenetic regulation of BWS HB networks

Another mechanism of gene regulation that plays a role in HB development is DNA methylation. At a global level, the HB methylome exhibits hypomethylation compared with nontumor livers; more specifically, while CpG islands become hypermethylated, the rest of the genome loses methylation.^[28] Global hypomethylation was observed in BWS HB compared with both BWS nontumor and controls, but was not statistically

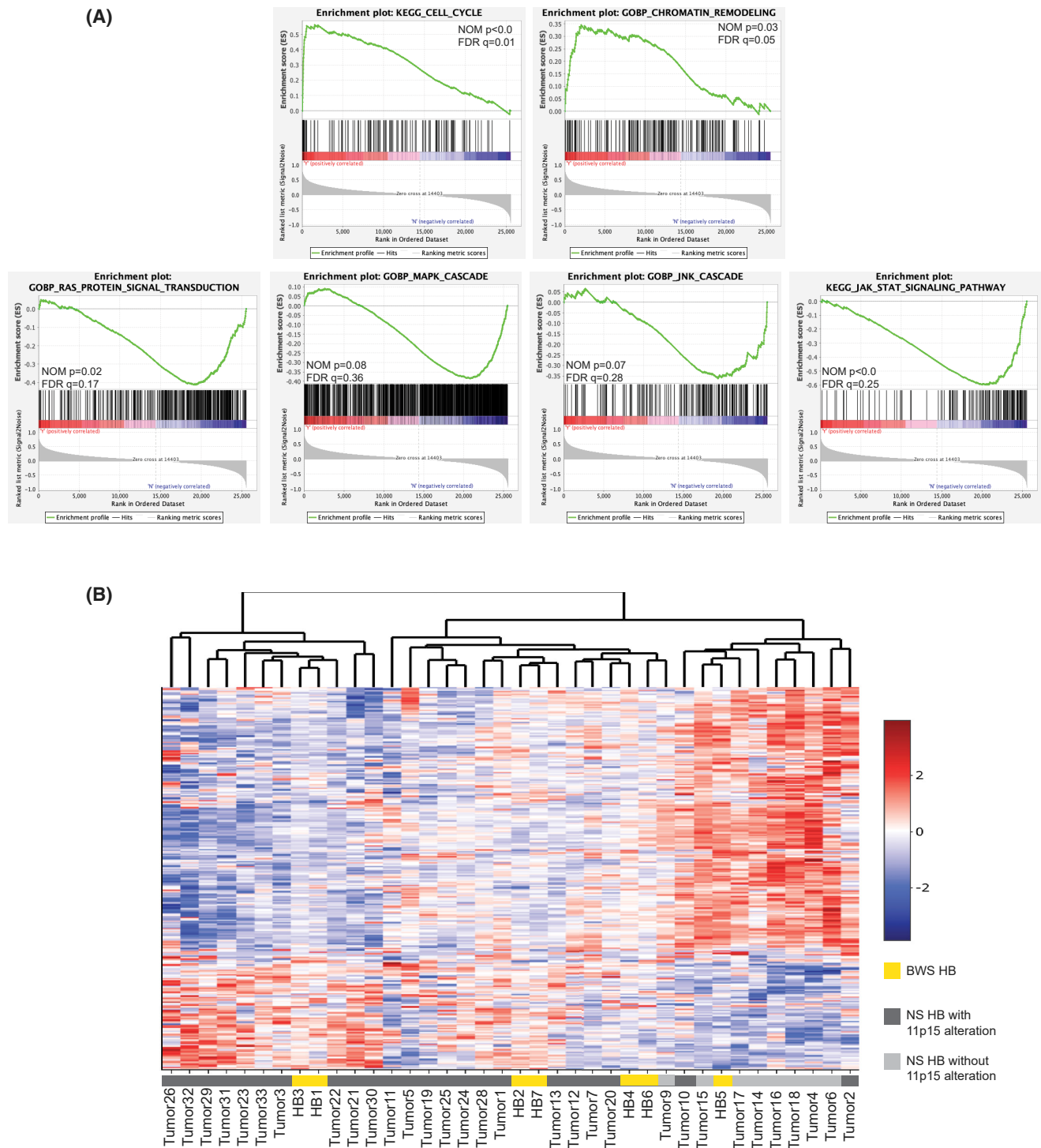


FIGURE 4 Transcriptome features of 11p15-altered HB. (A) Preranked GSEA plots of enriched signaling pathways comparing expression of BWS HB and nonsyndromic (NS) HB with 11p15 alterations versus nonsyndromic HB without 11p15 changes. FDR q value and NOM p values are displayed. (B) Heatmap of genes differentially expressed between HB groups stratified by 11p15 status identified through GSEA

significant (Figure 6A). For further classification of the BWS HB methylome, we looked at regions designated as CpG islands, shores, shelves, and open sea. While significantly differentially methylated probes at CpG islands were hypermethylated between BWS HB and nontumor, hypomethylation of significantly differentially

methylated probe mapping to shores, shelves, and open sea was observed (Figure 6B; Tables S3 and S4).

When we compared BWS HB methylation with that of nonsyndromic HB without 11p15 alterations, GSEA revealed that genes with histone H3 trimethylation of lysine 27 (H3K27me3) promoter-enrichment

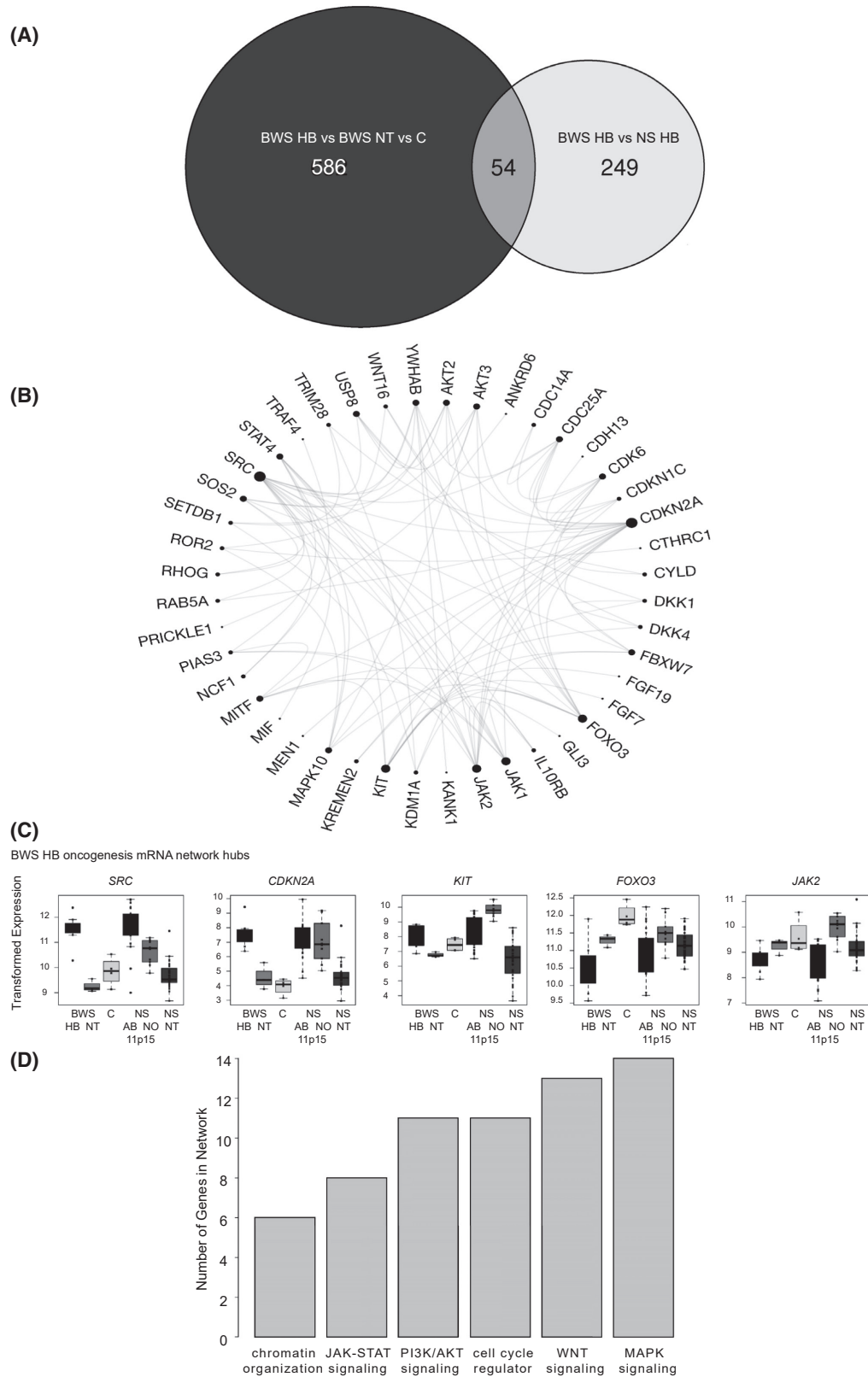


FIGURE 5 Features of expression factors comprising the BWS HB oncogenesis network. (A) Venn diagram comparing the genes identified in the pairwise comparison among BWS HB, BWS NT, and control (C) comparison (black), and in the BWS HB versus nonsyndromic (NS) HB comparison (white). (B) PPI of the 54 genes central to the Venn diagram. Hub genes are indicated by the proportionally large number of interactions. (C) Expression of hub genes in control, BWS NT and HB, and NS with normal (NO) or aberrant (AB) 11p15 methylation samples. Variance-stabilizing transformed read counts detected by RNA-seq are shown. (D) Pathways represented in the oncogenesis PPI network based on the number of genes attributed to the biological process. mRNA, messenger RNA

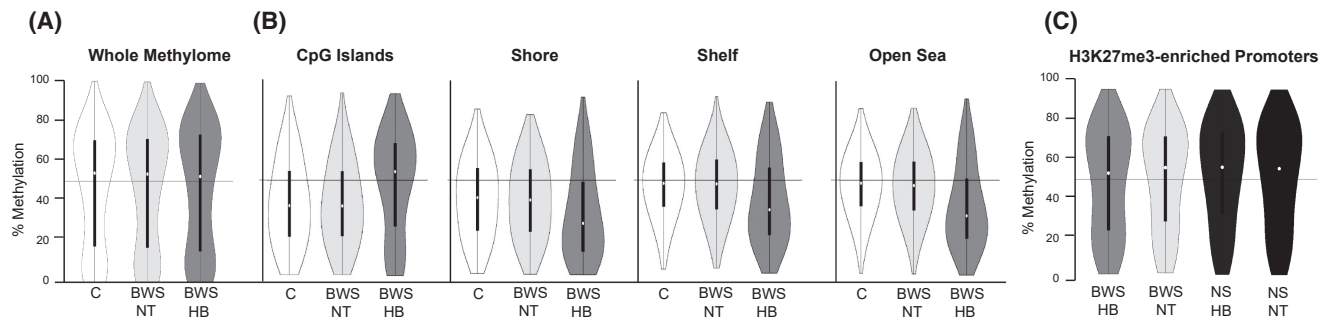


FIGURE 6 Methylation features of BWS HB. (A) Violin plot of the global methylation of BWS HB compared with NT and controls (one-way analysis of variance: $df = 2$, $F = 2.67$, p value = 0.07). (B) Violin plots of the methylation levels at CpG islands, as well as the shores, shelves, and at open sea. (C) Violin plots of the methylation levels of genes known to have H3K27me3 at the promoter in BWS HB and NT compared with NS HB and NT. White circle represents the average methylation level

also displayed statistically significant hypomethylation (Figure 6C and Table S5). At these probe sites, methylation levels in the matched nontumor samples from both BWS and nonsyndromic backgrounds were more similar to those of nonsyndromic HB (Figure 6C). However, these H3K27me3-promoter genes did not match those identified in the BWS HB predisposition or oncogenesis networks (Table S5). In fact, only four probes with differential methylation mapped to genes from these networks, and each mapped to a different gene (Tables S3 and S4). As such, it is unlikely that changes to the BWS HB methylome, aside from those at 11p15, drive BWS HB.

DISCUSSION

It has been established that changes to human chromosome 11p15 are associated with HB development [5–8,10–12]; however, a mechanistic link to the oncogenic process has yet to be elucidated. In this study, we present a multi-omic profile specific to HB occurring in the BWS background and the molecular changes associated with 11p15 alterations in liver oncogenesis. While our cohort is modestly sized compared with others describing the molecular nature of sporadic HB, this report represents an important step toward understanding the effect of 11p15 on growth regulation.

Data in this report point to BWS-associated 11p15 alterations acting in conjunction with cell cycle and chromatin regulators as well as *MYC* and its targets to create an environment of liver overgrowth (Figures 2 and 3). Up-regulation of *MYC* is a “hallmark” of general cancer development.^[38] *CTNNB1*, a commonly mutated driver of HB, is known to act as a transcription factor to increase levels of *MYC*; however, the 11p15-imprinted *KCNQ1* has been observed to regulate both factors.^[39] Decreased levels of *KCNQ1* may therefore contribute to increased *MYC* in BWS liver before altered *CTNNB1* activity (Figure S2C and Figure 3B). Fukuzawa et al. previously noted that nuclear localization of *CTNNB1*

was specific to BWS HB and was not observed in BWS nontumor samples.^[25] As such, *CTNNB1* was suggested to be a late-acting factor, rather than a key cause of hepatomegaly and BWS HB predisposition.^[25] Absent of DNA methylation changes, we propose that chromatin regulators, such as SET domain containing 6, protein lysine methyltransferase, *HDAC1*, *GFI1B*, *KMT2E*, and *ATRX*, along with cell-cycle restrictors such as *FBXW7*, *ARID4A*, *cullin 1/2/3/4b*, and 11p15-imprinted *CDKN1C*, function with *MYC*-targeted transcription in normal, nontumor BWS liver to create the landscape for liver overgrowth and cancer development (Figure 3 and Figure S3B).

Our previous study of BWS hepatocytes differentiated from patient-derived induced pluripotent stem cells indicated that 11p15 alterations and insulin signaling dysregulation were associated with differential expression of *MYC* and *FOXO1*.^[40] Our current investigation of primary BWS HB demonstrated loss of expression of *FOXO3* (Figure 5C). Generally, this family of transcription factors induces cell-cycle arrest and, subsequently, apoptosis.^[41] Conflictingly, both high and low levels of *FOXO3* have been associated with liver oncogenesis; in contrast, *MAPK10* is frequently down-regulated in liver cancer and therefore thought to function as a tumor suppressor.^[42–44] As *MAPK10* is a predicted binding target of *FOXO3* and was also down-regulated in BWS HB tissue, we posit that the loss of these factors cooperatively restricts cell death in BWS HB development (Figure 5 and Figure S5B).

In addition to these BWS HB data, we present an investigation of sporadic HB stratified by 11p15 status. Previously, studies by Sumazin et al. and Sekiguchi et al. identified WNT-antagonists *DKK1/4* as part of their HB signatures.^[30,31] However, we demonstrate that HB with 11p15 alterations largely drive this trend (Figure 5C and Figure S5B). We also observed that overexpression of the *DKK1/4* membrane receptor kringle containing transmembrane protein 2, as well as *WNT16*, was specific to 11p15-altered HB both in BWS and nonsyndromic backgrounds (Figures 4B and 5B; Figure S5B).

It has previously been noted that the function of WNT antagonists is abrogated in HB carrying a *CTNNB1* mutation.^[45] As such, *WNT16* may independently promote cell division.^[46] *DKK4* has also been shown to promote liver cell proliferation in a high glucose environment^[47]; as such, early dysregulation of 11p15-imprinted *IGF2*, as previously proposed by Honda et al.,^[48] may contribute to this pro-proliferative process (Figures S1 and S2C).

This study also provides evidence of the combination of genomic, transcriptomic, and epigenomic changes that distinguish BWS HB from that of sporadic HB. Regarding the genome, the TMB between BWS HB and other HB was similar, but BWS HB mutations did not recurrently include HB-common genes axis inhibition protein 2, nuclear factor erythroid 2-related factor 2, BCL6 corepressor like 1, or 8q gains^[8,28,34] (Figure 1). Regarding the epigenome, significant differences in methylation have been observed in genes such as Ras association domain family member 1 and *suppressor of cytokine signaling 1 (SOCS1)*, which are correlated with prognostic outcomes of HB.^[49] We did not observe differential methylation of either gene in BWS HB, nor others commonly implicated in HB (Tables S3 and S4). Regarding the transcriptome, we observed increases of *IL-6* and *KIT* in 11p15-altered HB, but not to the same extent as other HB (Figure 5C). The relatively moderate expression in BWS HB of these two genes, which normally increase liver progenitor cell proliferation, may contribute to decreased BWS-associated malignancy.^[50]

Lastly and importantly, we observed that the BWS subtypes observed in blood did not always match the BWS subtypes observed in liver samples (Table 1 and Figure S2A,B), suggesting that tumor-risk stratification by blood epigenotype in BWS is not necessarily sufficient. This unexpected result requires further investigation to better understand its frequency and effect. However, while these data presented herein represent several potential targets for future BWS HB surveillance and treatment development, further work and confirmational cohort information are required before clinical care for this patient population can be modified. Additionally, further research into the individual contributions of the 11p15-imprinted protein-coding genes, miRNAs, and noncoding RNAs to liver and HB development is essential to understanding the 11p15-HB relationship. Our multi-omic profiling provides a map for these future studies.

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CONFLICT OF INTEREST

Nothing to report.

AUTHOR CONTRIBUTIONS

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SUPPORTING INFORMATION

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