RESEARCH ARTICLE

Genome-Wide DNA Methylation Analysis Identifies Novel Hypomethylated Non-Pericentromeric Genes with Potential Clinical Implications in ICF Syndrome

L. Simo-Riudalbas1, A. Diaz-Lagares1, S. Gatto2, M. Gagliardi2, A. B. Crujeiras1, R. Matarazzo2, M. Esteller1, J. Sandoval1

1 Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Catalonia, Spain, 2 Institute of Genetics and Biophysics ABT, CNR, Naples, Italy, 3 Department of Physiological Sciences II, School of Medicine, University of Barcelona, Barcelona, Catalonia, Spain, 4 Institutio Catalana de Recerca i Estudis Avancats (ICREA), Barcelona, Catalonia, Spain

☯ These authors contributed equally to this work.
¤ Current Address: Development, Aging and Regeneration Program (DARe), Sanford-Burnham Medical Research Institute, La Jolla, California, United States of America
‡ Current Address: Laboratory of Molecular and Cellular Endocrinology, Health Research Institute of Santiago (IDIS), University Hospital of Santiago (XXIS/SERGAS), Santiago de Compostela University (USC), Santiago de Compostela and CIBER Fisiopatologia de la Obesidad y la Nutrición (CIBEROBN), Madrid, Spain

Abstract

Introduction and Results

Immunodeficiency, centromeric instability and facial anomalies syndrome (ICF) is a rare autosomal recessive disease, characterized by severe hypomethylation in pericentromeric regions of chromosomes (1, 16 and 9), marked immunodeficiency and facial anomalies. The majority of ICF patients present mutations in the DNMT3B gene, affecting the DNA methyltransferase activity of the protein. In the present study, we have used the Infinium 450K DNA methylation array to evaluate the methylation level of 450,000 CpGs in lymphoblastoid cell lines and untransformed fibroblasts derived from ICF patients and healthy donors. Our results demonstrate that ICF-specific DNMT3B variants A603T/STP807ins and V699G/R54X cause global DNA hypomethylation compared to wild-type protein. We identified 181 novel differentially methylated positions (DMPs) including subtelomeric and intrachromosomic regions, outside the classical ICF-related pericentromeric hypomethylated positions. Interestingly, these sites were mainly located in intergenic regions and inside the CpG islands. Among the identified hypomethylated CpG-island associated genes, we confirmed the overexpression of three selected genes, BOLL, SYCP2 and NCRNA00221, in ICF compared to healthy controls, which are supposed to be expressed in germ line and silenced in somatic tissues.
Conclusions

In conclusion, this study contributes in clarifying the direct relationship between DNA methylation defect and gene expression impairment in ICF syndrome, identifying novel direct target genes of DNMT3B. A high percentage of the DMPs are located in the subtelomeric regions, indicating a specific role of DNMT3B in methylating these chromosomal sites. Therefore, we provide further evidence that hypomethylation in specific non-pericentromeric regions of chromosomes might be involved in the molecular pathogenesis of ICF syndrome. The detection of DNA hypomethylation at BOLL, SYCP2 and NCRNA00221 may pave the way for the development of specific clinical biomarkers with the aim to facilitate the identification of ICF patients.

Introduction

The immunodeficiency, centromeric instability and facial anomalies syndrome (ICF) is a rare recessive disorder, with less than 60 cases reported worldwide. ICF syndrome is characterized by two peculiar signs: a variable immunodeficiency and a recurrent instability of pericentromeric heterochromatin, which usually leads to chromosome breakage in mitogen-stimulated lymphocytes. The chromosomal abnormalities are found exclusively in hypomethylated pericentromeric regions of chromosome 1, 16 and less frequently in 9. Other ICF symptoms count in facial anomalies, psychomotor and mental retardation and developmental delay [1].

The importance of ICF pathology, at the molecular level, relies on the fact that it is the only human disease showing mendelian inheritance of aberrant DNA methylation, caused by mutations in one of the three main DNA-methyltransferase genes, DNMT3B. Approximately 50% of the ICF cases, defined as ICF type1, present biallelic DNMT3B mutations located mainly in the catalytic domain of the protein, often leading to the impairment of its methyltransferase activity [2]. Among the rest of patients some carry nonsense mutations in zinc-finger and BTB domain-containing 24 gene (ZBTB24), designated as ICF2 patients, while a small group of them has still unknown etiology, and are designated as ICFX [2].

The biochemical defects in DNMT3B-mediated de novo DNA methylation have been recently assessed by in vitro studies of the ICF-associated DNMT3B variants [3]. These results reveal that catalysis by DNMT3B is much more complex than expected. In that context, ICF mutations cause a broad spectrum of biochemical defects in DNMT3B function, including defects in homo-oligomerization, SAM binding, SAM utilization and DNA binding [3].

Although it is well established in literature that all ICF1 patient derived cells exhibit targeted DNA hypomethylation of the pericentromeric heterochromatin at chromosomes 1, 16 and sometimes 9, the molecular defects in global DNA methylation caused by ICF-specific DNMT3B mutants remain relatively uncharacterized. Subtle differences in local DNA methylation patterns have been undetected by less sensitive assays previously employed, such as two-dimensional gel electrophoresis of methylation-sensitive digested genomic DNA and COBRA analysis [4],[5]. Recently, several studies suggested that DNA hypomethylation might be more extended than previously thought [6][7]. In this sense, previous genome-wide work from our laboratory, detected DNA hypomethylation in the inactive heterochromatic regions, satellite repeats and transposons associated to two heterozygous DNMT3B mutations in one ICF1 patient [8].
In this study, a state-of-the-art DNA-methylation high-resolution microarray (*Human-Methylation450k BeadChip*) from Illumina (San Diego, CA) that interrogates 450,000 CpGs sites in the human genome was used to find out the impact of four different ICF-specific DNMT3B mutant alleles (A603T/TP807ins and V699G/R54X) on DNA methylation at a global level. This will help us elucidate and understand the potential pivotal role of this heterogeneous epigenetic mechanism in ICF syndrome pathogenesis. The identification of potential biomarkers for ICF patients after validation in peripheral blood samples of ICF patients might allow us to design more effective strategies to address the diagnosis or treatment of this disease.

**Materials and Methods**

**Sample preparation**

The current analysis was performed by evaluating one lymphoblastoid (LCL) cell line and untransformed fibroblasts derived from two different ICF patients with compound and different heterozygous mutations in DNMT3B gene (PT5 and GM08747) compared to LCLs (XX and MS) and fibroblasts (3674) derived from 3 unrelated controls. PT5 sample comes from an ICF male with heterozygous DNMT3B mutations (V699G/R54X). GM08747 comes from a female diagnosed for ICF syndrome with heterozygous DNMT3B mutations (A603T/STP807ins). The healthy donors samples belonged to two females (XX, GM03674) and one male (MS). All of these cell lines were obtained from Dr. RS Hansen laboratory (University of Washington, USA) and the Coriell Cell Repository and have been used in a previous published article [9]. Additionally, DNA from mononuclear cells isolated from four cord blood donors (three males and one female) was kindly provided by Dr. D. Monk from PEBC (IDIBELL, Spain). For validation steps, we extended the cohort with three lymphoblastoid cell lines: GM08714 from the ICF female patient with mutations A603T/STP807ins (see above) and its related healthy donors (GM08728 and GM08729), identified as the GM08714’s mother (GM08728) and father (GM08729) and another primary fibroblast PT3 from the ICF male with heterozygous DNMT3B mutations (V699G/R54X). Another unrelated LCL control was used (LDA). All these cell lines were also obtained and purchased from the Coriell Cell Repository. The project has been approved by the local Ethical Committee of the IDIBELL Institution.

**Genome-wide DNA methylation analysis**

Genome-wide DNA methylation analysis was performed using the Infinium HumanMethylation450 BeadChip from Illumina. The 450K DNA methylation array by Illumina is an established, highly reproducible method for DNA methylation detection and has been validated in two independent laboratories [10],[11].

DNA from ICF patients and healthy donors were isolated using Phenol:Chloroform:isoamylalcohol (Sigma) and quantified by Quant-iT PicoGreen dsDNA Reagent (Invitrogen). The integrity was analyzed in a 1.3% agarose gel. Bisulfite conversion of 600 ng of each DNA sample was performed according to the manufacturer’s recommendation for Illumina Infinium Assay. Effective bisulfite conversion was checked for three controls that were converted simultaneously with the samples. Four μl of bisulfite converted DNA were used to hybridize on Infinium Human Methylation 450 BeadChip, following Illumina Infinium HD Methylation protocol. Chip analysis was performed using Illumina HiScan SQ fluorescent scanner. The intensities of the images were extracted using GenomeStudio (2010.3) Methylation module (1.8.5) software (San Diego, California). Methylation score of each CpG is represented as beta (β) value. The 450K DNA Methylation array includes 485,764 cytosine positions of the human genome that were filtered by sex chromosomes CpGs (avoiding sex link alterations) and non-valid CpGs (p-value<0.001). The intensities of the images were extracted and normalized.
using GenomeStudio (2011.1) Methylation module (1.9.0) software. Unsupervised (using the 5000 random CpGs) and supervised heatmaps were obtained using hierarchical clustering analysis with Manhattan metrics.

For determining differentially methylated CpGs a parametric analysis using an absolute difference in beta values of 0.65 and standard deviation <0.15 in ICF1 patients compared to controls were used for selecting the most relevant positions.

**Bisulfite genomic DNA sequencing**

The Methyl Primer Express v1.0 software (Applied Biosystems, Life technologies, Grand Island, New York) was used to identify the CpG islands in gene promoter regions and to design specific primers for the methylation analysis (S1 Table). DNA methylation status was established by bisulfite genomic sequencing. DNA was extracted from samples using the DNeasy tissue kit (Qiagen, Milan, Italy) and 1 μg of DNA was modified with sodium bisulfite using the EZ DNA methylation-gold kit (Zymo Research, CA USA) according to manufacturer’s instructions. Multiple clones were analyzed for each sample and the methylation frequency was calculated in each case.

**Gene expression analysis**

Total RNA from lymphoblastoid cell lines and untrasformed fibroblasts was extracted using TRizol reagent (Life Technologies. Grand Island, New York) and was reverse-transcribed using iScript cDNA Synthesis kit (Bio-rad). Quantitative real-time PCR (qRT-PCR) was performed using iQ Supermix SYBR Green 2X (Bio-rad. San Diego, California) on the Bio-Rad iCycler according to the manufacturer’s protocols. The $\Delta\Delta Ct$ method [12] was used to determine relative quantitative levels. GAPDH gene was used to normalize the data. Primer sequences for gene expression analysis are shown in (S2 Table). Statistical analysis was performed using Student t test.

**Results and Discussion**

ICF-specific DNMT3B variants cause a global decrease in DNA methylation profile

Seminal studies regarding DNA methylation in ICF1 patients have reported significant hypomethylation at pericentromeric satellite DNA sequences in ICF cells of chromosomes 1, 9, 16, Alu sequences, D4Z4 and NBL2 repeats. Heterogeneous hypomethylation has been described at few single copy loci, X-linked and imprinted genes, while the genomic hypomethylation in ICF1 patients has been thought to be involved only in a rather small percentage of the 5-methylcytosine residues [4],[13–17]. Recently, a genome-wide DNA methylation analysis has been performed in our laboratory with the limitation of using a unique patient sample [8].

Since the majority of ICF patients deal with mutations within the DNMT3B catalytic domain expected to variably interfere with the methyltransferase activity of the protein, the primary goal of this study was to describe the global DNA methylation profile affected by ICF specific DNMT3B mutant alleles. Here, we characterized the methylome of one lymphoblastoid cell line and of untrasformed fibroblasts derived from two different compound heterozygous ICF patients with the DNMT3B mutations V699G/R54X and A603T/STP807ins (PT5 and GM08747, respectively) compared to three control LCLs (XX, MS) and fibroblasts 3674 derived from healthy donors. Using this strategy that includes ICF patients derived from different tissue types, the variability and interference due to tissue-specific genes, will be reduced. The analysis, by calculating first the averaged Beta values for each CpG from the three controls and
average Beta values from the two ICF patients and later the delta values (average ICF-average controls), shows that ICFs globally contain more poorly methylated ($\beta$ value $<0.33$) and less highly methylated CpGs ($\beta$ value $>0.66$) compared to controls (Fig. 1A). In this sense, the accumulated number of poorly methylated CpGs ranging Beta values from 0 to 0.33 of ICF patients is 215,227; while for controls decrease to 202,003 (Delta ICF-Control = +13,224). However, an opposite pattern is obtained for highly methylated CpGs ranging from Beta values 0.66 to 1. In this case, control donor showed 143,108 highly methylated CpGs compared to lower number for ICF patients 138,873 CpGs (Delta ICF-Control = -4,235) (Fig. 1B). A more comprehensive representation is the scatter plot of the DNA methylation levels ($\beta$ value) of ICF patients compared to controls showing a higher accumulation of hypomethylated CpGs in ICFs than in controls, see triangle area in Fig. 1C. Confirming these results we observed, using a non-parametric Mann-Whitney U test after testing normality with the Shapiro-Wilk test, a significant decrease in methylation level in ICF samples compared to controls (Fig 1D). We provide

**Fig 1.** Genome-wide DNA methylation profiles in ICF patients and control samples. (A) Histograms shows bimodal distribution pattern of DNA methylation profiles in ICF patients and normal donors. The frequency of CpGs according to DNA methylation levels are depicted in the graph. (B) Table showing number of average poorly methylated (methylation levels $\beta$ $<0.33$) and average highly methylated (methylation levels $\beta$ $>0.66$). (C) Scatter plot represents comparison of DNA methylation levels of total CpG sites using the Infinium 450K DNA methylation assay. Green triangle selects hypomethylated area for ICF patients compared to controls. (D) Box plot displaying the distribution of $\beta$-values of total CpG sites of ICF versus healthy control donors. Normality was tested using the Shapiro-Wilk test and significance was evaluated with the Mann-Whitney U test and is indicated by three asterisks *** ($p<0.001$).

doi:10.1371/journal.pone.0132517.g001
individual histograms, scatter and box plots for all the hybridized samples. Individual methylation levels were consistent, although control 2 (XX) presented lower global levels than the other two controls (MS and GM03674) (S1 Fig). Therefore, our results are in agreement with previous studies reporting that ICF syndrome is a disease characterized by DNA hypomethylation and we further demonstrate that the combination of the specific DNMT3B variants A603T/STP807ins and V699G/R54X derives in a global loss of DNA methylation levels.

**Identification of differentially DNA methylated genes in ICF**

Previous studies, using candidate-gene approaches, have been searching for differentially methylated regions in ICF patients that would account for the severe clinical features that characterize the ICF patients. In this sense, Jin et al reported subtle but significant changes in DNA methylation levels associated with transcript level variations in a few genes involved in development, neurogenesis and immunological function of ICF patients using expression microarrays [6]. Moreover, high degree correlation between DNA methylation changes and gene expression patterns for a number of heterochromatic genes located at the pericentromeric region of chromosome 21 in ICF patients has been also reported [7]. Therefore, a genome-scale approach would contribute to identify new differentially methylated genes and increase the knowledge in the etiology and development of the disease.

Since we observed that ICF patients with DNMT3B mutations show a global reduction of DNA methylation, we focused on studying the CpG positions with loss of methylation. To gain robustness and reliability, we added to the previous set of ICF1 samples four new controls of peripheral mononuclear cells obtained from cord blood samples (CB10, CB13, CB20 and CB76), being aware of the limitation of the cell type heterogeneity in these samples. The unsupervised hierarchical clustering, using 5000 random CpGs mimicking the global methylome, shows that samples are grouped based on their tissue type. The methylome of the four peripheral mononuclear derived cells samples is homogeneous and clustered together. The immortalized cell lines derived from controls and ICF patients clustered in the same group, but in a separate subgroup. Finally, fibroblast cells clustered in an independent group (Fig 2A). These results indicate that the distance between samples is mainly due to tissue type.

We focused on ICF-associated regions which had loss of methylation, in order to obtain reliable and ICF-specific differentially methylated candidates. To achieve this, we used a restrictive threshold to overcome the limited number of samples and the heterogeneous effect of the mutations. Then, we performed a parametric analysis comparing average Beta values from ICF1 patients versus controls selecting those with differences in methylation levels higher than 65% (delta < 0.65) and a standard deviation value lower than 15% (Desvest < 0.15). Using this strategy, with restrictive cut-offs, we identified 181 hypomethylated CpGs in ICF1 patients compared to control group. A table with the 181 differentially methylated positions (DMPs) with their gene characteristics including target ID, name and Beta values for ICF1 and controls groups, chromosome location, genomic distribution and CpG context is provided (Table 1). The hierarchical clustering heatmap for all these selected 181 CpG sites clearly segregates both groups (Fig 2B). It is worth emphasizing that both ICF1 patients present different tissue types and DNMT3B mutations, thereby producing subtle distinct levels of DNA methylation defects. However, the selected approach allowed us to identify the epigenetic differences that are common to both ICF1 patients leading to the establishment of potential markers for the ICF1 disease. Furthermore, we aimed to compare our selected 181 DMPs with the previous published methylome in our laboratory by Heyn et al. of the LCL GM08714 from the same ICF patient from which the GM08747 fibroblasts derive [8]. Using whole genome bisulfite sequencing (WGBS) we previously obtained 296,964 differentially methylated regions (DMRs).
Consistently, we observed that a high number of 138 out of 181 (76.2%) of DMPs were inside a previously defined DMRs and therefore are common to both studies (S2A Fig). It is important to emphasize that although we are using different ICF1 samples from the previously published work, there is a high percentage of concordance that favors the reliability of our results.

Characterization of the genomic localization and gene features of obtained DMPs

ICF-specific DNA methylation changes have been previously reported mainly in pericentromeric regions of chromosomes 1, 9 and 16 [18]. However, when we analyzed the subchromosomal localization of our DMPs we observed that the majority of the DMPs are located in the intra and subtelomeric regions of the chromosomes (Figs 3 and 4A). These results support previous studies where hypomethylation of subtelomeric regions was associated in the ICF1 cells with advanced telomere replication timing and elevated levels of transcripts emanating from telomeric regions. These findings may explain the abnormal telomeric phenotype observed in ICF syndrome [19].

We further evaluated the association of the RNA transcripts with significant DMPs, finding that 39.8% and 2.2% were associated to coding and non-coding genes, respectively.
Table 1. List of Differentially Methylated CpGs comparing ICF patients and healthy controls.

| TargetID   | average(controls) | average(ICF) | Delta(ICF- controls) | CHR | MAPINFO  | Name       | Genomicdistribution | CpGisland |
|------------|------------------|--------------|----------------------|-----|-----------|------------|---------------------|-----------|
| cg03986562 | 0.84             | 0.03         | -0.82                | 16  | 7703893  | A2BP1      | Body                | Island    |
| cg12799314 | 0.76             | 0.07         | -0.69                | 10  | 1405193  | ADARB2     | Body                | Island    |
| cg01962727 | 0.86             | 0.14         | -0.72                | 16  | 46603059 | ANKR26P1   | TSS200              | Island    |
| cg07121021 | 0.85             | 0.18         | -0.67                | 10  | 37414597 | ANKR30A    | TSS200              | Island    |
| cg00381789 | 0.88             | 0.17         | -0.71                | 11  | 424667   | ANO9       | Body                | Shelf     |
| cg01669186 | 0.95             | 0.20         | -0.75                | 11  | 430919   | ANO9       | Body                | Island    |
| cg26217813 | 0.85             | 0.13         | -0.72                | 17  | 3378996  | ASPA       | 5'UTR               | Shelf     |
| cg10204707 | 0.95             | 0.10         | -0.85                | 19  | 53794711 | BIRC8      | 5'UTR               | Island    |
| cg00361562 | 0.92             | 0.08         | -0.84                | 2   | 19864977 | BOLL       | 5'UTR               | Island    |
| cg10547527 | 0.94             | 0.08         | -0.86                | 2   | 19865012 | BOLL       | TSS200              | Island    |
| cg13379983 | 0.94             | 0.12         | -0.82                | 2   | 19864977 | BOLL       | 5'UTR               | Island    |
| cg21300403 | 0.93             | 0.09         | -0.84                | 2   | 19865012 | BOLL       | TSS200              | Island    |
| cg00982548 | 0.92             | 0.08         | -0.84                | 2   | 19864978 | BOLL       | 5'UTR               | Island    |
| cg11031976 | 0.85             | 0.18         | -0.67                | 2   | 19864978 | BOLL       | 5'UTR               | Island    |
| cg04006718 | 0.88             | 0.19         | -0.69                | 10  | 1068667  | C10orf110  | Body                | Island    |
| cg05820861 | 0.77             | 0.04         | -0.73                | 13  | 24882296 | C1QTNF9    | TSS1500             | Island    |
| cg18167759 | 0.92             | 0.27         | -0.65                | 12  | 2017746  | CACNA2D4   | Body                | Island    |
| cg06839970 | 0.89             | 0.10         | -0.79                | 11  | 15093769 | CALCB      | TSS1500             | Shore     |
| cg03749198 | 0.84             | 0.12         | -0.72                | 19  | 51979766 | CEACAM18   | TSS200              | Island    |
| cg07369559 | 0.78             | 0.10         | -0.69                | 16  | 71559445 | CHST4      | TSS1500             | Island    |
| cg17754473 | 0.87             | 0.12         | -0.75                | 4   | 798611   | CPLX1      | Body                | Island    |
| cg12517410 | 0.75             | 0.09         | -0.66                | 2   | 11561209 | DPP10      | Body                | Island    |
| cg03272326 | 0.74             | 0.08         | -0.65                | 1   | 16613693 | FAM7B      | TSS1500             | Island    |
| cg01766885 | 0.77             | 0.12         | -0.65                | 17  | 21908498 | FLJ36000   | Body                | Island    |
| cg02432860 | 0.88             | 0.21         | -0.67                | 22  | 17499187 | GAB4       | TSS200              | Island    |
| cg00028598 | 0.91             | 0.12         | -0.79                | 15  | 27128633 | GABRA5     | Body                | Island    |
| cg17403731 | 0.76             | 0.13         | -0.70                | 6   | 33048502 | HLA-DPB1   | Body                | Island    |
| cg26645432 | 0.83             | 0.13         | -0.70                | 6   | 32729181 | HLA-DQB2   | Body                | Island    |
| cg19831369 | 0.89             | 0.10         | -0.79                | 22  | 26881026 | HPS4       | TSS1500             | Shore     |
| cg18515063 | 0.80             | 0.14         | -0.66                | 17  | 61616209 | KCHN6      | Body                | Shore     |
| cg11195059 | 0.80             | 0.09         | -0.71                | 7   | 10774104 | LAMB4      | Body                | Island    |
| cg11850943 | 0.79             | 0.10         | -0.70                | 7   | 55432402 | LANCL2     | TSS1500             | Shore     |
| cg15463284 | 0.76             | 0.07         | -0.68                | 11  | 18477534 | LDHAL6A    | 5'UTR               | Island    |
| cg03003467 | 0.88             | 0.05         | -0.83                | 14  | 10693828 | LOC100133469 | TSS200           | Island    |
| cg14404807 | 0.92             | 0.07         | -0.86                | 14  | 10693829 | LOC100133469 | TSS200           | Island    |
| cg16023283 | 0.82             | 0.04         | -0.78                | 14  | 10693823 | LOC100133469 | TSS200           | Island    |
| cg26117778 | 0.93             | 0.09         | -0.85                | 14  | 10693833 | LOC100133469 | TSS200           | Island    |
| cg17347810 | 0.84             | 0.16         | -0.68                | 19  | 42349010 | LYPD4      | TSS1500             | Shore     |
| cg08247921 | 0.90             | 0.18         | -0.72                | 4   | 10368311 | MANBA      | TSS1500             | Shore     |
| cg01320920 | 0.90             | 0.23         | -0.67                | 3   | 12597236 | MKNR2      | TSS1500             | Shore     |
| cg05013438 | 0.86             | 0.18         | -0.68                | 8   | 12563125 | MTSS1      | Body                | Island    |
| cg26954695 | 0.89             | 0.10         | -0.80                | 19  | 59085561 | MZF1       | TSS1500             | Shore     |
| cg26747317 | 0.74             | 0.05         | -0.70                | 6   | 31829793 | NEU1       | Body                | Shore     |
| cg18438823 | 0.75             | 0.08         | -0.67                | 11  | 4676365  | OR51E1     | 3'UTR               | Shore     |
| cg16599007 | 0.89             | 0.19         | -0.70                | 12  | 12164720 | P2RX4      | TSS1500             | Shore     |
| cg14995055 | 0.90             | 0.09         | -0.82                | 5   | 14024059 | PCDHs      | Body                | Shore     |

(Continued)
Table 1. (Continued)

| TargetID   | average(controls) | average(ICF) | Delta(ICF-controls) | CHR | MAPINFO       | Name     | Genomicdistribution | CpGisland |
|------------|-------------------|--------------|---------------------|-----|----------------|----------|--------------------|------------|
| cg00635356 | 0.71              | 0.05         | -0.66               | 5   | 140603942      | PCDHB14  | 1stExon            | Shore      |
| cg24403041 | 0.80              | 0.15         | -0.66               | 21  | 14982363       | POTED    | TSS200             | Island     |
| cg08691323 | 0.84              | 0.04         | -0.80               | 5   | 54826683       | PPAP2A   | Body               | Island     |
| cg16411279 | 0.94              | 0.02         | -0.92               | 5   | 54826768       | PPAP2A   | Body               | Island     |
| cg03473125 | 0.96              | 0.25         | -0.72               | 7   | 157550806      | PTPRN2   | Body               | Island     |
| cg22386389 | 0.98              | 0.13         | -0.85               | 2   | 238707268      | RBM44    | TSS200             | Island     |
| cg00399591 | 0.86              | 0.21         | -0.65               | 16  | 18802159       | RPS15A   | TSS1500            | Shore      |
| cg27258551 | 0.84              | 0.07         | -0.77               | 16  | 23197740       | SCN1G    | Body               | Island     |
| cg13924635 | 0.95              | 0.08         | -0.87               | 1   | 1168432        | SDF4     | TSS1500            | Island     |
| cg21396646 | 0.76              | 0.10         | -0.66               | 1   | 234039761      | SLC35F3  | TSS1500            | Shore      |
| cg04963199 | 0.81              | 0.11         | -0.69               | 16  | 87868947       | SLC7A5   | Body               | Island     |
| cg10700435 | 0.85              | 0.15         | -0.71               | 1   | 40838541       | SMAP2    | TSS1500            | Shore      |
| cg14608581 | 0.75              | 0.02         | -0.73               | 2   | 70122240       | SNRP27   | Body               | Shore      |
| cg10460168 | 0.82              | 0.10         | -0.72               | 5   | 112198022      | SRP19    | Body               | Island     |
| cg11258452 | 0.83              | 0.04         | -0.79               | 12  | 119594454      | SRRM4    | Body               | Shelf      |
| cg16266492 | 0.77              | 0.09         | -0.68               | 10  | 135379461      | SYCE1    | 5'UTR              | Island     |
| cg16654149 | 0.90              | 0.09         | -0.81               | 10  | 135379580      | SYCE1    | 5'UTR              | Island     |
| cg24678803 | 0.81              | 0.13         | -0.68               | 10  | 135378570      | SYCE1    | 5'UTR              | Island     |
| cg21950459 | 0.97              | 0.06         | -0.91               | 20  | 58508076       | SYCP2    | TSS1500            | Island     |
| cg02820072 | 0.92              | 0.12         | -0.79               | 19  | 33210552       | TDRD12   | TSS200             | Island     |
| cg18155407 | 0.92              | 0.11         | -0.81               | 19  | 33210662       | TDRD12   | TSS200             | Island     |
| cg19712189 | 0.79              | 0.11         | -0.68               | 19  | 33210851       | TDRD12   | 5'UTR              | Island     |
| cg12580503 | 0.89              | 0.18         | -0.72               | 12  | 130340287      | TEMEM132D| Body               | Island     |
| cg03862437 | 0.87              | 0.11         | -0.76               | 3   | 194353432      | TEMEM44  | Body               | Shore      |
| cg02203665 | 0.75              | 0.06         | -0.68               | 9   | 100262506      | TMOD1    | TSS1500            | Shore      |
| cg25691239 | 0.81              | 0.14         | -0.68               | 21  | 10991412       | TPTE     | TSS1500            | Island     |
| cg09612946 | 0.84              | 0.16         | -0.68               | 2   | 220121680      | TUBA4B   | Body               | Shelf      |
| cg02359511 | 0.81              | 0.11         | -0.70               | 4   | 186346156      | UFSP2    | Body               | Shore      |
| cg13913666 | 0.79              | 0.13         | -0.66               | 7   | 1198122        | ZFAND2A  | 5'UTR              | Shore      |
| cg00469395 | 0.90              | 0.17         | -0.73               | 16  | 89315104       |         |                   | Island     |
| cg00654159 | 0.80              | 0.10         | -0.70               | 16  | 32858241       |         |                   | Island     |
| cg00813343 | 0.83              | 0.14         | -0.68               | 7   | 109485219      |         |                   | Island     |
| cg00964333 | 0.89              | 0.05         | -0.83               | 10  | 15038045       |         |                   | Island     |
| cg01086526 | 0.88              | 0.16         | -0.72               | 5   | 3188191        |         |                   | Island     |
| cg01215328 | 0.92              | 0.10         | -0.82               | 16  | 34208792       |         |                   | Island     |
| cg01390039 | 0.93              | 0.22         | -0.71               | 16  | 89315293       |         |                   | Island     |
| cg01403596 | 0.89              | 0.10         | -0.79               | 2   | 92045973       |         |                   | Island     |
| cg01873249 | 0.88              | 0.23         | -0.65               | 16  | 34442602       |         |                   | Shore      |
| cg01893629 | 0.86              | 0.09         | -0.77               | 12  | 34494825       |         |                   | Island     |
| cg02052758 | 0.90              | 0.18         | -0.72               | 7   | 55430948       |         |                   | Shelf      |
| cg02491199 | 0.96              | 0.07         | -0.90               | 17  | 21901968       |         |                   | Island     |
| cg02506248 | 0.83              | 0.10         | -0.73               | 12  | 130502267      |         |                   | Island     |
| cg02587606 | 0.77              | 0.10         | -0.67               | 10  | 77352231       |         |                   | Island     |
| cg02797144 | 0.81              | 0.07         | -0.74               | 16  | 3245099        |         |                   | Shore      |
| cg02801570 | 0.85              | 0.13         | -0.72               | 10  | 15026284       |         |                   | Island     |
| cg02811702 | 0.87              | 0.08         | -0.78               | 13  | 24901961       |         |                   | Shore      |
| cg02985366 | 0.81              | 0.14         | -0.67               | 16  | 32360428       |         |                   | Island     |

(Continued)
Table 1. (Continued)

| TargetID     | average(controls) | average(ICF) | Delta(ICF- controls) | CHR  | MAPINFO | Name            | Genomicdistribution | CpGisland |
|--------------|------------------|--------------|----------------------|------|---------|-----------------|--------------------|-----------|
| cg03563298   | 0.86             | 0.07         | -0.79                | 2    | 242990263| Shore           |                    |           |
| cg03961510   | 0.87             | 0.07         | -0.80                | 10   | 2978438 | Shore           |                    | Island    |
| cg04523661   | 0.94             | 0.22         | -0.72                | 19   | 1302808 | Island          |                    | Island    |
| cg04696808   | 0.90             | 0.13         | -0.77                | 16   | 34257170| Island          |                    | Island    |
| cg04815577   | 0.72             | 0.03         | -0.69                | 5    | 51898   | Island          |                    | Island    |
| cg05136737   | 0.81             | 0.12         | -0.69                | 1    | 244924918| Island          |                    | Island    |
| cg05507257   | 0.86             | 0.17         | -0.69                | 10   | 135524081| Island          |                    | Island    |
| cg05585551   | 0.87             | 0.09         | -0.78                | 6    | 167314745| Island          |                    | Island    |
| cg06064212   | 0.80             | 0.13         | -0.67                | 6    | 28455048| Shelf           |                    | Island    |
| cg06217323   | 0.72             | 0.04         | -0.67                | 3    | 75445502| Island          |                    | Island    |
| cg06446408   | 0.83             | 0.12         | -0.71                | 16   | 73266679| Island          |                    | Island    |
| cg06766960   | 0.79             | 0.08         | -0.71                | 11   | 133703094| Island          |                    | Island    |
| cg06953577   | 0.84             | 0.13         | -0.70                | 11   | 113659952| Shore           |                    | Island    |
| cg07088555   | 0.93             | 0.07         | -0.86                | 16   | 89315186| Island          |                    | Island    |
| cg08198040   | 0.78             | 0.09         | -0.69                | 16   | 34429930| Shore           |                    | Island    |
| cg08363684   | 0.89             | 0.12         | -0.77                | 6    | 76154    | Island          |                    | Island    |
| cg08377526   | 0.82             | 0.13         | -0.70                | 2    | 92261659| Island          |                    | Island    |
| cg08617346   | 0.82             | 0.13         | -0.69                | 20   | 1716331 | Island          |                    | Island    |
| cg08761055   | 0.93             | 0.08         | -0.85                | 2    | 5846917 | Island          |                    | Island    |
| cg08767686   | 0.77             | 0.01         | -0.76                | 5    | 51426   | Island          |                    | Island    |
| cg08860346   | 0.82             | 0.09         | -0.73                | 2    | 75504008| Island          |                    | Island    |
| cg0915274    | 0.86             | 0.17         | -0.69                | 15   | 40422219| Island          |                    | Island    |
| cg09605095   | 0.79             | 0.04         | -0.76                | 2    | 5847414 | Island          |                    | Island    |
| cg09785512   | 0.78             | 0.09         | -0.69                | 17   | 21934840| Island          |                    | Island    |
| cg09817162   | 0.75             | 0.03         | -0.72                | 6    | 27185676| Island          |                    | Island    |
| cg09871679   | 0.87             | 0.08         | -0.79                | 7    | 61821180| Island          |                    | Island    |
| cg10399005   | 0.78             | 0.07         | -0.71                | 14   | 70316898| Island          |                    | Island    |
| cg10606029   | 0.86             | 0.20         | -0.65                | 5    | 37952598| Island          |                    | Island    |
| cg10854819   | 0.75             | 0.02         | -0.72                | 15   | 31515852| Island          |                    | Island    |
| cg11115267   | 0.84             | 0.15         | -0.68                | 10   | 38909894| Island          |                    | Island    |
| cg11350504   | 0.77             | 0.09         | -0.68                | 13   | 113121280| Shore          |                    | Island    |
| cg11476737   | 0.84             | 0.07         | -0.76                | 10   | 2815189 | Island          |                    | Island    |
| cg11837817   | 0.90             | 0.09         | -0.81                | 6    | 167314233| Island         |                    | Island    |
| cg11860886   | 0.93             | 0.07         | -0.85                | 14   | 106857312| Island         |                    | Island    |
| cg12109260   | 0.83             | 0.06         | -0.77                | 8    | 144850435| Shelf          |                    | Island    |
| cg12161959   | 0.84             | 0.19         | -0.65                | 2    | 240868337| Island         |                    | Island    |
| cg12492942   | 0.89             | 0.18         | -0.71                | 6    | 27510966| Island          |                    | Island    |
| cg12647837   | 0.91             | 0.16         | -0.75                | 7    | 61820842| Island          |                    | Island    |
| cg13396152   | 0.87             | 0.19         | -0.67                | 1    | 62005072| Island          |                    | Island    |
| cg14026325   | 0.93             | 0.02         | -0.90                | 8    | 144787867| Island          |                    | Island    |
| cg14218861   | 0.77             | 0.08         | -0.70                | 10   | 119443973| Island         |                    | Island    |
| cg14303972   | 0.84             | 0.05         | -0.79                | 12   | 34752640| Island          |                    | Island    |
| cg14341968   | 0.85             | 0.19         | -0.66                | 9    | 136075868| Shore          |                    | Island    |
| cg14493094   | 0.79             | 0.10         | -0.68                | 4    | 132896579| Island          |                    | Island    |
| cg15335669   | 0.77             | 0.05         | -0.72                | 15   | 22546908| Island          |                    | Island    |
| cg15646803   | 0.77             | 0.12         | -0.65                | 2    | 92261070| Island          |                    | Island    |
| cg16530595   | 0.81             | 0.07         | -0.74                | 8    | 144788492| Island          |                    | Island    |

(Continued)
Interestingly, there were 4 DMPs associated to a long non-coding RNA (Fig 4B). These results indicate that specific non-coding RNAs are target of DNMT3B and possibly regulated by DNA methylation. From the CpG content and neighborhood context standpoint (Fig 4C), the CpG island, which are regions with high dense number of CpGs, are the most extensively screened regions (48.6%) and are over-represented according to the design of the 450K Infinium array.

| TargetID  | average(controls) | average(ICF) | Delta(ICF- controls) | CHR  | MAPINFO | Name          | Genomicdistribution | CpGisland |
|-----------|-------------------|--------------|-----------------------|------|----------|---------------|--------------------|-----------|
| cg17024593| 0.75              | 0.06         | -0.68                 | 12   | 34490115 | Island        |                    | Island    |
| cg18304339| 0.83              | 0.16         | -0.67                 | 14   | 52213734 | Island        |                    | Island    |
| cg18315943| 0.79              | 0.12         | -0.67                 | 5    | 3188374  | Island        |                    | Island    |
| cg18480548| 0.87              | 0.09         | -0.78                 | 19   | 523642   | Island        |                    | Island    |
| cg18686665| 0.87              | 0.06         | -0.81                 | 2    | 629121   | Island        |                    | Island    |
| cg18709881| 0.75              | 0.07         | -0.69                 | 18   | 72837627 | Island        |                    | Island    |
| cg18710908| 0.83              | 0.18         | -0.65                 | 8    | 144787999| Island        |                    | Island    |
| cg18898632| 0.80              | 0.14         | -0.66                 | 2    | 24298856 | Island        |                    | Island    |
| cg18963811| 0.81              | 0.14         | -0.66                 | 14   | 52210597 | Island        |                    | Island    |
| cg18969117| 0.95              | 0.13         | -0.81                 | 1    | 22626917 | Shore         |                    | Shore     |
| cg19035254| 0.87              | 0.17         | -0.70                 | 8    | 4313260  | Island        |                    | Island    |
| cg19443750| 0.73              | 0.08         | -0.65                 | 13   | 11299658 | Island        |                    | Island    |
| cg19766591| 0.81              | 0.12         | -0.69                 | 10   | 2978190  | Island        |                    | Island    |
| cg20301678| 0.90              | 0.14         | -0.76                 | 6    | 16731326 | Island        |                    | Island    |
| cg20447730| 0.84              | 0.06         | -0.79                 | 10   | 45719880 | Island        |                    | Island    |
| cg21053539| 0.89              | 0.20         | -0.69                 | 6    | 28829503 | Shelf         |                    | Shelf     |
| cg21451653| 0.76              | 0.07         | -0.68                 | 12   | 34551994 | Island        |                    | Island    |
| cg21561672| 0.89              | 0.19         | -0.70                 | 14   | 102101551| Island        |                    | Island    |
| cg21918786| 0.90              | 0.10         | -0.80                 | 6    | 10961183 | Island        |                    | Island    |
| cg21941223| 0.95              | 0.14         | -0.81                 | 19   | 2926894  | Island        |                    | Island    |
| cg21973687| 0.82              | 0.12         | -0.70                 | 6    | 14814262 | Island        |                    | Island    |
| cg22243348| 0.84              | 0.13         | -0.71                 | 19   | 11874227 | Shelf         |                    | Shelf     |
| cg22778503| 0.90              | 0.09         | -0.82                 | 14   | 10685718 | Island        |                    | Island    |
| cg22834924| 0.83              | 0.09         | -0.74                 | 6    | 14039253 | Island        |                    | Island    |
| cg22973226| 0.90              | 0.21         | -0.69                 | 1    | 46705909 | Island        |                    | Island    |
| cg23193831| 0.83              | 0.11         | -0.72                 | 11   | 43177729 | Island        |                    | Island    |
| cg23207305| 0.83              | 0.12         | -0.71                 | 11   | 15012901 | Island        |                    | Island    |
| cg23771956| 0.73              | 0.03         | -0.70                 | 10   | 45720040 | Island        |                    | Island    |
| cg23972271| 0.87              | 0.21         | -0.66                 | 5    | 98541824 | Island        |                    | Island    |
| cg23997132| 0.86              | 0.02         | -0.84                 | 10   | 15038349 | Island        |                    | Island    |
| cg24854181| 0.80              | 0.02         | -0.78                 | 5    | 6823241  | Island        |                    | Island    |
| cg25606780| 0.87              | 0.20         | -0.68                 | 3    | 47397852 | Island        |                    | Island    |
| cg25885427| 0.77              | 0.08         | -0.69                 | 3    | 102333494| Island        |                    | Island    |
| cg26427818| 0.96              | 0.12         | -0.84                 | 11   | 15093613 | Shore         |                    | Shore     |
| cg26931296| 0.86              | 0.17         | -0.69                 | 7    | 150870852| Island        |                    | Island    |
| cg27399125| 0.78              | 0.05         | -0.72                 | 6    | 150378738| Shore         |                    | Shore     |
| cg27427743| 0.78              | 0.13         | -0.65                 | 13   | 23270850 | Island        |                    | Island    |
| cg27438152| 0.80              | 0.08         | -0.72                 | 15   | 31515761 | Island        |                    | Island    |
| cg27629454| 0.80              | 0.14         | -0.66                 | 1    | 228742687| Shore         |                    | Shore     |
| cg27660756| 0.82              | 0.08         | -0.74                 | 19   | 57103802 | Shelf         |                    | Shelf     |

doi:10.1371/journal.pone.0132517.t001
where CGs in islands represent only a 31% [10]. It is shown the classification according to functional genome distribution, indicating that the majority of the DMPs are located in intergenic regions (58%) leading to an over-representation of these regions regarding the design of the 450K infinium array where the percentage of probes in intergenic region is 24.6% (Fig 4D). Meanwhile, 24.3% of the DMPs correspond to promoter regions, defined as CpGs located at TSS1500, TSS200 and UTR regions. Interestingly this promoter group is under-represented based on the expected 38.9% from the 450K array [20]. Moreover, we took advantage of results published in [8] and compared the functional genomic distribution between our 181 DMPs and the 296,964 DMRs previously reported. We observed overall a discrepancy in promoter region (24.3% vs 4.5% in our study and Heyn et al, respectively) (S2B Fig, left panel). However, this striking result may be explained by the distribution of the CpGs in the array and in the whole genome that mimics our obtained pattern (S2B Fig, right panel). Finally, analyzing a subset of DMPs located in promoter regions only, we observed that 13 (87%) are associated with CpG islands or shores. Interestingly, these regions have been reported for being important
regulatory regions for disease [21],[22]. As a global conclusion, the majority of the hypomethylated DMPs in ICF patients are located at intergenic and CpG islands regions.

**DNA hypomethylation candidate validation**

To confirm the methylation results obtained by genome-scale techniques, we used a small scale, site-specific technique as targeted bisulfite sequencing. This technique takes advantage of the activity of sodium bisulfite that converts non-methylated cytosines into uraciles by deamination, while methyl-cytosines remain unaltered. We performed a technical validation using the same samples that were hybridized; the ICF fibroblasts and LCL (GM08747 and PT5) and 3 unrelated controls (XX, MS and 3674). Moreover, to gain robustness we also performed a biological validation using non-hybridized LCLs from the same ICF patient (GM08714) and ICF fibroblast compared with related controls (GM08728, GM08729 respectively mother and father of GM08714/GM08747) and the unrelated control (LDA). GM8714 was the ICF patient sample used in our previously paper [8].

---

**Fig 4. Genomic distribution and gene features of the 53 differentially methylated CpGs.** (A) Chromosomal sub-localization classified in different groups: subtelomeric, pericentromeric and intrachromosomal. (B) Associated RNA transcription classified in: coding, non-coding and intergenic. (C) CpG context and neighborhood classified in: island, shore, shelf and open sea/others. (D) Functional genomic distribution classified in: promoter (TSS1500, TSS200, 5’UTR), genic (1ºexon, body and 3’UTR) and intergenic.
Based on previous knowledge that epigenetic disruption of germ line function in somatic tissues has been associated with ICF [23][24], we selected four representative genes related to germ line specific pathways and/or are expressed exclusively in germ cells [25–27]. These four genes (BOLL, SYCP2, LDHAL6A and NCRNA00221) presented DNA methylation differences at crucial regulatory elements such as promoter with CpG islands. It is worth to mention that more than one DMCpGs in those important regulatory regions were identified in our analysis for BOLL and NCRNA00221, 6 and 4 respectively. Thus, coding genes such as, BOLL, SYCP2 and LDHAL6A, might be additional examples of DNMT3b target genes functionally regulated by DNA methylation in somatic tissues. Moreover, NCRNA00221 (Linc00221) is a long intergenic non-coding RNA (lincRNA). These non-coding genes are important regulators of gene expression that have been described in several diseases [28].

The pyrosequencing analysis confirmed, by comparing ICF patients with controls, the different methylation levels in the targeted promoter CpGs of the four genes (BOLL, SYCP2, LDHAL6A and NCRNA00221) (Fig 5, left panel). Both DNA methylation assays, bisulfite genomic sequencing and 450K infinium array, showed similar levels of methylation. Right panel of Fig 5 depicts CpGs DNA methylation values (including the CpGs obtained by 450K array analysis marked with a red arrow) located in the regions analyzed for the four genes in two representative samples: an ICF patient and a healthy control. DNA methylation values for all samples (ICF patients and control donors) and a schematic representation of the four selected gene regions are shown in (S3, S4, S5 and S6 Figs). Globally, we can conclude that not only the infinium-targeted CpGs are unmethylated in ICF patients (red arrow), but the surrounding CpGs also show the same pattern favoring the idea that global DNA demethylation landscape is maintained. This effect could indicate that the entire CpG island, and not only a single base demethylation, is altered as consequence of the DNMT3B deficiency in the ICF disease and might cause gene expression deregulation.

Association of gene expression and DNA methylation changes

The existence of aberrations in the DNA methylation patterns of ICF cells, particularly the hypermethylation of the CpG island sequences located in the promoter regions of key regulatory genes, that lead to gene silencing, have been extensively described in literature [29]. Conversely, DNA hypomethylation has been associated mainly with DNA methylation loss at genome-wide level, although it also occurs locally. In this light, the effect of DNA demethylation makes accessible the transcription machinery and hence facilitating gene activation, which have been mainly described in cancer, involving the role of oncogenes [22]. Although some hypomethylated genes have been found associated to ICF1 [23], the number of disrupted epigenetic genes identified is very limited and little is known about their association with the etiology of this disease. In line with this finding, we aimed to evaluate the impact of DNA methylation changes on the transcriptional activity and detect gene promoters that are highly methylated in healthy controls a suffer de novo established loss-of-methylation in ICF patients concordant with gene up-regulation.

Therefore, based on the established idea that promoter CpG islands (CGI) are the prominent and crucial regulatory regions for gene expression and with high variable methylation level between normal and diseased tissue [30],[31], we sought to evaluate gene expression of those previously validated genes. To elucidate the impact of DNA methylation on the transcriptional activity of SYCP2, BOLL, LDHAL6A and NCRNA00221, we analyzed by qPCR their gene expression in ICF patient cells (GM08714 and PT5) and control samples including related and unrelated healthy donors (GM08728, GM08729, MS and LDA). We also analyzed in parallel the untransformed fibroblasts form ICF patient (GM08747) and the healthy donor
The results clearly showed a significant up-regulation, using the unpaired Student t test, for three of four genes in ICF cells (except LDHAL6A that only show significance in GM08714, but not in PT5) compared to controls, indicating that the impaired DNA methylation at the identified DMPs is critical for controlling their gene activity (Fig 6).
expression levels between both ICF samples in SYCP2 and BOLL could be due to DNMT3 type of mutation and the idea that, the other factors together with DNA methylation changes may be involved in the complex regulation of gene expression. In line, consistent results were obtained in fibroblasts expression analysis, except for BOLL that we were not able to detect expression in any fibroblast. This could be explained due to a tissue-specific regulation beyond DNA methylation. Interestingly, even if at different extents overall comparing levels in LCLs with untransformed fibroblasts form an ICF patient and a healthy donor. Values were determined at least in triplicate. Statistic analysis was evaluated using student t test and significance symbols correspond to (* p<0.05; ** p<0.01 and *** p<0.001).
transcriptional repressor at their promoter region [32]. In this light, by identifying novel germ line genes, which are hypomethylated and inappropriately expressed, our results suggest that this phenomenon in the context of DNMT3B deficiency might be rather widespread. How this specific deregulation may contribute to ICF molecular pathogenesis remains to be established. Notably, the ectopic expression of meiotic genes in cancer cells has been functionally related to abnormal chromosome segregation and aneuploidy [33]. Because chromosomal instability is a hallmark of ICF syndrome, this raises the possibility that loss of silencing at particular germ line genes drives the typical cytological abnormalities seen in ICF patient lymphocytes.

It is known that early diagnosis of ICF syndrome is crucial since early treatment can improve the course of disease. However, ICF is probably underdiagnosed, especially in patients that present incomplete phenotype or born to families with no affected relatives [23]. Therefore, the DNA hypomethylation profile of NCRNA00221 especially, and in a minor extent SYCP2 and BOLL, could be further investigated and validated in peripheral blood in order to develop specific clinical biomarkers to facilitate the identification of ICF patients.

Conclusion

Our results contribute to elucidate how different mutations in DNMT3b result in deficiency of DNA methyltransferase activity, eventually causing ICF1 syndrome. It seems accepted that is the DNA methylation deficiency, and not other aspects of impaired DNMT3b activity, responsible for the ICF syndrome.

The regions with aberrant methylation in ICF patients were almost exclusive of pericentromeric regions of chromosome 1, 16, sometimes 9 and associated to repeated DNA sequences or heterochromatic genes. Although in vitro studies identified a spectrum of biochemical defects in the catalytic function associated to ICF-specific DNMT3B mutations, their impact on the genome-wide DNA methylation level in patient-derived cells is unsolved. Our results contributed to characterize the global defects of DNA methylation pattern in two heterozygous DNMT3B-mutant backgrounds, uncovering novel ICF-specific hypomethylated sites, outside the pericentromeric regions and in other chromosomes compared to those previously mentioned. Interestingly, we identified additional DNMT3B target loci whose expression must be restricted to germ cells. Establishment and maintenance of promoter DNA methylation in somatic tissues by DNMT3B is critical for their transcriptional repression. In addition, to provide further evidence on DNMT3B role in silencing germ line genes, these findings are of particular interest in the context of other human disease, like cancer. It is remarkable that the expression of catalytically inactive DNMT3b splice variants, the aberrant transcription of germ line genes and chromosomal instability are shared features. We thus believe that these genome-wide studies will help to elucidate the relationship between DNA hypomethylation and pathological phenotypes.

Finally, from the ICF syndrome point of view, our results contribute to further evaluate the utility of these potential biomarkers as diagnostic markers.

Supporting Information

S1 Fig. Individual genome-wide DNA methylation profiles in ICF patients and control samples. (A) Histograms showing bimodal distribution pattern of DNA methylation profiles in the two ICF patients (PT5 and GM8748) and normal donors (XX, MS and GM3674). (B) Table showing number of poorly methylated and highly methylated for each sample hybridized. (C) Individual scatter plots combining ICF patients and normal donors. (D) Individual box plots for ICF patients compared to normal donors. Normality was tested using the Shapiro-Wilk test and significance was evaluated with the Mann-Whitney U test and is indicated.
by three asterisks *** (p<0.001).

S2 Fig. Comparison of Hypomethylated regions reported by Heyn et al. and current differentially methylated positions. (A) Venn diagram illustrating the number of hypomethylated features. The intersecting region represents those CpGs that are common to both analyses. (B) Left panel: graph depicting percentages of differentially methylated features based on functional genomic distribution comparing both analyses (dark blue for our analysis and dark red for Heyn et al.). Right panel: graph showing percentages based on the 450K array design and the whole genome (light blue for 450K array and red for whole genome). Data obtained from [20].

S3 Fig. Bisulfite genomic sequencing for technical and biological validation of differentially DNA methylated gene BOLL. For technical validation two ICF (PT5 and GM08747) and two unrelated controls (XX, MS and GM03674) were used. For biological validation two ICF patient samples (GM8714 and PT3) and three controls (two related controls GM8728, GM8729 and one unrelated control LDA) were analyzed. CpG dinucleotides is shown in vertical lines. Multiple single clones are represented for each sample. Presence of unmethylated or methylated CpGs is indicated by white or black squares, respectively. Red arrows mark the localization of the differentially methylated CpGs by 450K array. The distance to Transcription Start Site (bp) is also indicated.

S4 Fig. Bisulfite genomic sequencing for technical and biological validation of differentially DNA methylated gene SYCP2. The design is similar to S3 Fig.

S5 Fig. Bisulfite genomic sequencing for technical and biological validation of differentially DNA methylated gene LDHAL6A. The design is similar to S3 Fig.

S6 Fig. Bisulfite genomic sequencing for technical and biological validation of differentially DNA methylated gene NCRNA00221. The design is similar to S3 Fig.

S1 Table. Primers for bisulfite genomic sequencing.

S2 Table. Primers for quantitative RT-PCR.

Acknowledgments
We thank Diana Garcia, Carles Arribas and Sebastian Moran for their technical support and Dr. Sylwia Leppert for her help during the early phase of the work.

Author Contributions
Conceived and designed the experiments: JS ME. Performed the experiments: LSR SG ADL MG ABC MM JS. Analyzed the data: JS ME. Contributed reagents/materials/analysis tools: JS ME MM. Wrote the paper: JS ME MM.
References

1. Ehrlich M, Jackson K, Weemaes C. (2006) Immunodeficiency, centromeric region instability, facial anomalies syndrome (ICF). Orphanet J Rare Dis 1: 2. PMID:16722602

2. Weemaes CM, van Tol MJ, Wang J, van Ostaijen-ten Dam MM, van Eggermond MC, Thijssen PE et al. (2013) Heterogeneous clinical presentation in ICF syndrome: correlation with underlying gene defects. Eur J Hum Genet. 21(11):1219–25. doi: 10.1038/ejhg.2013.40 PMID:23486536

3. Moarefi AH1, Chédin F. (2011) ICF syndrome mutations cause a broad spectrum of biochemical defects in DNMT3B-mediated de novo DNA methylation. J Mol Biol. Jun 24; 409(5):758–72. doi: 10.1016/j.jmb.2011.04.050 PMID: 21549127

4. Matthews AM, Buchanan KL, Tsien F, Jiang G, Sun B, Uicker W et al. (2001) DNA methyltransferase 3B mutations linked to the ICF syndrome cause dysregulation of lymphogenesis genes. Hum Mol Genet 10(25):2917–31. PMID:11741835

5. Jin B, Tao Q, Peng J, Soo HM, Wu W, Ying J et al. (2008) DNA methyltransferase 3B (DNMT3B) mutations in ICF syndrome lead to altered epigenetic modifications and aberrant expression of genes regulating development, neurogenesis and immune function. Hum Mol Genet. 17(5):690–709. PMID: 18029387

6. Kondo T, Bobek MP, Kuick R, Lamb B, Zhu X, Narayan A et al. (2000) Whole-genome methylation scan in ICF syndrome: hypomethylation of non-satellite DNA repeats D4Z4 and NBL2. Hum Mol Genet. 9(4):597–604. PMID:10699183

7. Miniou P, Jeanpierre M, Bourc'his D, Coutinho Barbosa AC, Blanquet V and Viegas-Péquignot E. (1997) Alpha-satellite DNA methylation in normal individuals and in ICF patients: heterogeneous methylation of constitutive heterochromatin in adult and fetal tissues. Hum Genet 99(6):738–45. PMID: 9187666

8. Miniou P, Jeanpierre M, Blanquet V, Bibikova M et al. (2011) Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 5(5):427–43. PMID: 20448464

9. Sandoval J, Heyn HA, Moran S, Serra-Musach J, Pujana MA, Bibikova M et al. (2011) High density DNA methylation array with single CpG site resolution. Genomics 98(4):288–95. doi: 10.1016/j.ygeno.2011.07.007 PMID: 21593595

10. Livak KJ and Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402–8. PMID: 11846609

11. Minioiu P, Jeanpierre M, Blanquet V, Bibikova M et al. (2011) Abnormal methylation pattern in constitutive and facultative (X inactive chromosome) heterochromatin of ICF patients. Hum Mol Genet 3(12):2093–102. PMID: 21593595

12. Bielinska E, Cimino A, Strazzullo M, Ferraro M, Mutarelli M et al. (2010) Epigenetic alteration of microRNAs in DNMT3B-mutated patients of ICF syndrome. Epigenetics. 5(5):427–43. PMID: 20448464

13. Matarazzo MR, De Bonis ML, Gregory RI, Vacca M, Hansen RS, Ferraro M et al. (2002) Allelic inactivation of the pseudoautosomal gene SYB1 is controlled by epigenetic mechanisms common to the X and Y chromosomes. Hum Mol Genet. 11(25):3191–8. PMID: 12444103

14. Matarazzo MR, De Bonis ML, Strazzullo M, Cerase A, Ferraro M, Vastarelli P et al. (2007) Multiple binding of methyl-CpG and polycomb proteins in long-term gene silencing events. J Cell Physiol. 210 (3):711–9. PMID: 17133344

15. Jeanpierre M, Turleau C, Aurias A, Prieur M, Ledeist F, Fischer A et al. (1993) An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet. 2(6):731–5. PMID: 8102570

16. Bielinska E, Cimino A, Strazzullo M, Ferraro M, Mutarelli M et al. (2010) Epigenetic alteration of microRNAs in DNMT3B-mutated patients of ICF syndrome. Epigenetics 5(5):427–43. PMID: 20448464

17. Matarazzo MR, De Bonis ML, Strazzullo M, Cerase A, Ferraro M, Vastarelli P et al. (2007) Multiple binding of methyl-CpG and polycomb proteins in long-term gene silencing events. J Cell Physiol. 210 (3):711–9. PMID: 17133344

18. Jeanpierre M, Turleau C, Aurias A, Prieur M, Ledeist F, Fischer A et al. (1993) An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet. 2(6):731–5. PMID: 8102570

19. Yehezkel S, Segev Y, Viegas-Péquignot E, Skorecki K and Selig S. (2008) Hypomethylation of subtelomeric regions in ICF syndrome is associated with abnormally short telomeres and enhanced...
transcription from telomeric regions. Hum Mol Genet. 17(18):2776–89. doi: 10.1093/hmg/ddn177
PMID: 18558631

20. Stirzaker C, Taberlay PC, Statham AL, Clark SJ. (2014) Mining cancer methylomes: prospects and challenges. Trends Genet. 30(2):75–84. doi: 10.1016/j.tig.2013.11.004 PMID: 24368016

21. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P et al. (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–86. doi: 10.1038/ng.298 PMID: 19151715

22. Sandoval J and Esteller M. (2012) Cancer epigenomics: beyond genomics. Curr Opin Genet Dev. 22 (1):50–5. doi: 10.1016/j.gde.2012.02.008 PMID: 22402447

23. Velasco G, Walton EL, Sterlin D, Hédouin S, Nitta H, Ito Y et al. (2014) Germline genes hypomethylation and expression define a molecular signature in peripheral blood of ICF patients: implications for diagnosis and etiology. Orphanet J Rare Dis. 9:56. doi: 10.1186/1750-1172-9-56 PMID: 24742017

24. Walton EL, Francastel C and Velasco G. (2014) Dnmt3b Prefers Germ Line Genes and Centromeric Regions: Lessons from the ICF Syndrome and Cancer and Implications for Diseases. Biology (Basel) 3(3): 578–605.

25. Li M, Liu C, Zhu H, Sun J, Yu M, Song W et al. (2013) Expression pattern of Boule in dairy goat testis and its function in promoting the meiosis in male germine stem cells (mGSCs). J Cell Biochem. 114 (2):294–302. doi: 10.1002/jcb.24368 PMID: 22930651

26. Zheng YH, Rengaraj D, Choi JW, Park KJ, Lee SI and Han JY. (2009) Expression pattern of meiosis associated SYCP family members during germline development in chickens. Reproduction 138 (3):483–92. doi: 10.1530/REP-09-0163 PMID: 19525366

27. Wang H, Zhou Z, Lu L, Xu Z and Sha J. (2005) Cloning and characterization of a novel intronless lactate dehydrogenase gene in human testis. Int J Mol Med 15(6):949–53. PMID: 15870898

28. Della Ragione F, Gagliardi M, D’Esposito M and Matarazzo MR. (2014) Non-coding RNAs in chromatin disease involving neurological defects Front Cell Neurosci. 8:54. doi: 10.3389/fncel.2014.00054 PMID: 24616662

29. Esteller M. (2008) Epigenetics in cancer. N Engl. J Med. 358(11):1148–59. doi: 10.1056/NEJMra072067 PMID: 18337604

30. Lopez-Serra P, Marciilla M, Villanueva A, Ramos-Fernandez A, Palau A, leal L et al. (2014) DERL3-associated defect in the degradation of SLC2A1 mediates the Warburg effect. Nat Commun. 5:3608. doi: 10.1038/ncomms4608 PMID: 24699711

31. Quintavalle C, Mangani D, Roscigno G, Romano G, Diaz-Lagares A, laboni M et al. (2013) MiR-221/222 target the DNA methyltransferase MGMT in glioma cells. Plos One 8(9):e74466. doi: 10.1371/journal.pone.0074466 PMID: 24147153

32. Velasco G, Hube F, Rollin J, Neulllet D, Philippe C, Bouzinba-Segard H et al. (2010). Dnmt3b recruitment through E2F6 transcriptional repressor mediates germ-line gene silencing in murine somatic tissues. Proc Nat Acad Sci US A 107:9281–9286. doi: 10.1073/pnas.1000473107 PMID: 20439742

33. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. (2005) Cancer/testis antigens, gametogenesis and cancer. Nat. Rev. Cancer 5: 615–625. PMID: 16034368