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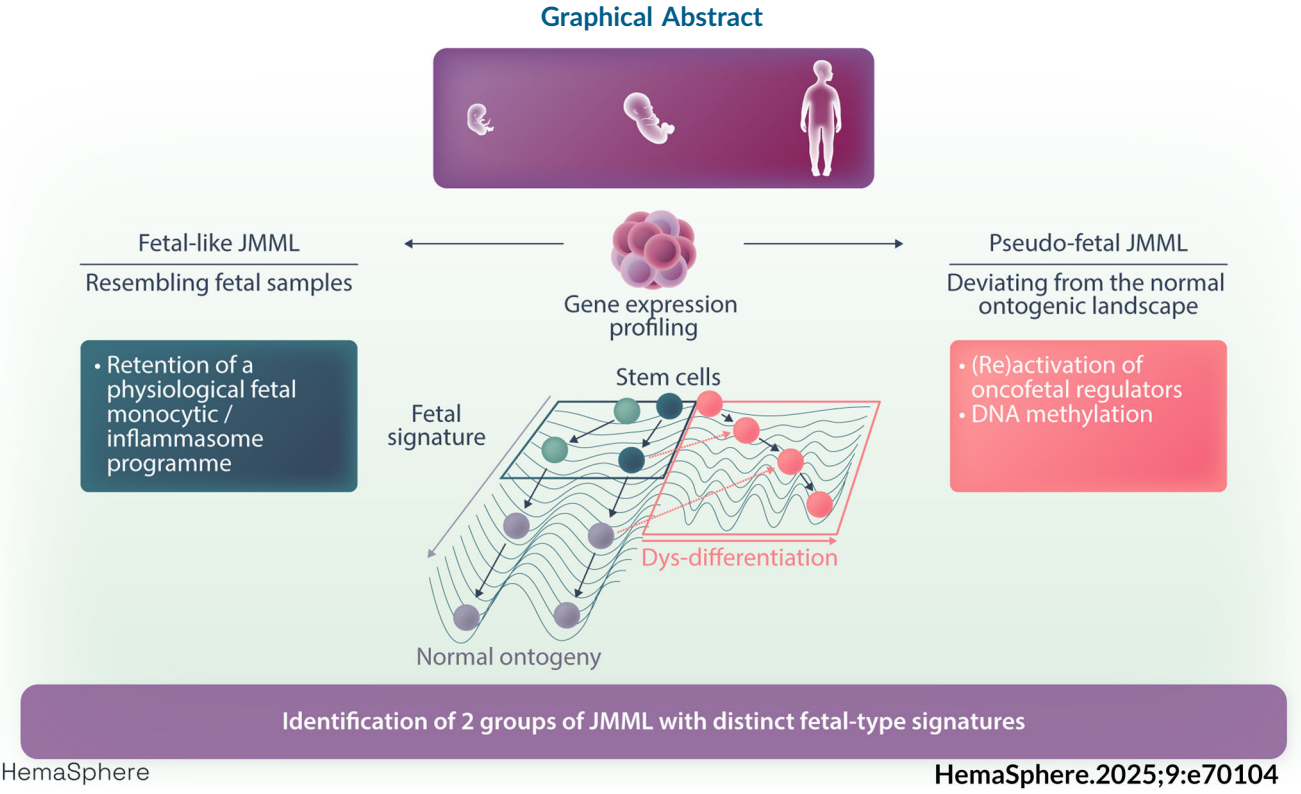
Graphical Abstract



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Two distinct fetal-type signatures characterize juvenile myelomonocytic leukemia

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Abstract

Juvenile myelomonocytic leukemia (JMML) is an aggressive clonal myeloproliferative neoplasm that affects infants and young children. The narrow window of onset suggests that age-related factors are involved in leukemogenesis. To investigate whether ontogeny-related features are involved in JMML oncogenesis, we compared the gene expression profile of hematopoietic progenitor cells isolated from JMML patients with that of healthy individuals at different stages of ontogeny. This analysis identified two main groups of JMML patients. In the first group, JMML progenitors exhibited a gene expression profile similar to that of embryo-fetal progenitors. Progenitors showed a strong monocytic identity as evidenced by the overexpression of monocytic/dendritic, inflammasome, and innate immune markers. This resembled the monocyte-predominant myelopoiesis characteristic of normal fetal hematopoiesis. However, in the second group, despite evidence of developmental dysregulation as indicated by the aberrant signature of the master oncofetal regulator LIN28B, JMML clustered separately from healthy prenatal and postnatal fractions. These findings highlight the intricate relationship between JMML and development, which will help inform future therapeutic approaches for this rare but severe form of leukemia.

It is increasingly recognized that a number of critical pathways and processes regulating developmental hematopoiesis are subverted to drive the initiation and/or evolution of hematological malignancies, particularly in children.[1] In juvenile myelomonocytic leukemia (JMML), a clonal aggressive myeloproliferative neoplasia (MPN) affecting infants and young children,[2,3] the need for a prenatal environment to support oncogenesis is suggested by narrow age window of onset, *in utero* initiation,[4] and low mutation burden.[5,6] Furthermore, fetal features such as high expression of fetal hemoglobin (HBF) and overexpression of *LIN28B*[7] a key developmental regulator that is highly expressed in fetal HSC[8] have been reported in a subset of JMML. Hematopoietic stem and progenitor cells in fetuses and adults adjust their functions through distinct transcriptional programs so as to best meet age-related needs, with a transcriptional switch occurring rapidly after birth.[8,9] To gain deeper insight into how the biology of JMML aligns with that of embryo-fetal hematopoietic cells, we conducted a comparative analysis of the gene expression profiles of JMML hematopoietic progenitor cells (HPCs) and their healthy counterparts across different developmental stages. This study was approved by our institutional review board (IRB-00006477), in accordance with the Helsinki declaration.

We established the transcriptomic profile of myeloid progenitor fractions (CMP, GMP, MEP) sorted from JMML ($n = 16$), fetal liver ($n = 3$), fetal bone marrow (BM) ($n = 2$) and children's BM ($n = 4$) (Figures S1 and S2A; Table S1) and validated them both transcriptionally and functionally (Figure S2B,C). Details are available in Supporting Information.

Unsupervised hierarchical clustering separated the samples into four groups (C1 to C4) (Figure 1A). Primary clustering was driven by ontogeny, with a first branching separating 14/15 healthy embryo-fetal fractions in C1 from healthy postnatal samples in C2 and C3. Strikingly, most JMML fractions clustered either in C1 with embryo-fetal

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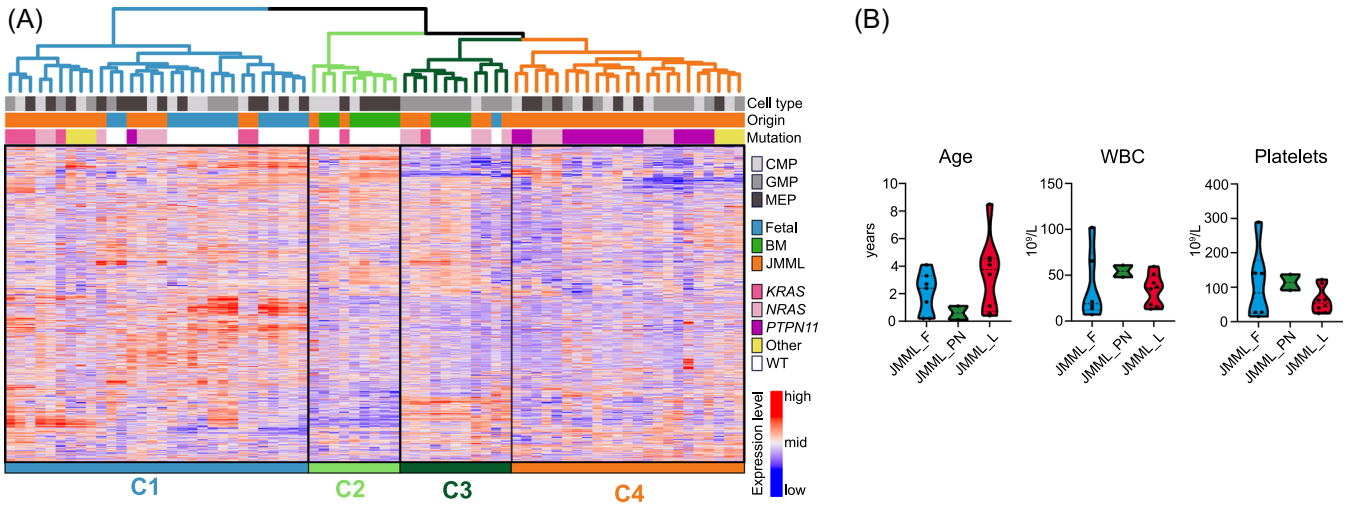

FIGURE 1 **Gene expression profile of JMML hematopoietic progenitors versus healthy prenatal and postnatal counterparts. (A)** Unsupervised hierarchical clustering of sorted JMML hematopoietic progenitors (CMP, GMP, MEP) and their pre- or postnatal counterparts according to gene expression profile. Four clusters (C1 to C4) were defined. C1 contains FL samples (n = 9/9), FBM samples (5/6), and 16/47 JMML samples (CMP n = 5, GMP n = 3, MEP n = 8) from 8/16 patients. C2 contains healthy BM, 7/7 samples (CMP n = 3/3, MEP n = 4/4), and 2 JMML samples (#12). C3 contains GMP from 4/4 healthy BM, 1/4 FBM, JMML GMP (5/16 samples), and 1 JMML CMP. C4 contains 23/47 JMML samples (CMP n = 8, GMP n = 8, and MEP n = 7) from 10/16 patients and no healthy fetal or postnatal tissue (see also Supporting Information S1: Table 5). BM, postnatal bone marrow; FBM, fetal bone marrow; FL, fetal liver; WBC, white blood cell count. **(B)** Patients' age and hematological features by JMML group (see also Supporting Information S1: Tables 1 and 2).

fractions (17/47 fractions from 8/16 patients) or in a separate group (C4) containing no healthy samples (23/47 samples from 10/16 patients). Only a few JMML fractions co-clustered with healthy BM. Removal of proliferation and cell cycle-associated transcripts (MSigDB_M2227) did not affect the clustering, indicating that the transcriptional proximity between some JMML and fetal HPC is not primarily driven by a higher proliferative state (data not shown).

Grouping of JMML patients according to preferential clustering (i.e., highest number of cell fractions in C1 with embryo-fetal fractions, in C2-3 with normal postnatal fractions, or in C4, respectively) allowed us to define three groups (Table S1). The first group, hereafter referred to as "JMML_Fetal" (JMML_F; 6/16), comprised JMML resembling embryo-fetal samples. A second group of JMML, hereafter named "JMML_later" (JMML_L; 8/16), comprised JMML that clustered apart from all healthy samples. A third JMML group contained only 2 JMML resembling healthy postnatal progenitors (JMML_Postnatal, JMML-PN).

We then analyzed in more detail and compared the two largest groups of cases: JMML_F and JMML_L, which together accounted for 14/16 JMML cases.

Patients with JMML_F tended to be younger and to display less severe hematological alterations (higher platelet count, lower WBC count, and lower dysplastic features) (Table S2; Figure 1B). Most (5/6) had a *RAS* mutation. Patients with JMML_L tended to be older, with a more severe presentation, systematic thrombocytopenia, and elevated HBF levels (TableS2; Figure 1B). All *PTPN11*-JMML were classified in this group.

In JMML_F progenitors, upregulated genes were strikingly dominated by components of the pyrin inflammasome and monocytic cell markers (Figure 2A; Table S3). Geneset enrichment analysis (GSEA) performed on MSigDB indexed pathways showed enrichment of 32/62 (52%), 294/485 (61%), and 61/95 (64%) genesets containing the terms "monocytes," "dendritic," and "inflammation," respectively (Table S4). Further analysis based on signatures classifying monocytes and dendritic cells (DC)[10] showed enrichment of JMML_F HPC in signatures of classic, non-classic, and intermediate monocytes and all types of conventional DC (Figure 2B; Table S5). Consistent with genes related to pyrin inflammasome being among the top upregulated genes, MSigDB-indexed inflammasome genesets were enriched in JMML_F progenitors, as well as an inflammasome signature recently reported to associate with oncogenic *KRAS*[11] (Figure 2B; Table S4 and S5).

Examination of monocytes, DC, and inflammation-related genes expressed in JMML_F HPC showed that some (*CD14*, *SCIMP*, *ARHGEF10L*, *CLEC10A*) were expressed in fetal liver HPC, whereas others (*CD300E*, *MEFV*) were physiologically absent from both prenatal and postnatal HPC (Figure 2C). A high level of correlation was found between overexpressed transcripts (Figure 2C), consistent with the activation of a physiological program.

Altogether, these analyses identify a subtype of JMML that resembles embryo-fetal healthy samples more than age-matched ones. This subtype is characterized by the persistence in HPC of a strong monocytic and pyrin inflammasome signature, which is present in physiological fetal hematopoiesis but normally disappears later in ontogeny. In these subtypes of JMML, the alleged *in utero* oncogenic RAS mutation is associated with abnormal postnatal retention of the transient HPC pyrin inflammasome activation that regulates fetal HSPC,[12] leading in turn to postnatal persistence of fetal-type differentiation characterized by a myelomonocytic bias in lineage output. It is remarkable that 3/6 of these JMML subtypes spontaneously resolved. Such a propensity to regress as the child grows, a well-recognized feature of congenital cancers,[1] is in line with the involvement of a physiological fetal developmental program in leukemogenesis.

Unlike JMML_F, the JMML_L group was not typified by a developmental origin and clustered apart from both prenatal and postnatal healthy samples. However, deregulation of fetal master regulators was prominent in this JMML subset, with *LIN28B* and *WT1* being in the top 3 upregulated genes (Figure 2A; Table S3). Both are considered fetal oncogenes as they are often overexpressed in malignancies and reactivate fetal pathways.[13] GSEA confirmed high

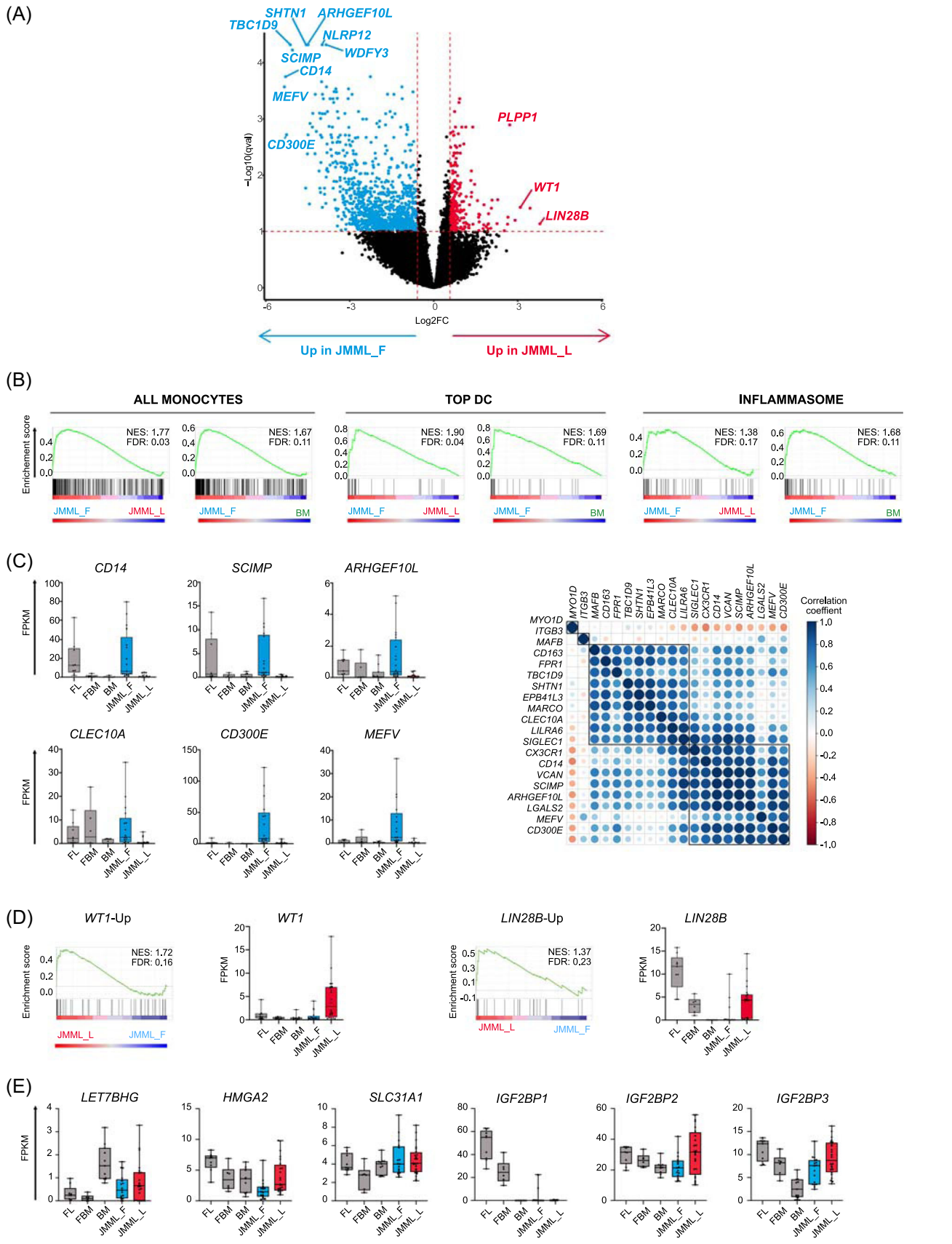

FIGURE 2 (See caption on next page).

FIGURE 2 **(A) Vulcano plot showing differentially expressed genes upregulated in JMML_F (left) or JMML_L (right) according to log2 fold change (*x*-axis) and *q*-value (*y*-axis).** Differential gene expression analysis between JMML groups evidenced 1052 upregulated genes with a fold change higher than 1.5 and a *q*-value lower than 0.1 in JMML_F versus 230 upregulated genes in JMML_L (listed in Table S3). Among genes most up-regulated in JMML_F, *MEFV*, *TBC1D9*, *NLRP12*, and *SCIMP* code proteins involved in the pyrin inflammasome whereas *CD14* and *CD300E* code monocytic markers. **(B–C) Gene expression profiling in the JMML_F group. (B)** GSEA plots show enrichment in monocyte, dendritic cells (TOP DC), and inflammasome signatures in the JMML_F group versus JMML_L (left) or versus healthy BM (right) (see also Table S4). **(C)** Histograms comparing gene expression (expressed as mean FPKM scores ±SD) of healthy samples across ontogeny (FL, FBM, BM), JMML_F and JMML_L (left panel). and correlation matrix of the 20 most upregulated genes in the JMML_F group (right panel). **(D, E) Gene expression profiling in the JMML_L group. (D)** GSEA plots show enrichment in LIN28B and WT1 signatures in JMML_L versus JMML_F. Histograms show expression levels (FPKM) of LIN28B (left) and WT1 (right) across ontogeny (FL, FBM, BM), JMML_F and JMML_L groups. **(C)** Histograms comparing gene expression of LIN28B transcriptional targets (expressed as mean FPKM scores ± SD) of healthy samples across ontogeny (FL, FBM, BM), JMML_F and JMML_L. BM, postnatal bone marrow; DC, dendritic cell; FBM, fetal bone marrow; FDR, false discovery rate; FL, fetal liver; FPKM, fragments per kilobase million; NES, normalized enrichment score; SD, standard deviation.

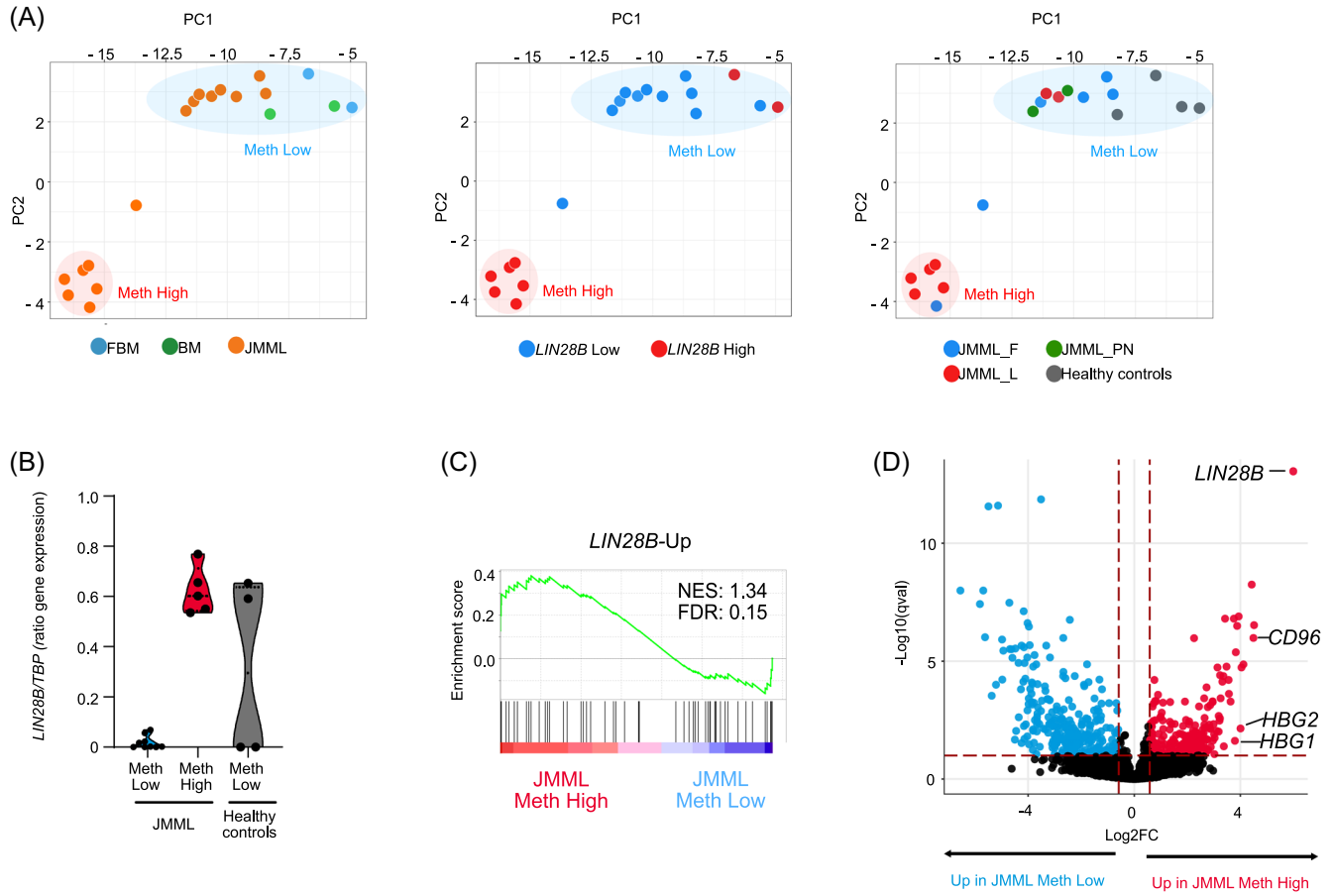

FIGURE 3 **DNA methylation analyses. (A)** Principal component analysis (PCA) of DNA methylation data obtained by RRBS for mononucleated cells from JMML samples ($n = 16$), fetal bone marrow (FBM; $n = 2$), and healthy postnatal bone marrow (BM; $n = 2$). The DNA methylation study distinguishes three groups of JMML according to low ($Meth^{low}$, $n = 8$), intermediate ($n = 1$), or high ($Meth^{high}$, $n = 6$) levels of methylation. JMML overexpressing *LIN28B* are all $Meth^{high}$ (medium panel). Right panel shows a relationship between methylation status and transcriptional-based groups. **(B)** LIN28B quantitation normalized by TPB expression using droplet digital PCR (ddPCR) shows that LIN28B expression is correlated with DNA methylation in JMML but not in healthy controls. **(C)** GSEA plot shows enrichment in the LIN28B signatures in $Meth^{high}$ JMML versus $Meth^{low}$ JMML. **(D)** Vulcano plot showing differentially expressed genes upregulated in $Meth^{low}$ JMML (left) or $Meth^{high}$ (right) according to log2 fold change (*x* axis) and *q*-value (*y* axis). BM, postnatal bone marrow; DC, dendritic cell; FBM, fetal bone marrow; FDR, false discovery rate; FL, fetal liver; FNES, normalized enrichment score; FPKM, fragments per kilobase million; SD, standard deviation.

enrichment of LIN28B[7,8] and WT1[14] expression signatures in JMML_L as compared with JMML_F (Figure 2D; Table S4).

LIN28B promotes the reprogramming of hematopoietic progenitors into a fetal-like state and thereby elevates HBF levels.[15] It determines the higher self-renewal potential of fetal HSC as part of the LIN28B-Let-7-HMGA2 axis.[8] Accordingly, Let7 tended to be downregulated and the top LIN28B targets (*HMGA2*, *IGFBP2*, *IGFBP3*) upregulated in the JMML_L, with mean expression levels comparable to those found in embryo-fetal fractions (Figure 2E). However, *IGF2BP1*, a major LIN28B target in fetal HPC[8] was not expressed in JMML_L (Figure 2E), although we confirmed it was the most differentially expressed gene between the prenatal and postnatal healthy HPC (Figure S3; Table S3). This observation reveals a partial discrepancy between the physiological LIN28B-driven fetal signature (Figure S3) and that found in JMML_L.

To investigate the overlap between our transcriptomic-defined JMML subsets and previously established JMML groups based on DNA

methylation,[16] we examined genome-wide DNA methylation using reduced representation bisulfite sequencing (RRBS) on total mononucleated cells from the 16 JMML, 2 healthy postnatal and 2 fetal BM (Figure S1). As expected, two main JMML groups (Meth^{high} and Meth^{low}) were delineated according to the level of hypermethylation (Figure 3A, left panel). JMML_F showed more often a Meth^{low} profile whereas JMML_L were mostly Meth^{high} (Table S1; Figure 3A right panel). Interestingly, similarly to the JMML_L group, the gene expression signature obtained in HPC from Meth^{high} JMML, was dominated by overexpression of *LIN28B*, and its signature (Figure 3B–D). Genes overexpressed in Meth^{high} patients included *HBG2*, *HBG1*, coding the gamma globin genes constitutive of HBF, and *CD96*[17] (Figure 3D; Table S3).

Remarkably, a strict correlation between DNA hypermethylation and *LIN28B* expression was observed in JMML samples but not in healthy fetal samples, which clustered with Meth^{low} JMML despite expressing *LIN28B* (Figure 3A, medium panel and 3B).

It has been postulated that *LIN28B* expression may be indicative of a "fetal-like" subtype of JMML.[7] Our data provide additional evidence for the existence of such a JMML subtype and its association with *PTPN11* mutations. However, it is striking that JMML with the most pronounced deregulation of *LIN28B* clustered apart from prenatal samples. In fact, comparison with healthy prenatal samples suggests that activation of the LIN28B-Let7-HMGA2 axis does not fully reactivate normal fetal physiology in these JMML subtypes. Indeed, as mentioned above, the LIN28B transcriptional signature in the JMML_L group differs from that of healthy fetal samples in subtle ways. Furthermore, the signature remains stable throughout the differentiation of HSPC and is associated with DNA hypermethylation, in contrast to what is observed in healthy fetal samples. Recent evidence that aberrant DNA methylation is a postnatal event, secondary to the genetic alterations in JMML patients,[4] suggests that LIN28B overexpression in JMML is mediated by a regulatory mechanism that is distinct from that observed during physiological development. This is reminiscent of a previous observation that in JMML, erythroid epigenetic changes in KLF1 lead to HBF overexpression by a mechanism distinct from that in healthy newborns.[18] These observations are consistent with the concept of "dys-differentiation,"[19] resulting from the misregulation of key developmental transcription factors.[19,20] The combination of regulatory elements from embryonic cells with those from other developmental stages results in "pseudo-fetal" JMML cells deviating from the normal ontogenic landscape.

Altogether, our findings show a strong involvement of ontogeny-associated features in JMML with at least two different patterns: either the retention of a physiological fetal signature or the aberrant activation of master oncofetal transcriptional regulators such as LIN28B.

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AUTHOR CONTRIBUTIONS

Conceptualization: Hélène Cavé, Michèle Souyri, and Dominique Bonnet. *Methodology*: Marion Strullu, Chloé Arfeuille, Aurélie Caye-Eude, Loïc Maillard, Elodie Lainey, Hélène Cavé, Michèle Souyri, and Dominique Bonnet. *Investigation/Resources*: Marion Strullu, Chloé Arfeuille, Aurélie Caye-Eude, Elodie Lainey, Florian Piques, Fabien Guimiot, Bruno Cassinat, Jean-Hugues Dalle, and André Baruchel. *Writing original draft*: Marion Strullu, Chloé Arfeuille, and Hélène Cavé. *Writing review and editing*: all authors. *Funding acquisition*: Hélène Cavé. *Supervision*: Hélène Cavé, and Michèle Souyri.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GEO at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE267628, reference number GSE267628.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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