

1 **Aberrant function of the C-terminal tail of HIST1H1E accelerates cellular senescence and**
2 **causes premature aging**

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1 **Abstract**

2 Histones mediate dynamic packaging of nuclear DNA in chromatin, a process that is precisely
3 controlled to guarantee efficient compaction of the genome and proper chromosomal segregation
4 during cell division, and accomplish DNA replication, transcription and repair. Due to these
5 important structural and regulatory roles, it is not surprising that histone functional dysregulation or
6 aberrant expression can have severe consequences for multiple cellular processes that ultimately
7 might contribute to cell transformation or affect development. Recently, germline frameshift
8 mutations involving the C-terminal tail of HIST1H1E, a widely expressed member of the linker
9 histone family facilitating higher order chromatin folding, have been causally linked to an as yet
10 poorly-defined syndrome with intellectual disability. We report that these mutations result in stable
11 proteins that reside in the nucleus, bind to chromatin, disrupt proper compaction of DNA, and are
12 associated with a specific methylation pattern. Cells expressing these mutant proteins have a
13 dramatically reduced proliferation rate and competence, hardly enter into the S phase, and undergo
14 accelerated senescence. Remarkably, clinical assessment of a relatively large cohort of subjects
15 sharing these mutations revealed a premature aging phenotype as a previously unrecognized feature
16 of the disorder. Our findings identify a direct link between aberrant chromatin remodeling, cellular
17 senescence and accelerated aging.

18

1 **Introduction**

2 In eukaryotic cells, nuclear DNA is organized in a complex and dynamical structure called
3 chromatin, which allows efficient packaging of the genome and proper chromosomal segregation
4 during mitosis, and controls its accessibility for essential processes, such as replication,
5 transcription and repair.¹ The basic unit of chromatin is the nucleosome, in which a stretch of about
6 147 bp of DNA wraps around an octamer of the core histones (*i.e.*, H2A, H2B, H3 and H4),
7 constituting the nucleosomal core particle (NCP).² A number of H1 linker histone isoforms bind to
8 short DNA segments at the entry and exit sites on the surface of the NCP, stabilizing the
9 nucleosomal structure and contributing to higher order chromatin folding.^{3,4} While H1 histones have
10 traditionally been associated with chromatin compaction and a regulatory function favoring
11 transcriptional repression,⁵ a more complex and plastic function of these proteins in the control of
12 accessibility to DNA has gradually been appreciated.⁶⁻⁸ In mammals, H1 histones are encoded by
13 11 genes. Among these, seven are expressed in somatic cells, while the remaining four are
14 transcriptionally active in germline cells.^{3,4} Of note, even among the seven somatic subtypes, the
15 levels of these proteins appears to be regulated during the cell cycle, development and in different
16 tissues and cell types, suggesting specific function of individual isoforms.⁹ On the other hand,
17 redundancy in their function has been reported.^{10,11} In higher eukaryotes, H1 histone isoforms share
18 a tripartite structure consisting of a central highly conserved globular domain and two less
19 conserved, unstructured *N*-terminal and *C*-terminal tails. The globular domain has high sequence
20 homology among H1 subtypes and mediates binding to the nucleosome. The two tails are
21 moderately conserved among orthologs but differ among isoforms, suggesting functional
22 specificity. Notably, both tails encompasses a number of residues subjected to reversible
23 modifications with regulatory function. Among these, the extent of phosphorylation in
24 serine/threonine residues at the *C*-terminal tail has been proposed to regulate chromatin dynamics in
25 interphase as well as chromosome condensation during mitosis.^{4,12}

1 Aberrant histone function, whether due to mutations in genes coding proteins participating in
2 histone-modifying complexes or mutations directly affecting histone-coding genes, has been
3 established to contribute to oncogenesis and cause multisystem syndromes affecting growth and
4 cognitive function.¹³⁻¹⁸ Recently, while this work was underway, germline frameshift mutations
5 affecting *HIST1H1E* (MIM: 142220), coding a member of the somatic, replication-dependent linker
6 histone subfamily, have been causally linked to an as yet poorly-defined syndrome with intellectual
7 disability (ID) (MIM: 617537).¹⁹ All mutations mapped at the C-terminal tail of HIST1H1E and
8 were predicted to have an equivalent functional impact by generating the same change in the
9 reading frame. The five affected individuals belonged to a cohort of subjects with overgrowth, and
10 were reported to have a similar facial appearance, but variable height, head circumference and
11 degree of ID.¹⁹ Notably, the growth pattern of these individuals appeared complex and
12 characterized by an above-average height during infancy progressively decreasing over time,
13 resulting in an average/short stature in adulthood. This peculiar pattern of growth has been
14 highlighted by a subsequent report, which also confirmed the association of this class of *HIST1H1E*
15 mutations with ID and specific facial features.²⁰

16 Here, we report that this homogeneous class of disease-causing frameshift mutations affecting
17 the C-terminal tail of HIST1H1E results in stable proteins that reside in the nucleus and bind to
18 chromatin but disrupt proper compaction of DNA, and are associated with a specific methylation
19 profile. We also provide data indicating that cells expressing these mutant proteins have a
20 dramatically reduced proliferation rate and competence, hardly enter into the S phase, and undergo
21 accelerated senescence. Remarkably, clinical assessment of 13 newly identified individuals
22 heterozygous for this class of mutations and those previously reported allowed to identify premature
23 aging as a previously unrecognized feature of the disorder. Collectively, these data highlight a strict
24 link between aberrant chromatin remodeling, cellular senescence and accelerated aging.

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1 **Subjects and Methods**

2 **Subjects**

3 This study was approved by the Committee for Medical Ethics, University of Antwerp, Antwerp,
4 and Ethical Committee, Ospedale Pediatrico Bambino Gesù, Rome. Clinical data and DNA
5 specimens from the subjects included in this study were collected following procedures in
6 accordance with the ethical standards of the declaration of Helsinki protocols and approved by the
7 Review Boards of all involved institutions, with signed informed consents from the participating
8 subjects/families. Explicit permission was obtained to publish the photographs of the subjects as
9 shown in Figure 1.

10 **Exome sequencing**

11 WES was performed using DNA samples obtained from leukocytes. In most cases, a trio-based
12 strategy was used. Exome capture was carried out using the Nimblegen SeqCap EZ Exome v3
13 (Roche) (subjects 1 and 5), SeqCap EZ VCRome v2.1 (Roche) (subjects 6 and 7), SureSelect
14 Human All Exon v4 (Agilent) (subjects 2, 3, 8, 9 and 13), SureSelect Human All Exon v5 (Agilent)
15 (subjects 4 and 12), SureSelect XT Human All Exon v6 (Agilent) (subjects 10 and 11) target
16 enrichment kits, and sequencing was performed on HiSeq 2000/2500/4000 and NexSeq550
17 platforms (Illumina), using paired-end. WES data processing, sequence alignment to GRCh37, and
18 variant filtering and prioritization by allele frequency, predicted functional impact, and inheritance
19 models were performed as previously described.²¹⁻²⁷ The *de novo* origin of the *HIST1H1E*
20 frameshifts was confirmed by Sanger sequencing in all cases.

21 **RNA stability**

22 Total RNA was isolated from circulating leukocytes of subjects 1 and 2 (S1 and S2, hereafter) by
23 RNeasyMiniKit (Qiagen). Reverse transcription was performed using the SuperScriptIII first strand
24 kit (Invitrogen), following the manufacturer's instructions. Primer sequences used to perform the
25 RT-PCR are available upon request.

26 **Constructs**

1 The c.441dupC (p.Lys148GlnfsTer48), c.464dupC (p.Lys157GlnfsTer39) and c.441_442insCC
2 (p.Lys148ProfsTer82) were introduced by site-directed mutagenesis in a *HIST1H1E* cDNA
3 (RefSeq: NM_005321.2, NP_005312.1) tagged with Xpress at the *N*-terminus cloned in
4 pcDNA6/His version C (Invitrogen).

5 **Cell Cultures and transfections**

6 Skin fibroblasts isolated from skin biopsy (subjects S1 and S2 and healthy donors), and COS-1 and
7 HeLa lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % heat-
8 inactivated fetal bovine serum (Gibco) and 1 % penicillin-streptomycin, at 37 °C with 5% CO₂.

9 **SCGE assay**

10 Samples were processed according to the alkaline SCGE assay protocol, as previously
11 described.²⁸ Briefly, cells were suspended in 0.7 % low melting agarose. Slides were prepared in
12 duplicates with control cells and fibroblasts derived from affected individuals placed on opposite
13 sides of the same slide, immersed in cold lysis solution and kept at 4 °C overnight. After lysis,
14 slides were transferred in alkaline buffer for 20 min. Electrophoresis was carried out for 20 min at
15 20 V and 300 mA (0.8 V/cm) at 4 °C. Basal level of nucleoids relaxation was explored applying
16 longer electrophoresis run-times (40 and 60 min). Slides were neutralized in 0.4 M Tris (pH 7.5) for
17 5 min, treated with absolute ethanol and stored at room temperature. Slides were then stained with
18 GelRed (Biotium Inc) and scored at a fluorescence microscope (Leica). To evaluate induced DNA
19 damage and DNA repair capability, fibroblasts were irradiated with 1 or 2 Gy γ -rays from a ¹³⁷Cs
20 source at a dose rate of 0.8 Gy/min. During treatment, cells were maintained at 0 °C to prevent
21 DNA repair. The kinetics of DNA repair were assessed by SCGE assay, as described above. The
22 residual DNA damage was measured after 15 and 30 min of incubation at 37 °C. For each
23 experimental point, at least 75 cells were analyzed.

24 **Histone modifications and nucleolus morphology assessment**

25 For immunofluorescence, fibroblasts from S1 and control subjects were seeded at a density of
26 20×10^3 in 24-well cluster plates onto 12-mm cover glasses. After 24 h of culture in complete

1 medium, cells were fixed with 3 % paraformaldehyde. Following permeabilization with 0.5 %
2 Triton X-100 (10 min at room temperature), fibroblasts were stained with anti-dimethyl-Histone H3
3 (lys4) rabbit polyclonal antibody, anti-trimethyl-histone H3 (lys9) rabbit polyclonal antibody, anti-
4 trimethyl-histone H3 (lys27) rabbit polyclonal antibody (Millipore), anti-heterochromatin protein-
5 1 β mouse monoclonal antibody (Chemicon) followed by the appropriate secondary antibody
6 (Invitrogen) and DAPI. To study nucleolus morphology, fibroblasts were stained with C23 (MS-3)
7 mouse monoclonal antibody (Santa Cruz) followed by the appropriate secondary antibodies
8 (Invitrogen) and DAPI. Observations were performed on a Leica TCS SP2 AOBS apparatus. Cells
9 stained only with the fluorochrome-conjugated secondary antibodies were used to set up acquisition
10 parameters. Signals from different fluorescent probes were taken in sequential scanning mode,
11 several fields (>200) were analyzed for each labeling condition, and representative results are
12 shown.

13 **Proliferation and cell cycle assays**

14 Cells were seeded at 200.000 cells/well in a 6-well plate and incubated a 37 °C. Cell numbers
15 (mean of three replicates \pm SD) were counted by trypan blue exclusion after four and seven days.
16 The percentage of cells in different cell cycle phases was determined by dual flow cytometry
17 analysis of BrdU-positive cells and stained with the fluorescent DNA probe propidium iodide.
18 Briefly, cells were incubated for 1 h with BrdU (Sigma Aldrich) at a final concentration of 30 μ M.
19 Then BrdU was removed, cells were rinsed with PBS prior to harvesting and permeabilized using
20 ice-cold 100 % EtOH. Cells were incubated with HCl 3N to denature DNA and 0.1 M sodium
21 tetraborate to stop this reaction. Fibroblasts were incubated with the anti-BrdU antibody
22 (Invitrogen) followed by goat anti-mouse Alexa Fluor 488 secondary antibody. Finally, cells were
23 resuspended in a buffer containing 10 μ g/ml RNase A and 20 μ g/ml propidium iodide and
24 immediately analyzed by FACSCalibur (BD Biosciences).

25 **SA- β -gal activity and P53 evaluation**

1 SA- β -gal activity was assessed as reported.²⁹ Briefly, cells were fixed with 3.6 % formaldehyde in
2 PBS for 4 min at room temperature. Fixed cells were washed and incubated overnight with freshly-
3 prepared staining solution at 37 °C in the absence of CO₂. After washing, the coverslips were
4 mounted by using the antifade Dako fluorescence mounting medium (Agilent Technologies). P53
5 endogenous levels were evaluated on fibroblast lysates collected at different passages using an anti-
6 p53 mouse monoclonal antibody (Invitrogen). Membranes were probed with an anti-GAPDH
7 mouse monoclonal antibody (Santa Cruz) to normalize protein content.

8 **Analysis of nuclear morphology**

9 After 24 h of culture in complete medium, fibroblasts were treated with 2 mM thymidine (Sigma)
10 for 24 h, washed with 1X PBS, recovered with complete medium for 3 h and then treated with 100
11 ng/ml nocodazole (Sigma) for 12h. Afterwards, fresh drug-free medium was added and recovery
12 was allowed for 120 min, by fixing the cells every 15 min using PHEMO buffer for 10 min at room
13 temperature, as previously described.³⁰ Finally, cells were stained with lamin A/C mouse
14 monoclonal antibody (Santa Cruz) followed by the appropriate secondary antibody (Invitrogen) and
15 DAPI. Observations were performed on a Leica TCS SP2 AOBS apparatus as described above.

16 **Evaluation of rRNA content**

17 Total RNA was extracted from the same amount of control fibroblasts and cells derived from
18 affected subjects (400,000 fibroblasts) at different cellular passages. Three μ l of total RNA was
19 loaded for size separation on 1 % agarose gel and stained with ethidium bromide.

20 **Evaluation of protein stability**

21 COS-1 cells were seeded in 6-well plates the day before transfection. Monolayer were transfected at
22 70 % confluency with Fugene 6 transfection reagent (Promega) with wild-type or mutant Xpress-
23 tagged *HIST1H1E* expression constructs. Forty-eight h after transfection, cells were treated with
24 cycloheximide (20 μ g/ml) for 8 and 16 h, or left untreated before lysis. Xpress-tagged HIST1H1E
25 levels were assessed by immunoblotting. Membranes were probed with an anti-GAPDH antibody
26 (Santa Cruz) to normalize protein content.

1 **CSK assay**

2 Twenty thousand fibroblasts and 30×10^3 HeLa cells were seeded on glass coverslip and maintained
3 in culture complete medium for 24 h. HeLa cells were transfected with 100 ng of vectors expressing
4 Xpress-tagged HIST1H1E. Forty-eight h after transfection, HeLa cells were either fixed with 3 %
5 paraformaldehyde, or treated with CSK buffer before being fixed.³¹ After permeabilization with 0.5
6 % TritonX-100 (10 min at room temperature), HeLa cells were stained with monoclonal antibody to
7 Xpress (Invitrogen) followed by the appropriate secondary antibody (Invitrogen) and DAPI for
8 DNA. Analyses were performed in three independent experiments on a Leica TCS SP2 AOBS
9 apparatus.

10 **Analysis of cell morphology**

11 Fibroblasts from subject S1 and a control individual were seeded in a 100 mm dish cell culture.
12 Optical microscope images at different passages were taken with Fluid Cell Imaging Station (Life
13 Technologies).

14 **γ H2AX immunofluorescence staining**

15 Cells were seeded on a glass in a petri dish for 24 h. The slides were fixed with 4 %
16 paraformaldehyde (Sigma Aldrich), permeabilized in 0.2 % Triton X-100 and blocked in PBS/BSA
17 1 % for 30 min at room temperature. Slides were incubated with a mouse mono-clonal anti-
18 phospho-histone H2AX antibody (Millipore) overnight at 4 °C, washed in PBS/BSA 1 % and then
19 exposed to the secondary Alexa 488-labelled donkey anti-mouse antibody (Invitrogen, Life
20 Technologies) for 1 h at 37 °C. After washes in PBS/BSA, 1 % DNA were counterstained by DAPI
21 (Sigma Aldrich) in Vectashield (Vector Laboratories). Cells were analyzed with fluorescence
22 microscopy using an Axio Imager Z2 microscope (Zeiss). The frequency of foci per cell were
23 scored in 150 nuclei in two independent experiments.

24 **Telomere dysfunction-induced foci (TIF) immunoFISH staining**

25 Cells were seeded on glass slides that were processed as reported for the γ H2AX
26 immunofluorescence staining up to the secondary antibody (Alexa 488-labelled donkey anti-mouse,

1 Invitrogen). Immediately after, slides were washed in PBS/Triton X-100 0.05 %, fixed in 4 %
2 formaldehyde for 2 min and dehydrated through graded alcohols (70 %, 80 % and 100 %). Slides
3 and probes (Cy3 linked telomeric PNA probe, DAKO) were co-denatured at 80 °C for 3 min and
4 hybridized for 2 h, at room temperature in a humidified chamber. After hybridization, slides were
5 washed twice for 15 min in 50% formamide, 10 mM Tris, pH 7.2, and 0.1 % BSA followed by
6 three 5 min washes in 0.1 M Tris, pH 7.5, 0.15 M NaCl and 0.08 % Tween 20. Slides were then
7 dehydrated with an ethanol series and air dried. Finally, slides were counterstained with DAPI
8 (Sigma Aldrich) in Vectashield (Vector Laboratories). Co-localisation between γ H2AX foci and
9 telomere, were analysed using an Axio Imager Z2 microscope (Zeiss). The frequency of co-
10 localisation dots per cell were scored in 150 nuclei in two independent experiments.

11 **Quantitative-fluorescence “in situ” hybridization analysis (Q-FISH)**

12 Chromosome spreads were obtained as reported above. Q-FISH staining was performed as
13 previously described,³² with minor modifications. Briefly, slides and probes (Cy3 linked telomeric,
14 PANAGENE, and chromosome 2 centromeric PNA probes, DAKO) were co-denatured at 80 °C for
15 3 min, and hybridized for 2 h at room temperature in a humidified chamber. Slides were
16 counterstained with DAPI (Sigma Aldrich) in Vectashield (Vector Laboratories). Images were
17 captured at a 63X magnification with an Axio Imager Z2 (Zeiss). The ISIS software (MetaSystems)
18 was used to assess the telomere size (defined as ratio between total telomeres fluorescence [T] and
19 fluorescence of the centromere of the two chromosomes 2 [C]). Data were expressed as T/C %.³³
20 Experiments were repeated two times, and at least 10-15 metaphases were scored each time.

21 **Detection of aneuploidy**

22 Chromosome spreads were obtained as described before. Metaphases were captured at 63X
23 magnification with an Axio Imager Z2 microscope (Zeiss). We considered euploidy the metaphases
24 with 46 chromosomes and aneuploidy all the metaphases that had a different number of
25 chromosome. The frequency of aneuploidy was scored in 40 metaphases. Two independent
26 experiments were performed.

1 **Chromosome condensation assay**

2 After 30 min incubation with 30 μ M Calyculin-A (Wako), a 28 min incubation at 37 °C with
3 hypotonic solution (75 mM KCl) and fixation in freshly prepared Carnoy solution (3:1 v/v
4 methanol/acetic acid), chromosome spreads were obtained. Slide were stained with DAPI (Sigma
5 Aldrich) in Vectashield (Vector Laboratories) and an Axio Imager Z2 microscope (Zeiss) was used.
6 To analyze the frequency of chromosomal condensation we counted the number of metaphases in
7 150 nuclei per sample in two independent experiments.

8 **Methylome profiling**

9 DNA methylation levels of one adult and 5 pediatric affected subjects were analyzed using genomic
10 DNA extracted from leucocytes by the Infinium Human Methylation EPIC BeadChip assay
11 (Illumina) to allow comprehensive genome-wide coverage. Methylation levels were measured as
12 Beta-values, or percentage of methylation at each CpG site, ranging from 0 (*i.e.*, no methylation) to
13 1 (full methylation). Raw data were processed with Bioconductor package ChAMP,³⁴ using default
14 parameters to filter low-quality signals and normalize data. Methylation profiles were compared
15 with those obtained from a series of 35 healthy adult male controls of European descent, including
16 31 subjects selected from a European cohort study (average age \pm SD: 56.1 \pm 7.4 years).³⁵ Using
17 unsupervised multidimensional scaling (MDS) analysis, taking into account the 1,000 most variable
18 probes amongst samples. Statistical analyses were conducted using R software (V. 3.5.0). Gene and
19 Pathway Enrichment analyses of the differentially methylated probes were performed using WEB-
20 based Gene Set Analysis online tool,³⁶ on the associated genes. Functional annotation was carried
21 out by means of GO terms, including biological processes, molecular functions and cellular
22 components classes. Pathway analysis was performed using KEGG. Statistical significance was
23 assumed at 0.05, following multiple testing adjustment (FDR).

24

25 **Results**

26 **Spectrum of *HIST1H1E* mutations and associated clinical features**

1 Using whole-exome sequencing (WES), we identified a novel *de novo* *HIST1H1E* frameshift
2 variant, c.441dupC (p.Lys148GlnfsTer48), in an adult individual with hypotrichosis, cutis laxa, and
3 ID (Figure 1A). Mutation analysis performed on primary skin fibroblasts and hair bulb epithelial
4 cells of the affected subject supported the germline origin of the frameshift. The variant had not
5 been reported in ExAC/gnomAD, and affected a relatively large portion of the encoded protein
6 highly conserved among orthologs. In the subject, WES data analysis excluded the presence of
7 other functionally relevant variants compatible with known Mendelian conditions based on the
8 expected inheritance model and clinical presentation. Through networking and GeneMatcher
9 search,³⁷ we identified 12 additional subjects with similar *de novo* frameshift *HIST1H1E* mutations
10 (Figure 1B, Table 1). All changes were short out-of-frame indels resulting in almost identical
11 shorter proteins containing a shared divergent C-terminal tail (Table S1), which was consistent with
12 previous reports.^{19,20,38,39} None of the frameshifts had been reported in gnomAD, all were predicted
13 to dramatically impact protein function, and two of them had already been reported in ClinVar as
14 disease-causing mutations (Table 1).

15 We analyzed the clinical records of the 13 identified subjects (S1 to S13) and collected the
16 information available for the seven previously published individuals (S14 to S20). All subjects had
17 variable developmental delay (DD)/ID and a distinctive facies characterized by bitemporal
18 narrowing (100%), prominent forehead (93%) and high anterior hairline (88%) as main features,
19 which were reminiscent of Pallister-Killian syndrome (MIM: 601803) (Figure 1A). In four children,
20 Sotos syndrome (MIM: 117550) had been suggested, while Weaver syndrome (MIM: 277590),
21 Simpson-Golabi-Behmel syndrome (MIM: 312870) and Pallister-Killian syndrome had been
22 suspected in single cases (Table S2). Half of the individuals had scaphocephaly and sparse
23 frontotemporal hair. The craniofacial appearance was characterized by hypertelorism (91% of
24 individuals), downslanted palpebral fissures (67%), broad nasal tip (83%), and low-set and
25 posteriorly rotated ears (57%). Forty-three percent of subjects showed small, widely spaced teeth.
26 Skin hyperpigmentation was present in one third of individuals. Neonatal problems included

1 hypotonia, feeding difficulties, failure to thrive, jaundice, congenital hypothyroidism, and
2 micrognathia. Three showed single palmar creases possibly related with prenatal hypotonia. Finger
3 abnormalities included short fourth metacarpals, camptodactyly, fifth finger clinodactyly,
4 brachydactyly, broad and low-set thumbs. Toe abnormalities included long halluces, broad toes and
5 fifth toes overlapping fourth toes. Two individuals had pectus excavatum. Growth parameters at
6 birth were unremarkable. At last evaluation, only one subject (S10) had macrosomy (> 3.65 SD),
7 while 12 individuals (63%) had a large head circumference ($> +2.0$ SD), including two children
8 with relative macrocephaly at the age of 4 years. Developmental delay was present in all
9 individuals. They were mildly to moderately intellectually disabled. Fourteen cases have speech
10 delay, and gross motor delay was a common feature. In young individuals, hypotonia and a stiff,
11 clumsy and uncoordinated gait were relatively common findings. The 49-year-old individual (S1)
12 had gait ataxia. Behavioral features include ADHD (two individuals), autistic features (five
13 individuals), and psychotic episodes (one individual). One child had focal seizures in early
14 childhood, another child was treated for recurrent status epilepticus. Brain MRI reveals aspecific
15 abnormalities like mild inferior vermian hypoplasia, delayed myelination, partial agenesis of the
16 corpus callosum, and mild to moderately enlarged third and lateral ventricles. Five individuals
17 presented with mild hearing loss. Visual problems included hypermetropia, myopia, astigmatism,
18 and strabismus. Sixty percent of the individuals had feeding or eating difficulties, ranging from
19 satiety problems at younger age to problems swallowing fluids or learning to eat solid food. One
20 child was fed by G-tube at the age of 2.5 years. She had gastro-esophageal reflux disease and
21 rumination disorder. Other remarkable features in this cohort include pancytopenia accompanying
22 systemic lupus erythematosus in the oldest individual. A summary of the major features are
23 reported in Table 2, while a more detailed description for each subject is available in Table S2.

24 Notably, the oldest individuals of the present cohort (S1 and S3) and two previously
25 reported subjects (S15 and S17) had a facial appearance compatible with a much more advanced
26 age. When we evaluated the clinical presentation of the entire cohort of affected individuals,

1 including those previously reported, we noticed an overrepresentation of features that are generally
2 less common for individuals in this age group but more commonly found in elderly subjects,
3 including hypotrichosis, ptosis, cutis laxa, hyperkeratosis, skin hyperpigmentation, dry skin, nail
4 abnormalities, hearing loss, cataracts, diabetes mellitus and osteopenia (Table S3). One girl was
5 diagnosed with childhood hypophosphatasia (osteopenia and advanced bone age that was
6 investigated after presenting with multiple small stress fractures of the lower limbs). Three other
7 children also had advanced bone age. A fifth child has multiple fractures after minor trauma.
8 Several children had dental problems including missing permanent teeth and small, fragile teeth.

9 **Functional characterization of *HIST1H1E* mutations**

10 Based on the unique narrow spectrum of mutations, we hypothesized a specific disruptive impact on
11 *HIST1H1E* function as a consequence of the frameshift. Haploinsufficiency was considered
12 unlikely since endogenous *HIST1H1E* mRNA levels from two unrelated affected subjects (S1, 49
13 yrs; S2, 4 yrs) were comparable to controls and transient transfection experiments using Xpress-
14 tagged *HIST1H1E* constructs in COS-1 cells documented an increased stability of the mutant
15 proteins compared to the wild-type protein (Figure S1). Furthermore, confocal microscopy analysis
16 performed in transiently transfected HeLa cells showed proper nuclear localization and stable
17 binding to chromatin of the tested mutants (Figure S2). These data and the finding of a mutant
18 generated to express the third open reading frame at an equivalent position of the C-terminus (not
19 occurring in affected subjects) was characterized by compromised chromatin binding indicating
20 loss-of-function (Figure S2), strongly suggested a dominant negative or neomorphic effect as the
21 mechanism of disease.

22 H1 linker histones are core chromatin components, and bind to short DNA segments at the
23 entry/exit sites on the surface of nucleosomes, stabilizing their structure and regulating chromatin
24 folding.⁴ The C-terminal tail of H1 histones contains a number of serine/threonine residues that
25 undergo reversible phosphorylation, modulating the dynamics of chromatin compaction, which are
26 lost in disease-associated *HIST1H1E* mutants. Of note, while partial phosphorylation of the C-

1 terminal tail allows chromatin relaxation during interphase, full phosphorylation is required for
2 maximal chromatin condensation during mitosis.^{4,12} To assess chromatin compaction, single cell gel
3 electrophoresis (SCGE) assay was performed in primary fibroblasts from subjects S1 and S2.
4 Increasing run times performed to allow DNA loops to extend under the electrophoretic field
5 showed a significantly increased nucleoid relaxation in fibroblasts from affected individuals
6 compared to control cells, quantified as “tail moment” values (Figure 2A). Since chromatin
7 compaction is associated with a specific pattern of histone modifications, including methylation of
8 histone H3 at specific lysine residues,⁴⁰ the methylation profile of histone H3 was assessed in the
9 two fibroblast lines. Consistent with the SCGE data, a decrease in H3K4me2, H3K9me3, and
10 H3K27me3 staining was documented by immunofluorescence analyses (Figure 2B). In line with
11 these findings, reduced levels of HP1 β , a member of the HP1 family mediating
12 heterochromatinization by binding to methylated H3K9, and whose expression is reduced in cells
13 with a defective heterochromatin state,⁴¹ was also observed (Figure 2C). Finally, a caliculin-induced
14 premature chromosome condensation assay documented a significant reduction of condensed
15 chromosomes in fibroblasts from affected subjects (11.3 % of analyzed cells) compared to control
16 cells (19.6 %) ($P < 0.01$, χ^2 test). Overall, these data indicated a more relaxed state of chromatin in
17 cells expressing the disease-causing HIST1H1E mutants.

18 **Methylome profiling**

19 Since altered chromatin condensation is expected to impact DNA methylation,⁴² a genome-wide
20 methylation profiling analysis was used to investigate perturbations at the epigenome level. To this
21 aim, a subset of DNA samples obtained from leukocytes of six affected subjects were compared to
22 those referring to a control group including 35 healthy individuals of European descent by Infinium
23 MethylationEPIC BeadChip profiling. Considering the entire set of assayed target probes, the
24 analysis did not highlight a substantial change in methylation pattern (data not shown). However,
25 multidimensional scaling analysis carried out considering the information associated to the 1,000
26 most differentially methylated probes among all samples (independently from their group

1 classification) indicated clear-cut divergent methylation profiles between healthy controls and
2 affected individuals, the latter clustering according to the age of subjects (Figure 2D). Among these
3 informative probes, 170 differentially methylated CpGs were located within the UCSC CpG Islands
4 (from 200bp to 1500bp from TSS) (Table S4). KEGG and GO Cellular Component enrichment
5 analyses revealed a significant enrichment of genes linked to pathways mainly related to
6 neurological, immunological, and cell adhesion/membrane function, which represent cellular
7 processes relevant to the clinical phenotype of affected subjects (Table S5). A separate clustering of
8 affected pediatric individuals from controls was also observed by using a recently defined
9 episinature set modeled to successfully profile 14 developmental disorders (data not shown),⁴³
10 further supporting the occurrence of a specific methylation profile in subjects with *HIST1H1E*
11 frameshift mutations. These findings are in line with previous studies performed in cells with
12 defective histone H1 function documenting a minor impact on global DNA methylation. In these
13 cells, changes rather involved specific CpGs within regulatory domains of regulated genes,⁴⁴
14 indicating that chromatin rearrangement in these cells does not impact globally the methylation
15 status of DNA, but affects specific subsets of genes and cellular processes.

16 ***HIST1H1E* mutations cause accelerated cellular senescence**

17 Based on the occurrence of phenotypic features suggestive of premature aging and the fact that a
18 more relaxed compaction of chromatin has been functionally linked to replicative senescence,⁴⁵ a
19 feature occurring in progeroid syndromes,⁴⁶ we hypothesized that the aberrant function of the
20 *HIST1H1E* mutants might promote accelerated senescence. To test this hypothesis, proliferative
21 competence and morphological and biochemical markers of cellular senescence were evaluated.
22 Trypan blue exclusion assay documented a variable but consistently reduced proliferation rate in
23 cells from affected subjects (Figure 3A). Of note, a complete proliferative arrest was observed in S1
24 fibroblasts at relatively early passages. Consistent with this finding, flow cytometry analysis
25 documented a block in cell cycle transition from G0/G1 to S phase (Figure 3B). Compared to
26 control fibroblasts, those derived from affected individuals were larger and had a flattened and

1 irregular shape. This was apparent at early passages and became more pronounced with passage of
2 cultured cells (Figure S3). Additionally, compared to age/passage-matched control cells, fibroblasts
3 endogenously carrying the *HIST1H1E* mutant allele were characterized by a significantly
4 augmented SA- β -gal activity (Figure 4A), which is an established marker of cellular senescence.⁴⁶
5 Furthermore, enhanced levels of TP53, whose amount increases during replicative senescence,⁴⁷
6 were observed at early passages (Figure 4B). These data demonstrate a direct link between aberrant
7 HIST1H1E function and replicative senescence.

8 In premature aging disorders, replicative senescence can be caused by multiple events,
9 including DNA double-strand breaks (DSBs), telomere dysfunction and/or accelerated shortening,
10 as well as lamin defects.⁴⁸⁻⁵⁰ To evaluate the telomere status we first performed Q-FISH analysis,
11 which showed no difference in telomere length between control cells and fibroblasts from affected
12 subjects (Figure S4A). Immunofluorescence analysis designed to identify γ H2AX foci, a marker of
13 DSBs,⁵¹ showed an increased number of positive cells and foci *per* cell among those carrying the
14 HIST1H1E mutant compared to control cells (Figure S4B), indicating increased spontaneous DNA
15 damage. ImmunoFISH analysis also documented a higher frequency of co-localization between
16 γ H2AX foci and telomeres in cells from affected individuals compared to controls (Figure S4C),
17 highlighting the presence of dysfunctional telomeres in these cells. Fibroblasts carrying the mutated
18 *HIST1H1E* allele also showed a higher sensitivity to low doses of γ -ray irradiation (Figure 2E),
19 documenting augmented susceptibility to DNA damage. These findings suggest that the
20 proliferative arrest and senescent phenotype of cells expressing the mutant HIST1H1E may result,
21 at least in part, from ineffective DNA repair and persistent activation of DNA damage response
22 (DDR) signaling.^{52,53} To explore this possibility, the fraction of residual DNA damage was
23 evaluated by SCGE assay post- γ -ray irradiation. Kinetics data documented defective/delayed DNA
24 repair of single and DSBs in mutant fibroblasts compared to control cells (Figure S5).

25 Cells carrying *LMNA* (MIM: 150330) gene mutations driving cellular senescence exhibit
26 characteristic morphological nuclear abnormalities due to altered mechanical properties of the

1 lamina,⁵⁴ a network of structural filaments that interacts with chromatin to participate in chromatin
2 remodeling and organization, and DNA replication and transcription.⁵⁵⁻⁵⁸ Thus, we explored
3 possible changes in nuclear morphology and architecture. Immunofluorescence analysis showed
4 aberrant lamin A/C morphology in cells from affected individuals that were induced to divide after
5 thymidine/nocodazole treatment (Figure 5A). Aberrant morphology ranged from abnormal nuclear
6 shapes to nuclear blebbing. Of note, an increased number of aberrantly shaped nuclei in fibroblasts
7 carrying the *HIST1H1E* frameshift was observed at early passages compared to control cells, which
8 became more pronounced with passages ($p < 0.0000001$; two-tails Fisher exact test) (Figure 5B).
9 This feature is reminiscent of changes in nuclear shape occurring in progeroid syndromes, and
10 confirms the senescent phenotype associated with aberrant HIST1H1E function.

11 ***HIST1H1E* mutations trigger nucleolar instability**

12 Heterochromatin plays a role in maintaining nucleolar stability and controlling rRNA synthesis,⁵⁹
13 and decreased heterochromatin levels have been associated with nucleolar instability and
14 upregulated transcription of rRNA genes that, in turn, has been suggested to enhanced protein
15 synthesis and contribute to overgrowth and accelerated aging.^{60,61} Besides functioning in ribosome
16 biogenesis, the nucleolus is involved in cell cycle control,⁶² and linker histones have been reported
17 to interact with multiple nucleolar proteins implicated in various nucleolar functions.⁶³ Based on
18 these considerations, we explored whether *HIST1H1E* mutations trigger nucleolar instability. As
19 shown, S1 fibroblasts displayed fragmentation of the nucleolus, as revealed by loss of the nucleolar
20 marker nucleolin (Figure 6A), and showed increased amount of 18S and 28S rRNA levels (Figure
21 6B), further documenting the pleiotropic effect of *HIST1H1E* mutations. Notably these findings
22 suggest that besides affecting nucleolar function, dysregulated rRNA synthesis driven by
23 *HIST1H1E* frameshift mutations might represent the molecular event causally linked to
24 macrosomia, a recurring feature among young children with this class of mutations.^{19,20} **This**
25 **hypothesis is in line with other observations correlating tissue plasticity to ribosomal biogenesis, as**

1 documented for the skeletal muscle plasticity induced by seasonal acclimatization in *Cyprinus*
2 *carpio*.⁶⁴ **Aneuploidy is a major feature of cells bearing *HIST1H1E* frameshift**
3 Finally, because maximal chromatin compaction is required for proper chromosomal segregation
4 and aneuploidy is a marker of chromosomal instability characterizing the senescent
5 phenotype, we expected the occurrence of aneuploidy in cells expressing the disease-causing
6 *HIST1H1E* mutants. Indeed direct count of chromosomes in metaphases revealed a remarkably high
7 proportion of aneuploidy in early-passage fibroblasts from S1 (41.2 % of analyzed cells), which was
8 significantly higher even when compared to late-passage control fibroblasts (18.9 % of cells)
9 ($P < 0.01$, χ^2 test).

10

1 **Discussion**

2 Here we show that a specific class of dominantly acting frameshift mutations affecting the C-
3 terminal tail of HIST1H1E disrupts chromatin structure and nuclear lamina organization, and drives
4 cellular replicative senescence. By assessing the clinical records of a relatively large cohort of
5 subjects carrying these mutations we also document that this endophenotype is mirrored by features
6 suggestive of accelerated aging.

7 HIST1H1E is one of the members of the linker histone family, which function as structural
8 components of chromatin to control the extent of DNA compaction and contribute to the regulation
9 of gene expression and DNA replication, recombination, and repair.^{4,6,8,44} Linker histones are
10 encoded by multiple genes in the mammalian genomes, including paralogs characterized by a
11 diverse expression pattern during development and having either an ubiquitous distribution or an
12 expression restricted to specific cell types.^{9,65} Among these, HIST1H1E has been reported to be
13 expressed ubiquitously at a high level,⁶⁵ and similarly to the other replication-dependent linker
14 histones, is synthesized during the S phase to assemble chromatin with newly replicated DNA.⁶⁶ As
15 the other linker histones, HIST1H1E is relatively depleted from active promoters and other
16 regulatory regions controlling transcription and enriched in portions of the genome carrying
17 repressive histone marks.⁶ Notably, previous studies provided evidence that inactivation of any of
18 this class of histones does not significantly perturb murine development, which is compromised
19 only when a concomitant inactivation of multiple subtypes occurs, resulting in an extensive
20 reduction of these proteins.^{10,11} Consistent with this functional redundancy, our data do not support
21 haploinsufficiency as the molecular mechanism implicated in pathogenesis (see below), but point to
22 a specific, dominantly acting effect causing a profound perturbation of multiple cellular processes
23 directly and indirectly controlled by chromatin remodeling. Such pleiotropic effects converge
24 toward genome instability, epigenetic modifications, inefficient DNA repair ability, improper
25 chromosome compaction and segregation, nucleolar fragmentation, cellular senescence, and
26 replicative impasse.

1 Similarly to what is observed in progeroid disorders (*e.g.*, Hutchinson-Gilford progeria,
2 Werner syndrome, lipodystrophy syndromes), the cellular processes affected in the disorder
3 associated with aberrant HIST1H1E function link cellular senescence to premature aging. The
4 clinical profile of the affected subjects with *HIST1H1E* mutations, however, differs from progeroid
5 disorder and is quite peculiar, with a distinctive facies characterized by bitemporal narrowing,
6 macrocephaly, prominent forehead, high anterior hairline, sparse frontotemporal hair,
7 hypertelorism, downslanted palpebral fissures, broad nasal tip, and low-set and posteriorly rotated
8 ears being the main features. DD/ID invariably occurs, while overgrowth, which had been
9 originally reported as a key feature of the disorder, was not reported in any of the subjects at last
10 assessment, suggesting that enhanced growth may represent a feature characterizing infancy. While
11 the facial gestalt can help in recognition of the disorder, we noted that no pathognomonic features
12 can be used for a definitive clinical diagnosis. In early childhood, a tentative differential diagnosis
13 should include Pallister-Killian syndrome and mild phenotypes within the spectrum of Weaver
14 syndrome, Werner syndrome and other progeroid disorders.

15 Besides documenting the multiple events contributing to cellular senescence, the present
16 findings provide a mechanistic model for the multifaceted impact of this class of *HIST1H1E*
17 mutations. Our data further document the specific and narrow spectrum of disease causative
18 mutations affecting this gene. The long, lysine-rich C-terminal tail of HIST1H1E encompasses
19 multiple regulatory residues that are substrates for cyclin-dependent kinases, with the extent of
20 phosphorylation controlling proper compaction required for chromosome segregation, and local
21 chromatin decondensation needed to promote DNA transcription, replication and repair. The
22 phosphorylation of this region during the cell cycle has been theorized to contribute to the control of
23 the chromatin state by a twofold process.^{3,4,12,67} Partial phosphorylation is attained during interphase
24 (G0-S phase) allowing dynamical chromatin relaxation and access to DNA.⁶⁸⁻⁷⁰ On the other hand, a
25 maximal phosphorylation is attained during mitosis (M phase), which is considered to be required
26 for chromatin condensation and segregation of chromosomes into daughter cells during cell

1 division.⁷¹⁻⁷⁵ In this context, the functionally equivalent disease-causing frameshift events are the
2 only mutations that are predicted to not significantly affect the capability of the mutant protein to
3 retain efficient chromatin binding with concomitant loss of the regulatory sites required for
4 modulation of higher-order chromatin architecture. Consistent with this model, the *HIST1H1E*
5 truncating variants reported in gnomAD affect regions of the protein that do not overlap the
6 mutational hotspot here defined, but are much more proximal to the *N*-terminus or close to the *C*-
7 terminus (Figure S6). The former are predicted to result in truncated proteins with defective binding
8 to chromatin and/or expected to undergo accelerated degradation, while the latter retain the
9 regulatory serine/threonine residues and are not expected to have a dominant negative effect. The
10 complexity by which the *C*-terminal tail controls HIST1H1E function, the high stability of these
11 mutants, and their ability to bind to chromatin strongly support a dominant negative effect of these
12 mutants. However, neomorphism/gain-of-function as alternative mechanism(s) of disease cannot be
13 ruled out *a priori*, and dedicated studies are required to experimentally test the different models.

14 Taken together, the present findings provide evidence that dominantly acting functional
15 dysregulation of a linker histone causes a complex cellular phenotype characterized by replicative
16 senescence and results in a neurodevelopmental disorder characterized by accelerated aging.

17

18 **Supplemental Data**

19 Supplemental Data include six figures and five tables.

20

21 **Declaration of Interests**

22 The authors declare no competing interests.

23

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9

10 **Web Resources**

11 ExAC database, <http://exac.broadinstitute.org/>;

12 GnomAD, <http://gnomad.broadinstitute.org/>;

13 Gene, <http://www.ncbi.nlm.nih.gov/gene/>;

14 Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>;

15 Combined Annotation Dependent Depletion (CADD), <http://cadd.gs.washington.edu/>;

16 Database for nonsynonymous SNPs' functional predictions (dbNSFP),

17 [https://sites.google.com/site/jpopgen/dbNSFP](https://sites.google.com/site/jpopgen/dbNSFP;);

18 Gene SeT AnaLysis, <http://www.webgestalt.org/option.php>.

19

20 **Accession Numbers**

21 All mutations identified in this work have been submitted to ClinVar (Submission ID:

22 SUB5860205).

23

24

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- 7

1 **Figure Titles and Legends**

2

3 **Figure 1. Facial appearance of subjects with *HIST1H1E* frameshift mutations and protein**
4 **structure (A)** In affected subjects, facial appearance is characterized by a high anterior hairline,
5 prominent forehead, bitemporal narrowing, sparse temporal hair, hypertelorism, hooded eyelids,
6 short palpebral fissures, high and broad nasal bridge with full nasal tip, small, widely spaced teeth,
7 and low-set ears. A facial appearance compatible with a more advanced age (*e.g.*, hypotrichosis and
8 ptosis) is evident in S1 and S3 (S1, 49 yrs; S3, 30 yrs; S4, 14 months; S5, 12yrs; S6, 3yrs; S7,
9 12yrs; S9, 2yrs; S11, 6yrs; S12, 4yrs). **(B)** Schematic diagram representing the *HIST1H1E*
10 structure, which is composed by a globular domain flanked by *N*- and *C*-terminal tails. **The position**
11 **of the disease-causing frameshift mutations is shown above the cartoon (novel mutations are**
12 **highlighted in red). The number of independent cases identified in the present study is in brackets.**
13 **The domain boundaries and cyclin-dependent kinase phosphorylation sites (black triangles) are**
14 **reported below the cartoon. All mutations are expected to result in a shorter protein with an**
15 **identical divergent *C*-terminal tail (the new stop codon is shown below the cartoon, 194*).**

16

17 **Figure 2. SCGE assay, immuno-fluorescence studies, and methylation profiling analysis (A)**

18 Increasing electrophoresis run times (20, 40 and 60 min) highlighted significant differences in
19 relaxation of DNA supercoiling between fibroblasts from control and affected subjects. DNA
20 migration was quantified as Tail moment values, which is defined as the product of the tail length
21 and the fraction of total DNA in the tail (upper panel). Nucleoids of cells from subjects S1 and S2
22 showed a significantly higher Tail moment value (* $P < 0.05$, ** $P < 0.01$; two-tailed Student's *t*-
23 test). For each experimental point, at least 75 cells were analyzed. Values are mean \pm SEM of three
24 independent experiments. Representative images of nucleoids from fibroblasts from control and
25 affected individuals at each run time are shown (lower panel). **(B)** Confocal laser scanning

1 microscopy (CLSM) observations document an overall decreased amount of H3K4me2, H3K9me3,
2 and H3K27me3 staining (green) in S1 cells compared to control cells. Nuclei were stained with
3 DAPI. Images are representative of >200 analyzed cells. Bars correspond to 8 μm . (C) Fibroblasts
4 from subject S1 show a decreased amount of HP1 β compared to control cells (WT). Cells were
5 stained using antibodies against HP1 β (red) and HIST1H1E (H1.4) (green); DNA are DAPI-stained
6 (blue). Scale bars represent 7 μm . (D) Multidimensional scaling plot of genome-wide methylation
7 analysis using the top 1,000 most variable probes among samples. The plot shows the distinct
8 methylation profiles of pediatric (open circles) and adult (S1, duplicate) (filled circles) affected
9 subjects, compared to healthy controls (filled squares). (E) DNA damage was induced by 1 or 2 Gy
10 γ -ray irradiation. Tail moment values indicate the amount of radiation-induced DNA damage
11 measured by SCGE assay immediately after treatment. S1 and S2 fibroblasts showed a higher
12 sensitivity to γ -ray irradiation (* $P < 0.02$, ** $P < 0.001$; two-tailed Student's t-test). For each
13 experimental point, at least 75 cells were analyzed. Values are mean \pm SEM of three independent
14 experiments.

15

16 **Figure 3. Proliferation assay and cell cycle progression.** (A) Cells were seeded at 200,000
17 cell/well in a 6-well plate and incubated at 37 $^{\circ}\text{C}$. Cell numbers (mean of three replicates \pm SD) were
18 counted by Trypan blue exclusion assay after four and seven days. A significantly decreased
19 proliferation rate was observed in the fibroblast lines from the two unrelated affected individuals
20 (S1 and S2). Cells from subject S1 show a permanent cells growth arrest. (B) Cell cycle phases of
21 S1/S2's (right) and control (left) fibroblasts as measured by BrdU incorporation and propidium
22 iodide (PI) flow cytometry analysis. The upper box identifies cells incorporating BrdU (S phase),
23 the lower left box identifies G0/G1 cells and the lower right box represents G2/M cells. One of
24 three independent experiments is reported with the percentage of cells in different cell cycle phases.

25

1 **Figure 4. Defective HISTH1E function results in altered SA-β-gal activity and p53 expression**
2 **level.** (A) Representative images (left) and quantification (right) of SA-β-gal activity evaluated on
3 S1 and control (C) fibroblasts at different culture passages. The significance was measured by one-
4 way Anova with Tukey's multiple comparison test (* $P < 0.01$, ** $P < 0.0001$). (B) Compared to
5 control cells, enhanced TP53 protein levels were observed in S1/S2's fibroblast lysates at earlier
6 passages. Representative blots (left) and mean \pm SD densitometry values (right) of three
7 independent experiments are shown (* $P < 0.05$, ** $P < 0.002$; two-tailed Student's t-test).

8
9 **Figure 5. Defective HISTH1E function results in aberrant nuclear morphology that is**
10 **exacerbated over cell culture passages.** (A) CLSM analysis was performed in steady state (left)
11 and synchronized (right) skin fibroblasts induced to divide after thymidine/nocodazole treatment
12 and recovered with fresh medium. The panels show an aberrant nuclear morphology in cells from
13 subject S1. While control cells proceed through mitosis (representative metaphases are shown), S1
14 fibroblasts fail to progress. Experiments were carried out at early passages (passage 3). Cells were
15 stained using an antibody against lamin A/C (red) and DAPI (blue). Images are representative of
16 >200 analyzed cells. Scale bars represent 7 μm . (B) CLSM analysis was performed on S1 and
17 control fibroblasts seeded at different culture passages. The panels show an aberrant nuclear
18 morphology in cells from subject S1 at early passages compared to control cells, which were seeded
19 at late passages (passage 16). Percentages refer to the number of cells with aberrant nuclear
20 morphology. Cells were stained as above. Images are representative of > 200 analyzed cells. Scale
21 bars represent 27 μm .

22
23 **Figure 6. Defective HISTH1E function results in nucleolar fragmentation and increased 18S**
24 **and 28S rRNA levels.** (A) CLSM observations were performed on S1 and control (C) fibroblasts.
25 Panels show nucleolar fragmentation in S1 fibroblasts as revealed by a significant decrease in C3
26 clone antibody staining. Nuclei were stained with DAPI. Images are representative of > 200

1 analyzed cells. Scale bars represent 9 μm . **(B)** Total RNA was extracted from the same amount of
2 S1 and control (C) cells at different cellular passages. Three μl of total RNA was loaded for size
3 separation on 1% agarose gel and stained with ethidium bromide. An increased amount of both 28S
4 and 18S rRNA codifying for ribosomal subunits is evident in fibroblasts from subject S1 compared
5 to control cells.

6

7

1 **Table 1. Frameshift *HIST1H1E* mutations identified in this study.**

Nucleotide Change	gnomAD	ClinVar	Amino acid change	Domain	CADD ¹	Number of cases	Origin
c.408dupG	-	-	p.Lys137GlufsTer59	C-terminal tail	34	1	<i>de novo</i>
c.414dupC	-	-	p.Lys139GlnfsTer57	C-terminal tail	35	2	<i>de novo</i> (1)
c.425_431delinsAGGGGGT	-	-	p.Thr142LysfsTer54	C-terminal tail	31	1	<i>de novo</i>
c.425delinsAG	-	-	p.Thr142LysfsTer54	C-terminal tail	29	1	undetermined
c.430dupG	-	reported	p.Ala144GlyfsTer52	C-terminal tail	26.8	2	undetermined
c.441dupC	-	reported	p.Lys148GlnfsTer48	C-terminal tail	34	4	<i>de novo</i>
c.447dupG	-	-	p.Ser150GlufsTer46	C-terminal tail	34	1	<i>de novo</i>
c.464dupC	-	-	p.Lys157GlufsTer39	C-terminal tail	35	1	<i>de novo</i>

2

3 Nucleotide numbering reflects cDNA numbering with 1 corresponding to the A of the ATG translation initiation codon in the *HIST1H1E* reference
 4 sequence (RefSeq: NM_005321.2, NP_005312.1).

5

6 ¹ CADD v1.4.

1 **Table 2. Summary of the clinical features occurring in subjects carrying *de novo***
 2 ***HIST1H1E* frameshift mutations.**

3

Clinical features	Frequency ¹
DD/ID	18/18² (100%)
Motor delay	12/12 (100%)
Walking independently, range (mean), months	15-66 (31)
Speech delay	14/14 (100%)
Hypotonia	11/15 (73%)
Autistic features	5/14 (36%)
Craniofacial features	19/19 (100%)
Macrocephaly	12/19 (63%)
Scaphocephaly	6/11 (54%)
Sparse hair	6/12 (50%)
High anterior hairline	14/16 (87%)
Prominent forehead	13/14 (93%)
Hypertelorism	10/11 (91%)
Downslanted palpebral fissures	8/12 (67%)
Full nasal tip	10/12 (83%)
Low set ears	8/14 (57%)
Aging appearance	20/20 (100%)
Skin hyperpigmentation	5/15 (33%)
Hypotrichosis	6/20 (30%)
Skin hyperpigmentation	5/20 (25%)
Nail abnormalities	6/20 (30%)
Dental problems	6/20 (30%)
Advanced bone age	4/20 (20%)
Other	9/15 (60%)
Feeding or eating difficulties	9/15 (60%)
Hearing loss	5/14 (36%)
Strabismus	8/15 (53%)

4

5 ¹ The reported cohort includes 13 presently described subjects and 7 previously reported cases.

6 ² Subjects showed variable ID: 5 mild, 9 moderate, and 2 severe. In 2 cases the severity of ID was
 7 unspecified.

8

9