Introduction

Hutchinson-Gilford Progeria syndrome (HGP; OMIM:176670) and Werner Syndrome (WS; OMIM:277700) are two distinct premature aging diseases defined by aging related phenotypes occurring early in life. As naturally aged individuals, HGP and WS patients present hair graying, skin thinning, atherosclerosis and osteopenia, but also show unique features such as subcutaneous tissue loss or laryngeal atrophy (high voice). Genetically, the disease could be traced back to mutations in \( \text{LMNA} \)\(^2\)\(^,\)\(^3\) and the \( \text{WRN} \)\(^4\) genes. However, the downstream causal events still remain elusive. In addition, a number of patients presenting a HGP or WS phenotype do not present any known genetic aberrations.

Herein, epigenetics could provide additional information by adding a level of gene regulation independent from genetic alterations. Epigenetics, understood in general terms as the inheritance of genome activity that does not depend on the strict DNA sequence, is known to be dynamic and to adapt to the surrounding circumstances.\(^5\) In addition, epigenetics provides an explanation for the phenotypic differences of genetically identical beings, such as the examples of monozygotic twins,\(^6\)\(^,\)\(^7\) cloned animals\(^8\) or the Agouti mice.\(^9\)\(^,\)\(^10\) To address the issue of whether epigenetic variants can be associated with the disease phenotypes of premature aged patients, we analyzed the DNA methylation (the most recognized and studied epigenetic mark) profiles of HGP and WS samples. We approach both diseases from an epigenetic point of view assuming missregulation to take place beyond the genetic blueprint.

Results and Discussion

We performed the Infinium DNA methylation BeadChip platform (Illumina), analyzing more than 450,000 CpG sites genome-wide. DNA was extracted from Epstein-Barr virus (EBV) immortalized B-cells (lymphoblastoid cell lines; LCLs) obtained from two \( \text{WRN} \) (AG07896, AG11385) and one \( \text{LMNA} \) (AG19911) mutant WS patient (Table 1).\(^11\) In addition, we analyzed one non-mutant WS patient (AG03364) and three non-mutant HGP patients. It is of note that the non-mutant HGP samples consist of direct relatives, represented by a father (AG15693) and its two daughters (AG15694, AG15695). The DNA methylation data are freely available at the Gene Expression Omnibus (GEO) database: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xlcj6eweoa&acc=GSE42865.

Noticing an effect of the EBV immortalization on the epigenome of the analyzed control LCL samples, as illustrated by absolute DNA methylation levels of naive CD19\(^+\) B-cells and a LCL, Fig. 1A, we initially filtered for CpG sites without variability between healthy LCLs (\( n = 3 \)) and healthy naive B-cells (\( n = 3 \) (\( \bar{\beta} \)-value < 0.05). To further exclude an impact of blood cell composition of the analyzed LCL samples, especially...
imported when comparing LCLs and peripheral blood mononuclear cells (PBMC) data from a previous aging study, we additionally excluded CpG sites revealing variability between naive B-cells (n = 3) and samples obtained from PBMC (n = 3; $\delta$ average $\beta$-value < 0.05). After further exclusion of CpG sites on gender chromosomes and those containing single nucleotide polymorphisms in their detection probe or interrogated CpG site (Caucasian population, 1000 Genome Project, > 1% frequency) and poor detection quality (detection $p$-value > 0.01), we analyzed 272,290 CpG sites in the here presented study. Following filtering for probes detecting here unmodelled sources, we observed a very high correlation ($R^2 > 0.99$) among all analyzed healthy control samples, as exemplary illustrated in Figure 1B.

**Table 1. Patient and healthy samples analyzed on the Infinium DNA methylation BeadChip**

| Disease | Sample ID | Gender | Type          | Relation  | Status            |
|---------|-----------|--------|---------------|-----------|-------------------|
| HGP     | AG15694   | Female | Immortalized  | Daughter   | Non-mutant        |
| HGP     | AG15695   | Female | Immortalized  | Daughter   | Non-mutant        |
| HGP     | AG15693   | Male   | Immortalized  | Father    | Non-mutant        |
| WS      | AG19911   | Female | Immortalized  | None      | LMNA mutant       |
| WS      | AG03364   | Male   | Immortalized  | None      | Non-mutant        |
| WS      | AG07896   | Female | Immortalized  | None      | WRN mutant        |
| WS      | AG11385   | Male   | Immortalized  | None      | WRN mutant        |
| PBMC    | PBMC11    | Male   | Naive         | None      | Healthy donor     |
| PBMC    | PBMC12    | Female | Naive         | None      | Healthy donor     |
| PBMC    | PBMC14    | Female | Naive         | None      | Healthy donor     |
| LCL     | LCL5      | Female | Immortalized  | None      | Healthy donor     |
| LCL     | LCL6      | Female | Immortalized  | None      | Healthy donor     |
| LCL     | LCL7      | Female | Immortalized  | None      | Healthy donor     |
| B-cells | Bcell01   | Female | Naive         | None      | Healthy donor     |
| B-cells | Bcell02   | Male   | Naive         | None      | Healthy donor     |
| B-cells | Bcell03   | Male   | Naive         | None      | Healthy donor     |

HGP, Hutchinson-Gilford Progeria syndrome; WS, Werner syndrome; LCL, lymphoblastoid cell line, LMNA, lamin A, WRN, Werner syndrome RecQ helicase.

Mutant patients exhibit global DNA methylation differences. Genome-wide DNA methylation variation (Fig. 2A) and unsupervised clustering analysis (Fig. 2B) of the DNA methylation profiles of HGP and WS patient DNA revealed profound differences between mutant and non-mutant samples. The WRN and LMNA mutant samples clearly clustered separately from the controls, suggesting mutation-specific DNA methylation profiles with possible causality on disease onset. Single samples comparisons revealed globally similar DNA methylation profiles with a fraction of CpG sites gaining methylation (in a healthy unmethylated context) and losing methylation in previous hypermethylated regions (Fig. 2C–E). Importantly, we noticed an overlap of differentially methylated CpG sites. However, each sample revealed additional unique alterations (Fig. 2C–E).

The non-mutant WS and HGP samples presented DNA methylation profiles that were poorly distinguishable between healthy and diseased samples (Fig. 2A and B). Interestingly, the non-mutant sample of the father (AG15693) revealed an increase of variability compared with the two daughters (AG15694, AG15695), likely reflecting the fact that, besides presenting the HGP phenotype, the father was 28 y older at sample acquisition.

Differentially methylated sites in mutant patients. In order to extract particular disease-related differences between controls and samples obtained from premature aging patients, we determined differentially methylated CpG sites (dmCpGs) between the controls (n = 3) and diseased samples ($\delta$ average $\beta$-value > 0.2).

Initially, we were interested in epigenetic alteration associated to the disease-related gene mutations of LMNA or WRN. As mutations in LMNA are causing both HGP and WS, whereas WRN mutations are exclusively related to the latter, we analyzed both mutations separately. We observed that the mutant samples presented a number of overlapping alterations; however, there was a high proportion of sample-specific alteration (Fig. 2C–E) and inter-sample variation (Fig. 2B). Using the aforementioned selection criteria, we determined 3,544 consistent differentially methylated CpG sites associated to mutation in the WRN gene (13,501 for AG11385 and 17,506 for AG07896). Although affected by the same genetic defect the WRN mutant samples revealed a high variability, which might be explained by the nature of the genetic defect. The Werner syndrome RecQ helicase gene is involved in DNA replication, recombination and DNA repair (3’ to 5’ exonuclease activity). Impaired function of WRN eventually results in double strand breaks and accumulation of mutations. The here observed epimutations could be a direct consequence of mutant CpG sites or CpG methylation-associated sequences in their proximity (methylation quantitative trait loci). Considering the stochastic distribution of such events, which is supported by the equal genome-wide distribution (Fig. 2A), we expected a few driver events masked by a mass of passenger events on genetic and epigenetic level. To address
this critical point, we assumed that, due to the phenotype similarities to normal aging, the disease causing driver events were also detectable in natural aged samples. Consequently, we integrated DNA methylation data obtained from a set of newborn and centenarian samples previously analyzed in our laboratory for aging specific changes. Here, 144 CpG showed differential methylation in the natural aged and in the WRN mutant sample sets and thus represent potential driver candidates for the premature aging phenotype of the WS patients (Table S1). These sites include gene promoters involved and significantly enriched in I-kappaB kinase/NF-kappaB signaling (CASP8, IL1RL1 and LGALS1) and the proteinaceous extracellular matrix formation (ADAMTS4, LGALS1, PODNL1 and ZP3) (Fisher’s exact test: \( p < 0.01 \)). All of these candidates are likely to be involved in phenotypic changes observed during aging.

While patients with WRN mutation have to cope with an accumulation of DNA damage, LMNA mutant cells are associated to severe structural alterations in the nucleus, leading to chromatin reorganization and subsequent epigenetic alterations. Therefore, most of the detected variation in DNA methylation will be passenger events, not directly related to disease onset. Consequently, we applied the aforementioned selection criteria and comparisons to define dmCpGs for the WS patient harboring a LMNA mutation. Here, we identified 18,480 dmCpGs, of which 485 also revealed differential methylation in the natural aging data set (Table S2). Interestingly, the NF-kappaB nucleus import mechanism was a significantly enriched ontology term among the differentially methylated genes (Fisher’s exact test: \( p < 0.01 \); PRKCG, NLRP12), suggesting this inflammation-related process plays a crucial role in the aging process of healthy and diseased individuals.

Non-mutant patients revealed consistent changes in DNA methylation. Analyzing non-mutant premature aging samples, we took advantage of the unique properties of the three HGP family members. Assuming a similar genetic background of the samples, we aimed to extract common dmCpGs and suggested those to present a particular association to the premature aging disease. Here, we observed 78 sites with consistent changes in DNA methylation (Table S3). Most strikingly, the promoter of the non-coding RNA LOC149837 harbored four dmCpGs in the close proximity of the transcription start site (−51 bp), forming a differentially methylated region with high potential as an important factor for gene regulation (Fig. 3A). LOC149837, also annotated as LINC00654, is defined as long intergenic non-protein coding RNA (lincRNA) with to date unknown biological function. Interestingly, the transcript is located in a cluster of PIWI-associated RNAs (piRNAs) (piRNA bank ID: 103; chr20:5461000–5500000), suggesting the region is a so far poorly recognized piRNA host transcript with association to premature aging. The approximately 30 nt-sized small RNAs participate in germline-specific regulatory processes, including silencing of transposable elements by direct interaction or epigenetic repression. As a subset of piRNAs, including the here identified, do not overlap transposable sequences, an additional function of the small RNAs is suspected. As the epimutation of LOC149837 is present in all seven analyzed premature aged samples (including the mutation driven phenotypes, Fig. 3B), we suggest the differential promoter hypomethylation to be a common downstream event with crucial function in disease onset. We further propose the inappropriate promoter hypomethylation of LOC149837 and subsequent piRNA activation as causative epigenetic event for the development of HGP and WS.

In order to determine yet unidentified disease-related genetic alteration in addition to the previously identified (WRN and LMNA), we screened differentially methylated gene promoters of the non-mutated samples for common regulatory mechanisms. Here, the 25 transcripts associated to disease-specific CpG sites were enriched for transcription factor binding sites for MYB (\( z \)-test; TRANSFAC: FDR < 0.05), a transcription factor associated to aging,15 including the response to oxidative stress of aged cells.16 It is of note that the DNA polymerase POLD3 is among the MYB targets, whose deregulation, due to its DNA repair activity, might have a direct impact on the disease phenotypes as it was observed for the DNA repair gene WRN (Fig. 3C). The significant enrichment of MYB binding sites in differentially methylated gene promoters led us to hypothesize if the transcription factor itself is targeted by genetic alterations, eventually gene mutation, causing the HGP phenotype. Although speculative at.

Figure 1. Probe filtering results in consistent DNA methylation profiles between naïve and immortalized samples. (A) DNA methylation level of 448,376 CpG sites (after filtering for high quality autosomal probes not overlapping SNPs) of a representative lymphoblastoid cell line (LCL) and a naïve B-cell sample. (B) DNA methylation levels of 272,290 CpG sites of the same samples after excluding probes affected by Epstein-Barr virus immortalization and cell composition differences. The correlation coefficient (\( r^2 \)) was calculated using Pearson’s correlation analysis.
MicroBeads (Miltenyi Biotec) were applied following the manufacturer’s instructions. DNA was extracted using Phenol:Chloroform:Isoamylalcohol (Sigma). B-cells were immortalized by Epstein-Barr virus (EBV) applying previous published protocol. Infinium HumanMethylation450 BeadChip. All DNA samples were assessed for integrity, quantity and purity by electrophoresis in a 1.3% agarose gel, picogreen quantification, and nanodrop measurements. All samples were randomly distributed into 96 well plates. Bisulfite conversion of 500 ng of genomic DNA was performed using EZ DNA methylation kit (Zymo Research) following manufacturer’s instructions. 200 ng of bisulfite converted DNA were used for hybridization on the HumanMethylation450 BeadChip (Illumina). Briefly, samples were whole genome amplified followed by an enzymatic end-point fragmentation, precipitation and resuspension. The resuspended samples were hybridized onto the beadchip for 16 h at 48°C and washed. A single nucleotide extension with labeled dideoxy-nucleotides was performed and repeated rounds of staining were applied with a combination of labeled antibodies differentiating between biotin and DNP.

Data were normalized using GenomeStudio V2010.3 (Illumina). The DNA methylation level is displayed as β-values ranging from 0 to 1. Methylation level (β-value) for each of the 485,577 CpG sites were calculated as the ratio of methylated signal divided by the sum of methylated and unmethylated signals.
plus 100. After normalization step, probes related to X and Y chromosomes were removed as well as those containing a SNPs with a frequency > 1% (Caucasian population; 1000 Genome project) in the probe sequence or interrogated CpG site. Samples were clustered by hierarchical clustering using Manhattan distances.

Transcription factor binding enrichment. Transcription factor binding enrichment analysis was performed using PSCAN.\(^\text{18}\) Transcription factor binding annotations were extracted from TRANSFAC. The gene promoter region was defined as -1,000 and +0 bp to the transcription start site. Z-test p values were corrected for multiple hypotheses testing using the Bonferroni method.

Functional enrichment analysis. Gene Ontology (GO) enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7).\(^\text{19,20}\)

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
The research leading to these results has received funding from the European Research Council (ERC) grant EPINORC under the agreement n° 268626, the MICINN Project—SAF2011-22803, the Cellex Foundation, the European Community’s Seventh Framework Programme (FP7/2007–2013) by the grant HEALTH-F5-2011-282510-BLUEPRINT and the Health and Science Departments of the Generalitat de Catalunya. M.E. is an ICREA Research Professor.

Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/epigenetics/article/23366

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