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ORIGINAL ARTICLE

Antioxidant treatment ameliorates phenotypic features of *SMC1A*-mutated Cornelia de Lange syndrome *in vitro* and *in vivo*

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Abstract

Cornelia de Lange syndrome (CdLS) is a rare disease characterized by cognitive impairment, multisystemic alterations and premature aging. Furthermore, CdLS cells display gene expression dysregulation and genomic instability. Here, we demonstrated that treatment with antioxidant drugs, such as ascorbic acid and riboceine, reduced the level of genomic instability and extended the *in vitro* lifespan of CdLS cell lines. We also found that antioxidant treatment partially rescued the phenotype of a zebrafish model of CdLS. Gene expression profiling showed that antioxidant drugs caused dysregulation of gene transcription; notably, a number of genes coding for the zinc finger (ZNF)-containing Krueppel-associated box (KRAB) protein domain (KRAB-ZNF) were found to be downregulated. Taken together, these data suggest that antioxidant drugs have the potential to ameliorate the developmental phenotype of CdLS.

Introduction

Cornelia de Lange syndrome (CdLS) is a rare multisystem disease characterized by pre- and postnatal growth delay, craniofacial abnormalities, intellectual impairment, limb anomalies, hypertrichosis, defects in the cardiopulmonary and gastrointestinal systems, and indications of premature physiological aging (1–3). CdLS is genetically heterogeneous and is caused by mutations in the *NIPBL*, *SMC1A*, *SMC3*, *RAD21* and *HDAC8* genes belonging to the cohesin pathway (2,4–8). Although cohesin was first identified as playing a critical role in holding sister chromatids together (9), experimental evidence indicates that cohesin also plays a role in both regulating gene expression and safeguarding the genome (10–16). These noncanonical roles have been supported by observations that most cohesin localizes to

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intergenic and intron/exon regions, and notably, about 60% of cohesin co-localizes with the CCCTC-binding factor (CTCF), a zinc finger DNA-binding protein. The interplay between cohesin and CTCF sites is involved in the long-range chromatin fiber interactions critical for mediating functional connections between promoters and their distal enhancers (17–21). Furthermore, mutations in cohesin genes have been shown to occur frequently in cancers characterized by genome instability, such as colorectal cancer, urothelial bladder carcinomas, Ewing sarcoma, acute myeloid leukemia, myelodysplastic syndrome and acute megakaryoblastic leukemia (22–26).

Cellular and developmental models of CdLS, including cell lines, mice, *Drosophila* and zebrafish display modest perturbations in gene expression (27–30). CdLS cells also exhibit a high degree of genome instability, which can manifest itself as both structural and numerical chromosome aberrations, premature centromere separation and a delay in DNA repair following genotoxic treatments (31). Recently, we showed that CdLS cell lines carrying mutations in the *SMC1A* gene present with decreased energy production and downregulation of proteins involved in detoxification and antioxidant pathways (32). However, the molecular mechanism underlying the CdLS phenotype remains incompletely characterized. It is well established that high levels of oxidative stress promote cell growth arrest, apoptosis, genome instability and premature aging (33–35). Given that all of these traits have been found in CdLS, reducing the cellular burden of reactive radical oxygen molecules presents a potential therapeutic approach for ameliorating the CdLS phenotype. Here, we treated *SMC1A*-mutated cells with two antioxidant drugs, ascorbic acid and riboceine, which are able to protect the cells from oxidative stress in two different ways. The major functional roles ascribed to ascorbic acid, vitamin C, and to riboceine, a mixture of D-ribose and L-cysteine, are the ability to scavenge free radicals through formation of a more stable ascorbyl radical and to increase the production of ATP and the antioxidant glutathione, respectively (36,37). In this study, we found that cells with mutations of the cohesin structural component SMC1A showed an increase in oxidative stress, spontaneous genome instability and premature cellular aging. Treatment with antioxidant drugs was found to decrease the degree of oxidative stress, reduce genome instability and prolong the lifespan of cells. RNA-seq analysis showed that treatment with riboceine and ascorbic acid led to changes in gene expression. Of note, antioxidant drugs decreased the expression of genes encoding the zinc finger (ZNF)-containing Krueppel-associated box (KRAB) protein domain, a domain known to be involved in cell proliferation, cell cycle regulation and DNA repair. Finally, zebrafish larvae carrying an *smc1a* heterozygous mutation to model CdLS showed increased DNA damage and a partial reversal in the mutant phenotype following riboceine treatment. These findings open a new avenue for further studies directed at exploring the potential clinical applications of antioxidant drug treatment in CdLS.

Results

Oxidative stress and genomic instability in *SMC1A*-mutated CdLS cell lines

CdLS patients show premature aging when compared with their chronological age (1,3). However, whether this phenomenon is correlated to a shortened cell life has never been investigated. To address this issue, the CdLS 417 and CdLS 565 cell lines, harboring the mutations c.3146 G>A and c.2046–2048delAGA in *SMC1A* gene, respectively, were cultured *in vitro*. These mutations did not affect the level of protein synthesis (data not shown). We found that CdLS cells became senescent at the 26th or 27th passage with a considerable decrease (about 40%) in their *in vitro* lifespan compared with three control cell lines (Fig. 1A). This senescence was confirmed with a β-galactosidase assay (Fig. 1B) with positive cells ranging from 78% to 82% in control and around 90% in CdLS cells. Because replicative senescence has previously been related to telomere shortening in the literature (38), we investigated telomere length in young and presenescent cells. FISH experiments using a telomere-PNA probe showed no difference in fluorescence intensity (data not shown). However, because the number of metaphase spreads and their quality were very low in pre-senescent cells, we measured the fluorescence intensity of the telomeres in 200 interphase nuclei and we found no difference in telomere length (data not shown). Next, we analyzed the level of oxidative stress during cell progression through *in vitro* culture. In order to study global oxidative stress, we measured the level of protein carbonyls by ELISA. At passage 14, the protein carbonyl content in CdLS cells was significantly higher than control cells (Fig. 1C). At the same passage, the frequency of spontaneous chromosome aberrations was also found to be significantly higher in the *SMC1A*-mutated cell lines (Figs 1D and E). As an additional marker of chromosomal instability, we investigated γ-H2AX focus formation. We found that the number of γ-H2AX foci was higher in the CdLS cells after 30 min of exposure to 2 Gy of irradiation when compared with the controls (Fig. 1F, $P < 0.05$). Altogether, these results suggest that the reduced *in vitro* lifespan of CdLS cells might result from a high degree of oxidative stress and genomic instability.

Effects of antioxidant drug treatment on CdLS cells

To investigate the effect of reducing the level of oxidative stress on the phenotype of CdLS cells, we treated the CdLS cells with ascorbic acid and riboceine. We found that both antioxidant drugs independently increased the number of passages the cells made *in vitro* by about 50%, using untreated CdLS cell as control for lifespan (Fig. 1A). These data are further supported by the decrease in both the number of β-galactosidase positive cells (data not shown) and their staining intensity (Fig. 1B). Drug treatment brought the protein carbonyl content down from 0.48 to 0.44 nmol/mg in the untreated CdLS cells to control levels at 0.21–0.19 nmol/mg with ascorbic acid and 0.18–0.17 nmol/mg with riboceine (Fig. 1C). Finally, analysis of the metaphase spreads revealed a strong decrease in chromosomal aberrations following antioxidant treatment. The frequency of chromosomal aberrations in the CdLS cells was higher than in control cells at 0.23–0.26, the treatment partially rescued this increase, bringing chromosome aberrations down to 0.10–0.14 per cell (Fig. 1E). These data indicate that antioxidant drugs reduce the level of oxidative stress and genomic instability, potentially contributing to a prolonged lifespan of CdLS cells *in vitro*.

Effect of antioxidant treatment on gene expression

We next asked whether antioxidant treatments affected gene expression in CdLS cells by performing RNA-seq. The ascorbic acid treatment dysregulated the expression of 2548 and 5332 genes in CdLS 417 cells and CdLS 565 cells, respectively (Table 1, Fig. 2A). Riboceine treatment altered the expression of 1840 and 2962 genes in CdLS 417 and CdLS 565 cells, respectively (Table 1, Fig. 2A). However, when CdLS cell lines were given ascorbic acid or riboceine treatments there were only 1715 and 1118 genes

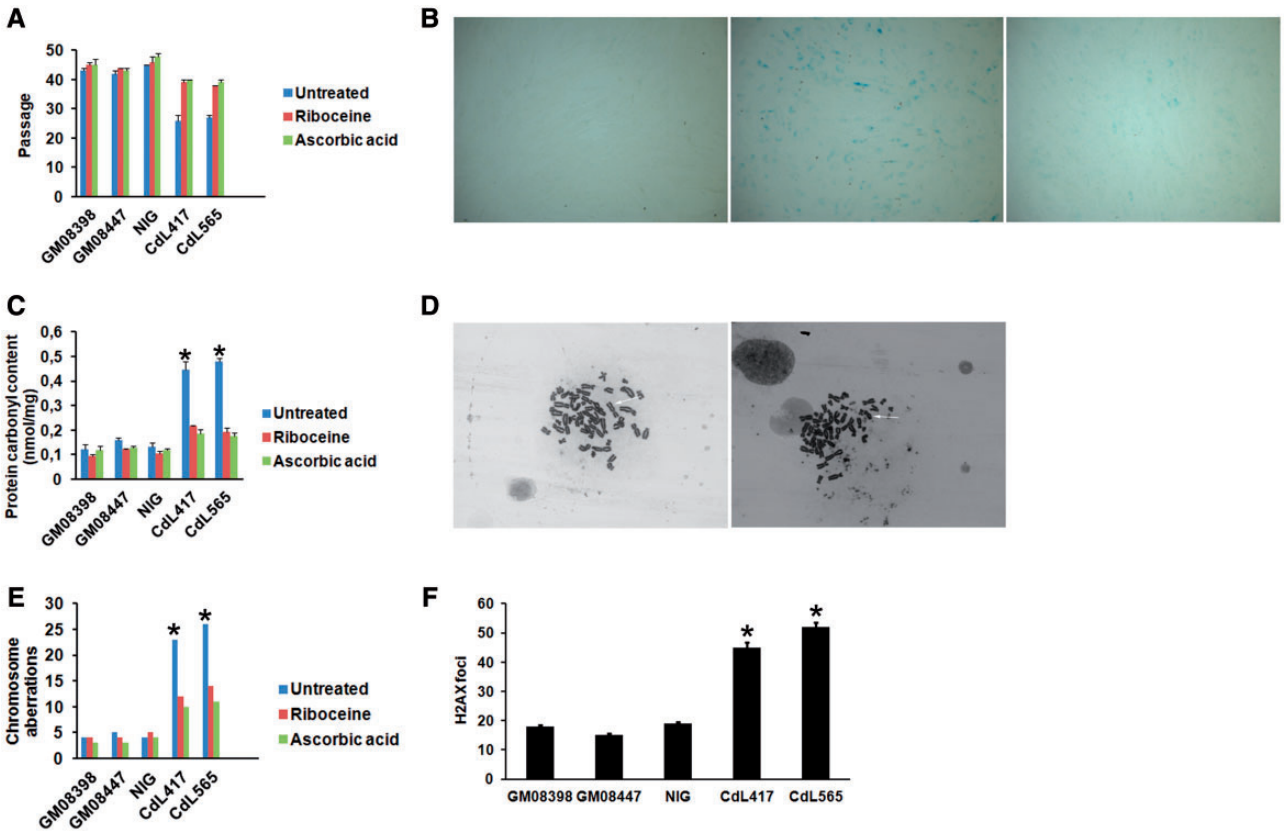

Figure 1. Effect of ascorbic acid and riboceine treatments in *SMC1A*-mutated cell lines. (**A**) Effects of antioxidant drug on replicative senescence in *SMC1A*-mutated fibroblasts and control cell lines. (**B**) CdLS 417 fibroblasts at early passage (left), senescent at passage 26 (medium) and at passage 26 following ascorbic acid (right) were subjected to *in situ* β-galactosidase staining. (**C**) Protein carbonyl content, as marker of oxidative stress, was measured in both CdLS and control cells with and without drug treatment. (**D**) Chromosome aberrations (indicated by an arrow) during *in vitro* cell culture progression of CdLS 417 (left) and CdLS 565 (right) cells. (**E**) Number of chromosome aberrations found in 100 Giemsa-stained metaphase spreads with and without antioxidant treatment. (**F**) Mean of gamma-H2AX foci following irradiation with 2 Gy. Bar: Standard error. $^{*}P < 0.05$.

Table 1. Effects of antioxidant drug treatment on gene expression

Cell line	Treatment					
	Ascorbic acid		Total	Riboceine		Total
	Down-regulated	Up-regulated		Down-regulated	Up-regulated	
CdLS 417	1614	934	2548	1485	355	1840
CdLS 565	3339	1993	5332	2126	836	2962

altered in both cell lines, respectively (Fig. 2A). Gene Ontology (GO) enrichment analysis on the differentially expressed genes revealed that the biological process terms 'regulation of transcription-DNA-templated' (GO: 0006355) and 'transcription-DNA-templated' (GO: 0006351) were significantly over-represented in both of the CdLS cell lines after ascorbic acid treatment (Supplementary Material, Table S1). Riboceine treatment was found to alter genes involved in regulating transcription, but the GO term 'regulation of gene silencing' (GO: 0060968) was found for both CdLS cell lines (Supplementary Material, Table S1). There were 741 genes differentially expressed in both treatments and both CdLS cell lines (Fig. 2A). With the exception of a few genes, most genes displayed only minor fold changes that ranged from +3 to −3. Significant over-representation of transcription pathway (R-HAS-212436 2436) involving 36 down-regulated zinc family genes (Supplementary Material, Table S2), as well as transcription-DNA-templated (GO: 0006351) and regulation of transcription-DNA-templated (GO: 0006355) GO biological process terms were detected (Fig. 2B).

Among the set of enriched genes within both treatments and both CdLS cell lines, 56% (51 out of 91) of genes were found to be ZNF coding genes, of which 86.3% (44 out of 51) contain a KRAB domain (Fig. 2C). It is worthy to note that quantitative PCR data showed that ZNF coding genes were not dysregulated in untreated CdLS cell lines (Fig. 3A) whereas they were down-regulated after antioxidant treatment (Supplementary Material, Fig. S1). Also down-regulated with both drug treatments and in both CdLS cell lines was the Cbp/p300 interacting transactivator with Glu/Asp-rich carboxy-terminal domain 4 (*CITED4*) gene.

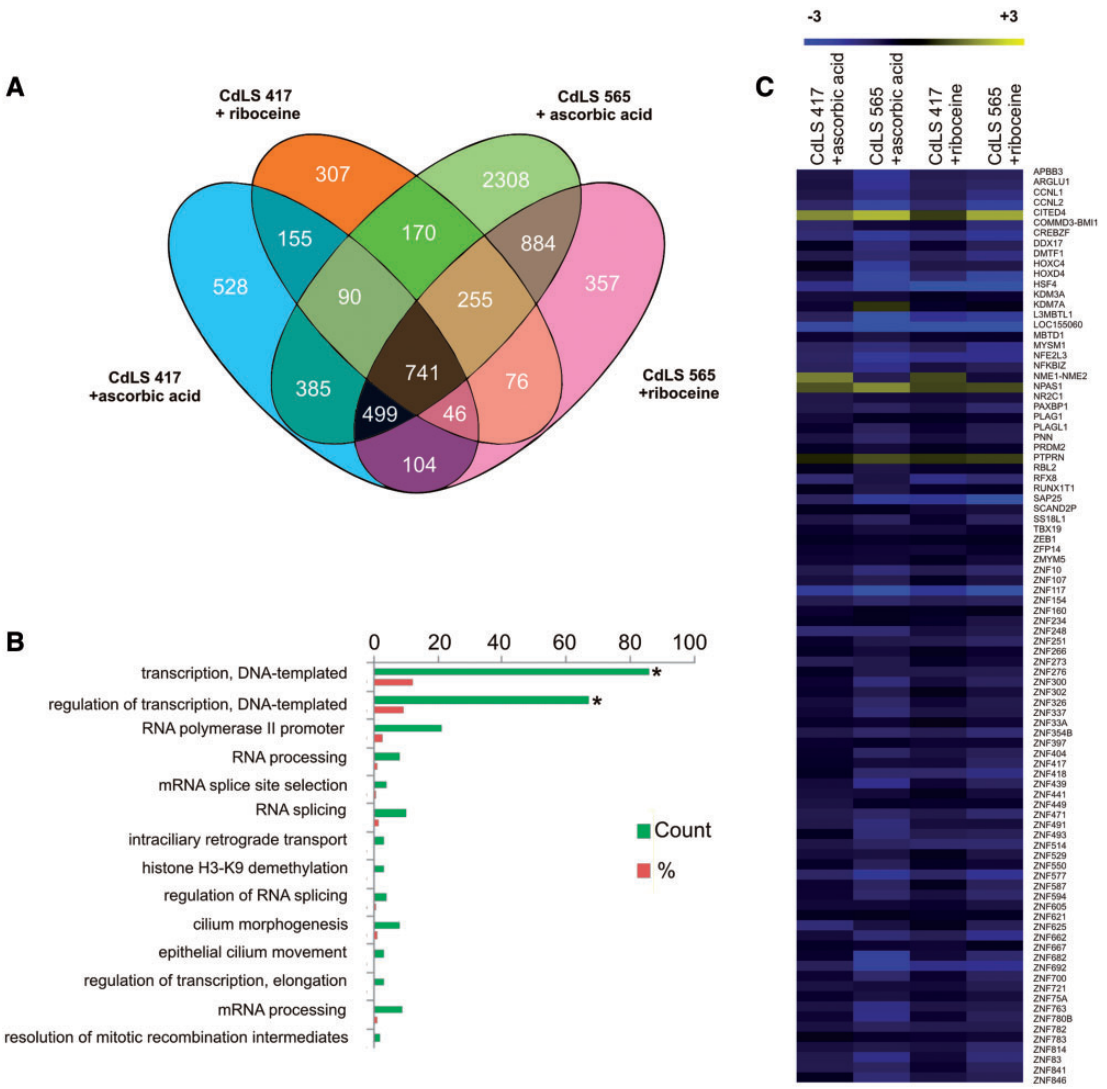

Figure 2. RNA-seq analysis after treatment with antioxidant drugs. (**A**) Venn diagram of differentially expressed genes following ascorbic acid and riboceine treatments. (**B**) GO term enrichment analysis of biological process that were significantly over-represented when considering differentially expressed genes in both treatments and both CdLS cell lines. (**C**) Heatmap of identified enriched genes. Enrichment analysis was performed using differentially expressed genes in both treatments and both CdLS cell lines for pathway and biological process enrichment analysis. *FDR < 0.05

Up-regulated with both drug treatments and in both CdLS cell lines were neuronal PAS domain protein 1 (*NPAS1*), protein tyrosine phosphatase, receptor-type N (*PTPRN*), histone methyl-lysine-binding protein (*L3MBTL1*) and heat shock transcription factor 4 (*HSF4*). The expression of these genes was analyzed by quantitative PCR and used to validate the RNA-seq data (Fig. 3B). Taken together, these data indicate that both ascorbic acid and riboceine affect gene transcription and highlight the potential of antioxidant drugs to rescue the CdLS cell phenotype.

Effect of antioxidant in a zebrafish model of CdLS

To test the effects of antioxidant exposure in an *in vivo* model we used the zebrafish *smc1a*^{hi1113a} mutant, which is null for *smc1a* (39,40). Zebrafish lacking Smc1a die by around 3 days post-fertilization (dpf) with multiple developmental abnormalities including small eyes, cardiac edema and loss of circulation. In homozygous mutants, which have a complete lack of Smc1a, death is caused by cell cycle arrest resulting from a failure in sister chromatid cohesion. Up until that time, embryos can still undergo cell division using maternally deposited Smc1a RNA and protein.

Although *smc1a*^{hi1113a} heterozygotes appear to be phenotypically normal, they were found to have a higher background level of DNA damage than wild-type animals, as assessed by comet assay (Fig. 4A, Supplementary Material, Fig. S2). This finding indicates that insufficiency of Smc1a has a genotoxic effect in a live animal model, just as was observed in the *SMC1A* mutant cell lines (Fig. 1). However, enzymatically cleaving sites of 8-oxoguanine (8oxoG) did not reveal a proportionally higher level of DNA oxidation in the *smc1a*^{hi1113a} heterozygote larvae (Fig. 4A, Supplementary Material, Fig. S2). To determine if an antioxidant can ameliorate the mutant phenotype of *smc1a*^{hi11113a}, we applied the antioxidant riboceine to homozygous mutants. Although riboceine could not rescue the death resulting from cell cycle arrest, we did observe that the riboceine-treated fish had less cardiac edema by 48 hours post-fertilization (hpf)

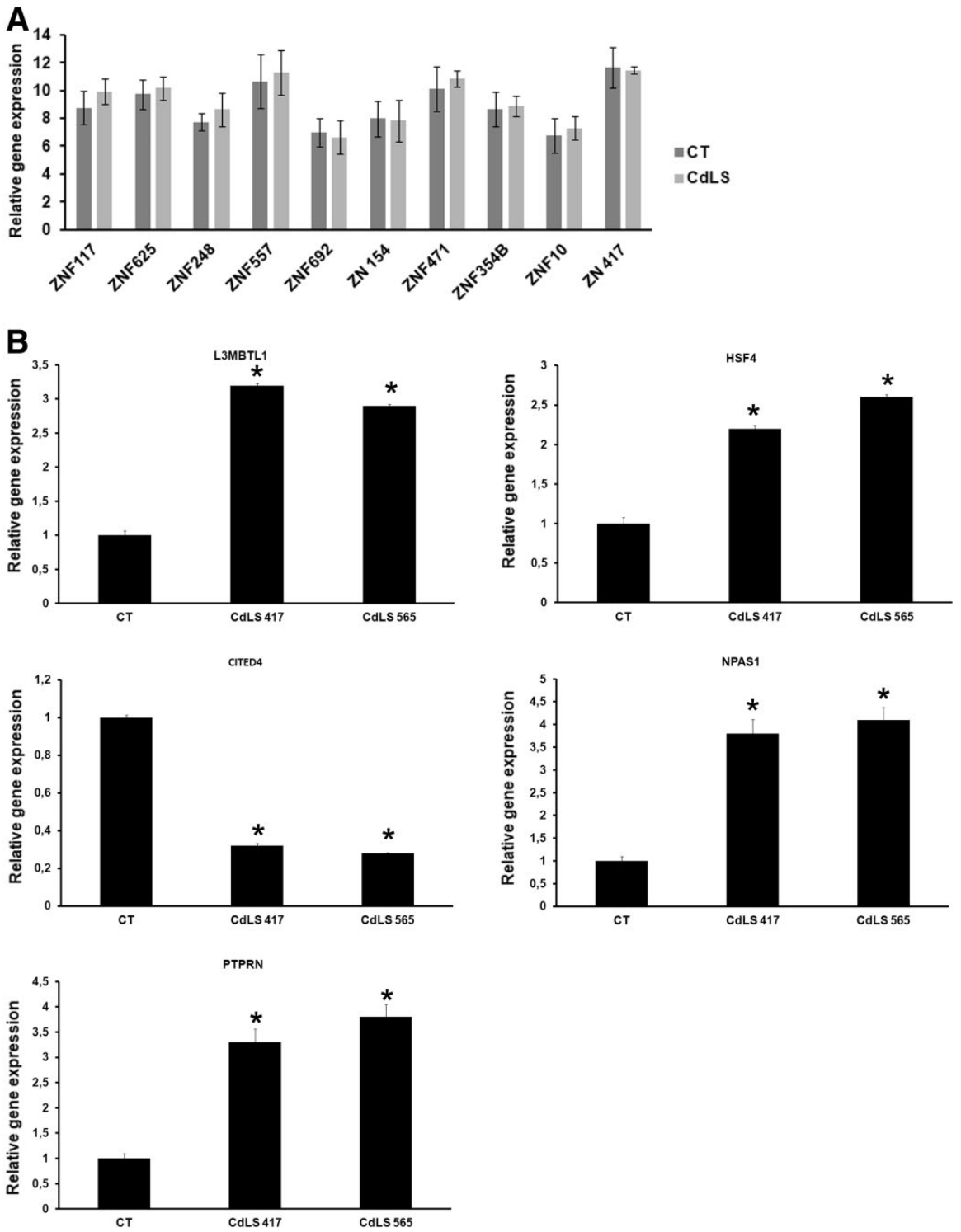

Figure 3. Expression of ZNF coding genes in untreated CdLS cells and RNA-seq data validation. (**A**) Selected ZNF coding genes identified as down-regulated after treatment with ascorbic acid or riboceine in CdLS cell lines. Analysis revealed that gene expression of these ZNF coding genes does not differ between CdLS and control cell lines. Error bars represent standard deviation (SD; $n = 3$). (**B**) Gene expression profile data was validated for *CITED4*, *NPAS1*, *PTPRN*, *L3MBTL1* and *HSF4* genes by RT-qPCR. *$P < 0.05$.

and a modest improvement in circulation relative to the DMSO-treated controls (Fig. 4B). In addition, *l3mbtl1*, *hsf4* and *ptprn* genes were up-regulated as in CdLS cells, though their differences were not statistically significant (Supplementary Material, Fig. S3). Only a modest rescue was observed in zebrafish homozygous for the *smc1a*^{hi1113a} mutation, which show severely compromised survival. However, the results appear to suggest that antioxidants could be of benefit when Smc1a is only partially compromised.

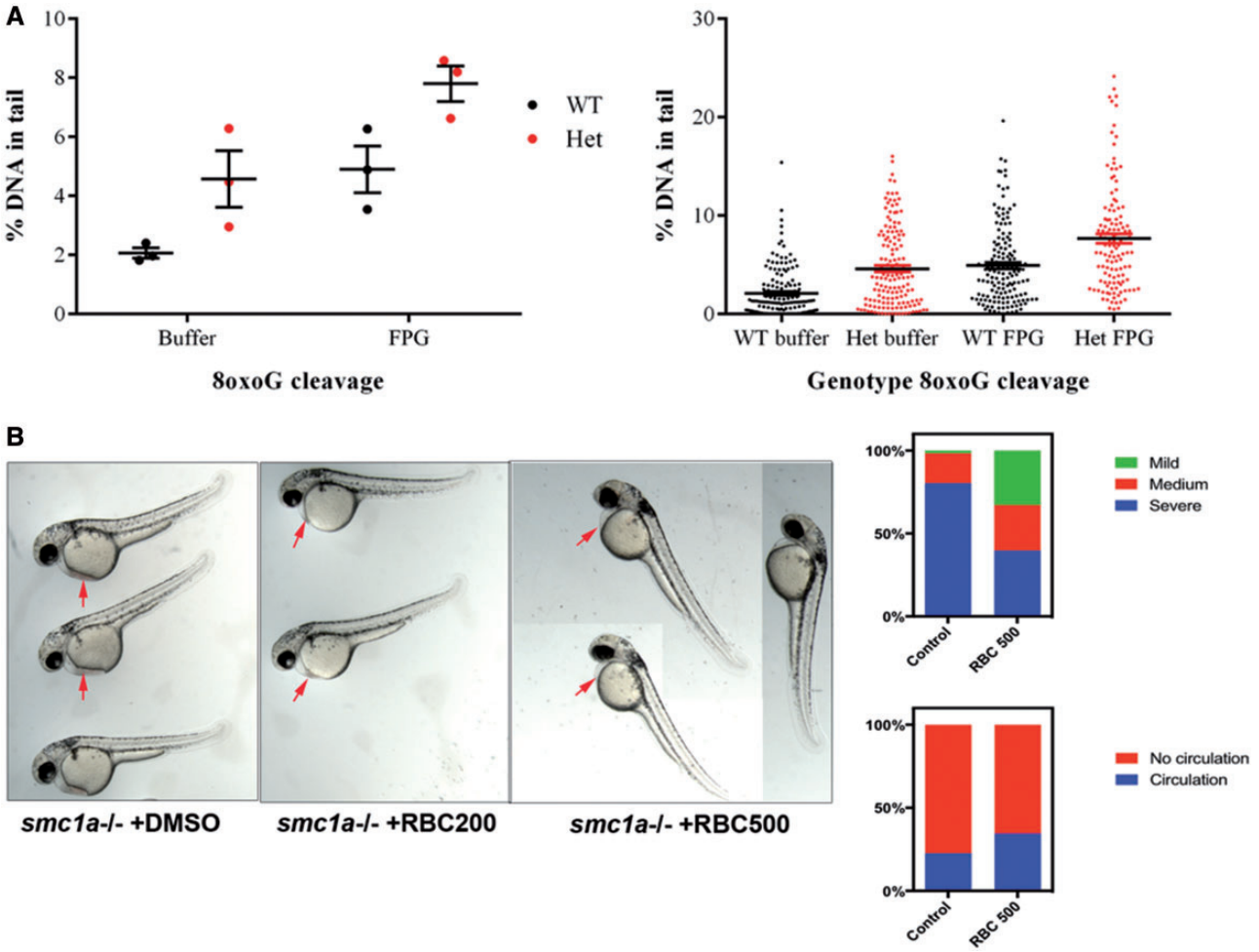

Figure 4. *smc1a*-mutant zebrafish have higher levels of DNA damage and show partial phenotypic rescue with the antioxidant riboceine. (**A**) comet assay analysis of wild-type (black points) versus *smc1a*^{hi1113a} heterozygotes (red points). Left, combined data from three independent experimental replicates. Right, combined datapoints from all replicates. Slides were either treated with FPG to reveal 8oxoG sites or a buffer to analyze double-strand breaks. (**B**) *smc1a*^{hi1113a}-mutant zebrafish exhibit mild phenotypic rescue when treated with riboceine. Cardiac edema (red arrows) is noticeably reduced when mutant animals are exposed to riboceine at 200 μM (+RBC200) and 500 μM (+RBC500) relative to controls (+DMSO). Graphs show rescue according to severity of dysmorphology (top) or presence of blood circulation (bottom). Graphs represent a combination of three replicates: $n = 66$ for controls and $n = 58$ for riboceine treated.

Discussion

CdLS patients display a plethora of phenotypic effects, including intellectual disability, craniofacial alterations, multiorgan defects, and premature aging when compared with their chronological age (1,3). Though the molecular mechanisms underlying CdLS are poorly understood, it has been recognized that CdLS cells show genome instability (41–43) suggesting that cohesin plays a critical role in safeguarding the genome. In addition, proteomic analysis had identified alterations to oxidative stress pathways in CdLS cells (32). Here, we saw that mutations in *SMC1A* predisposed the cells to protein oxidation, DNA damage, genomic rearrangements and cellular senescence. These data suggest that an accumulation of cellular damage and a cellular aging phenotype contribute to the etiology of CdLS.

Antioxidant drugs are able to protect the DNA from damage and prevent redox-induced death, as was shown in cerebellar cells using *N*-acetyl-cysteine (NAC) (44,45). The links between oxidative stress and genome instability in neurodegeneration and senescence (46–48) suggest that antioxidant therapy could provide a means of improving some of the phenotypic features of CdLS. Here, we provide the first evidence that antioxidant drugs can prolong the *in vitro* lifespan of CdLS cells and ameliorate CdLS-like phenotypes during development. Our data indicate that the protective effects of antioxidant therapy might be mediated by changes in gene expression.

Treatment with the antioxidants ascorbic acid and riboceine resulted in down-regulation of KRAB-ZNFs. While the gene targets of KRAB-ZNFs have not been identified, an interaction is known to occur with KAP1, which results in the KRAB-ZNF/KAP1 complex (49,50). Interestingly, KRAB-ZNF/KAP1 is required at sites of DNA damage where it has a role in modifying the chromatin environment and recruiting DNA repair proteins (51,52). Therefore, ascorbic acid and riboceine might help to activate the DNA damage response carried out by KRAB-ZNFs.

Since the function of KRAB-ZNFs depends on their ability to induce chromatin modification, we infer that antioxidant drug treatment resulted in global chromatin modification. This idea is further supported by the observation that the drug treatments also changed the levels of *CITED4*, *L3MBTL1* and *NPAS1*: genes that regulate gene transcription via chromatin remodeling (53–56). Cohesin has an important role in gene regulation (27,30), a function performed, in part, by mediating changes in

3D chromatin structure. As such, CdLS cell rejuvenation may involve structural rearrangement of the nucleus to impart gene expression dysregulation and protect against genomic instability.

The treatments also affected the expression *HSF4*: a gene known for its ability to modulate the cellular response to stress by specific gene expression regulation suggesting that antioxidant exposure triggered an environmental stress response (57–60). This study recorded a general decrease in protein oxidation, suggesting that a stress response was initiated by changes in the redox status of cellular signaling proteins. While specific protein targets of antioxidant-induced reduction in oxidative stress were not identified, a previous study suggests that cytoplasmic peroxiredoxins may be involved (32). These data show that targeting cell stress pathways have the potential to ameliorate cellular phenotypes associated with CdLS thus identifying an opportunity for potential treatment development for features of CdLS using antioxidant therapy.

Materials and Methods

Cell culture

All experiments were performed using the CdLS 417 and CdLS 565 cell lines, carrying the mutations c.3146 G>A and c.2046–2048delAGA in the *SMC1A* gene respectively. Normal human primary fibroblasts, named NIG, derived as described previously (61), GM08398 and GM08447, purchased from Coriell Cell Repositories, were used as control cell lines. Cells were grown in Dulbecco's minimal essential medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1% L-glutamine and 10% fetal calf serum in a humidified 5% CO_2 atmosphere.

Ethics statement

The CdLS 417 and CdLS 565 patients were enrolled under an IRB-approved protocol of informed consent at the Children's Hospital of Philadelphia. Zebrafish were maintained under standard conditions. All zebrafish research was approved by the University of Otago Animal Ethics Committee.

Riboceine and ascorbic acid treatment

Fibroblasts were treated with 50 μM of riboceine or 50 μM of ascorbic acid (Sigma Aldrich). Treatments were performed every 3 days to over 15 days.

Cytogenetic analysis

Colcemid was added to the cultures for 60 min, followed by a 30 min incubation in 0.075 M KCl hypotonic solution at 37°C and multiple changes of Carnoy's fixative. One hundred Giemsa-stained metaphases were analyzed by Leica DM2500 microscope.

γ-H2AX foci

In order to investigate DNA repair following irradiation, fibroblasts were exposed to 2 Gy by a linear accelerator (Siemens Primus) with a 6 MV photon energy source. Cells were fixed in 2% paraformaldehyde for 10 min, permeabilized in 0.2% Triton X-100 and blocked in PBS with 1% BSA. Thereafter, cells were incubated with primary antibody γ-H2AX (Trevigen), and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes). γ-H2AX foci were scored manually from at least 200 cells by Leica DM2500 microscope.

β-Galactosidase staining

The senescence assay was performed using the Senescence cell histochemical staining kit (Sigma Aldrich) following the manufacturer's instructions.

Measurement of oxidative stress

Protein carbonyl content was assayed with enzyme-linked immunosorbent assay (ELISA) after derivatization with 2,4-dinitrophenylhydrazine using Protein Carbonyl ELISA Kit (Cell Biolabs) and following the manufacturer's instructions.

Telomere fluorescence in situ hybridization (FISH)

Telomere FISH was performed with Telomere PNA FISH kit (Agilent) according to manufacturer's instructions. Cells were analyzed using a Leica DM2500 microscope.

Comet assay

Comet assays were performed as described previously (62), but with minor changes. Briefly, 2 dpf dechorionated embryos from each zebrafish cross were mechanically homogenized in Ringer's solution containing 0.13 Wünsch units/ml Liberase TL and incubated at 37 °C for 2 h. Cell suspensions were washed twice with PBS and passed through a 40 μm cell strainer. Cells were mixed in a 1: 1 ratio with 1% low melting point agarose and added to slides precoated with 1% agarose. For cell lysis, slides were covered with N_1 solution (2% sarkosyl, 0.5 mg/ml proteinase k, 0.5 M Na_2EDTA) and incubated at 37°C overnight. Slides were then rinsed with N_2 solution (90 mM Tris buffer, 90 mM boric acid, 2 mM Na_2EDTA, pH 8.5). For measurement of oxidative DNA damage, slides were incubated with 1/3000 formamidopyrimidine-DNA glycosylase for 45 min at 37°C, to cleave oxidatively modified DNA. The slides were then rinsed and electrophoresed in N_2 solution at 20 V for 20 min. The DNA on the slides was then stained with 2 μg/ml propidium iodide for 2 h at room temperature. Comets were visualized under RFP light on an inverted microscope. Photos of comets were analyzed using CometScore freeware v1.5 (TriTek).

Zebrafish riboceine treatment

smc1a^{hi1113a} heterozygotes were incrossed and fertilized eggs were collected and incubated in E3 embryo medium until 6 hpf. Embryos were then split into two petri dishes in which half were treated with DMSO carrier and half with riboceine at 200 or 500 μM. At 48 hpf, mutant embryos were identified and scored for rescue phenotype.

Library preparation and RNA sequencing

Library preparation was obtained using the TruSeq Stranded mRNA Sample Prep kit (Illumina). The poly-A mRNA was fragmented for 3 min at 94°C and every purification step was performed using 1× Agencourt AMPure XP beads. Both RNA samples and final libraries were quantified using the Qubit 2.0 Fluorometer (Invitrogen) and the quality was tested using the Agilent 2100 Bioanalyzer RNA Nano assay (Agilent). Libraries were then processed with Illumina cBot for cluster generation on the flowcell, following the manufacturer's instructions and

sequenced on single-end mode on HiSeq 2500 (Illumina). The CASAVA 1.8.2 version of the Illumina pipeline was used to process raw data for both format conversion and de-multiplexing.

RNA-seq analysis

To avoid low-quality data, adapters were removed by Cutadapt1 and lower quality bases were trimmed by ERNE2. For the analysis of differentially expressed genes, the quality-checked reads were processed using the TopHat version 2.0.0 package (Bowtie 2 version 2.2.0) as FASTQ files 3, 4, 5. Reads were mapped to the human reference genome GRCh37/hg19. Read abundance was evaluated and normalized by using Cufflinks3 for each gene and Cuffdiff from the Cufflinks 2.2.0 package was used to calculate the differential expression levels and evaluate the statistical significance of detected alterations. Only protein-coding genes were considered and gene level expression values were determined by read per kilobase of exon per million fragments mapped (RPKM). All genes with RPKM > 1 were designated as expressed.

Pathway analysis and function

The differentially expressed genes were functionally analyzed for biological processes using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov; date last accessed May 14, 2018).

cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Human cells

Total RNA was extracted by RNAeasy Mini-kit (Qiagen) and cDNA was synthesized with SuperScript™ II reverse transcriptase using oligo dT (Invitrogen). PCR analyses were performed using Rotor Gene 3000 (Corbett). qPCR reactions were run in duplicate and normalized with respect to HPRT. Since no difference was found in control cell lines, data was pooled. Primers used for mRNA expression analysis are listed in Supplementary Material, Table S3.

Zebrafish cells

Total RNA was isolated from control and RBC (500 uM) treated *smc1a*-mutant zebrafish embryos at 48 h post-treatment using the NucleoSpin RNA kit (Macherey-Nagel). cDNA was synthesized with qScript cDNA SuperMix (Quanta Biosciences). *l3mbtl1*, *hsf4* and *ptprn* expression were measured using TaKaRa SYBR Premix Ex Taq™ (Clontech) on a LightCycler400 (Roche Diagnostics). Primers *l3mbtl1* F: 5′-ACATGCACATGACGGAGCTA-3′ and R: 5′-AGCCTCAGATTTCTCGTCCA-3′, *hsf4* F: 5′-AGTGCA GTCATGCAGGAGAA-3′ and R: 5′-AAATCTGCCTTGGTCGAAAA-3′ and *ptprn* F: 5′-TGGAGAGCCCTGTTTTTGAT-3′ and R: 5′-AT GTCTGGAGCTACGGCACT-3′ were used for amplification. Gene expression analyses were carried out on qBase Plus (Biogazelle) and were normalized relative to the mean of reference elongation factor 1a (*ef1a*) and ribosomal protein L17 (*rpl17*).

Statistics

Data was analyzed by Student's *t*-test, *P*-values <0.05 were considered statistically significant.

Accession Number

Short reads have been deposited into NCBI Sequence Read Archive under BioProject accession PRJNA391403.

Supplementary Material

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. None declared.

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