Epigenetic Silencing of Lysyl Oxidase-Like-1 through DNA Hypermethylation in an Autosomal Recessive Cutis Laxa Case

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We have recently reported a case of cutis laxa caused by a fibulin-5 missense mutation (p.C217R). Skin fibroblasts from this individual showed an abnormal pattern of expression of several genes coding for elastic fiber-related proteins, including *lysyl oxidase-like-1* (*LOXL1*). In this study we intended to elucidate the mechanism responsible for *LOXL1* downregulation in these fibulin-5-mutant cells. We identified a proximal region (−442/−342) of the human *LOXL1* promoter in which two binding sites for the transcription factor specific protein 1 (Sp-1) are required for gene activity in normal fibroblasts. Binding of Sp-1 to these sequences was dramatically reduced within cutis laxa cells, although Sp-1 expression was normal. Further analysis of the promoter sequence found increased methylation levels in cutis laxa cells compared with cells from unaffected individuals. When DNA methyltransferase activity was transiently inhibited in cutis laxa cells using the 5-aza-2′-deoxycytidine, we found a significant increase in *LOXL1* expression. In conclusion, besides changes caused by the fibulin-5 mutation, *LOXL1* gene regulation is affected by an epigenetic mechanism that can be reversed by an inhibitor of DNA methyltransferase activity. It is not yet known whether *LOXL1* gene expression is affected in all cases of cutis laxa arising from fibulin-5 mutation.

Journal of Investigative Dermatology (2010) **130,** 2594–2601; doi:10.1038/jid.2010.186; published online 8 July 2010

INTRODUCTION

Lysyl oxidase-like 1 (LOXL1) enzyme belongs to the lysyl oxidase (LOX) family involving five related members: LOX, LOXL1, LOXL2, LOXL3, and LOXL4 (Kagan and Li, 2003). They show highly similar sequences in their catalytic C-terminal regions, whereas their N-terminal regions are unique. This would suggest identical enzymatic activities but different patterns of expression and secretion. LOX, the best characterized isoform, was first identified as the enzyme responsible for crosslinking type I collagen and elastin *in vitro* (Kagan and Trackman, 1991). More recently, the role of LOXL1 in elastin crosslinking has also been shown *in vitro* (Borel *et al.*, 2001) and its association with human skin elastic fibers confirmed (Noblesse *et al.*, 2004). Mice deficient in LOXL1 do not deposit normal elastic fibers and present many tissue defects, such as enlarged airspaces of the lung, loose skin, and vascular abnormalities. In addition, post partum reconstruction of uterine elastic tissue is impaired in $LOXL1^{-/-}$ female mice and prolapse of the pelvic organs is observed (Liu *et al.*, 2004). Immunohistochemical studies during development and growth of mouse tissues underlined the fact that despite LOXL1 being present in most major organs in late fetal and newborn mice, it generally diminishes as animals age (Hayashi *et al.*, 2004). A decrease in LOXL1 levels has also been correlated with aortic fragility in the rat because of a defect in collagen and elastic fibers remodeling (Behmoaras *et al.*, 2008). These data highlight the importance of LOXL1 in elastogenesis during development and aging.

For normal assembly of elastic fibers, the N-terminal region of LOXL1 is required to localize the enzyme to elastic fibers (Thomassin *et al.*, 2005) and to bind fibulin-5, which facilitates elastin polymerization onto the microfibril scaffold (Liu *et al.*, 2004; Kielty, 2006).

Cutis laxa-related homozygous inherited mutations in the gene for fibulin-5 are considered as null mutations with respect to the role of fibulin-5 in elastic fiber formation because of decreased interaction between tropoelastin and fibrillin-1 (Hu *et al.*, 2006). We have reported two cutis laxa cases for a brother and sister from a consanguineous Lebanon family with autosomal recessive transmission (Claus *et al.*, 2008). The 9-year-old boy presented with bilateral inguinal hernias at birth. At 1 month, the parents noticed redundant

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Abbreviations: 5aza-dC, 5-aza-2′-deoxycytidine; DNMT, DNA methyltransferase; LOXL1, lysyl oxidase-like-1; Sp-1, specific protein 1

Received 30 September 2009; revised 11 May 2010; accepted 20 May 2010; published online 8 July 2010

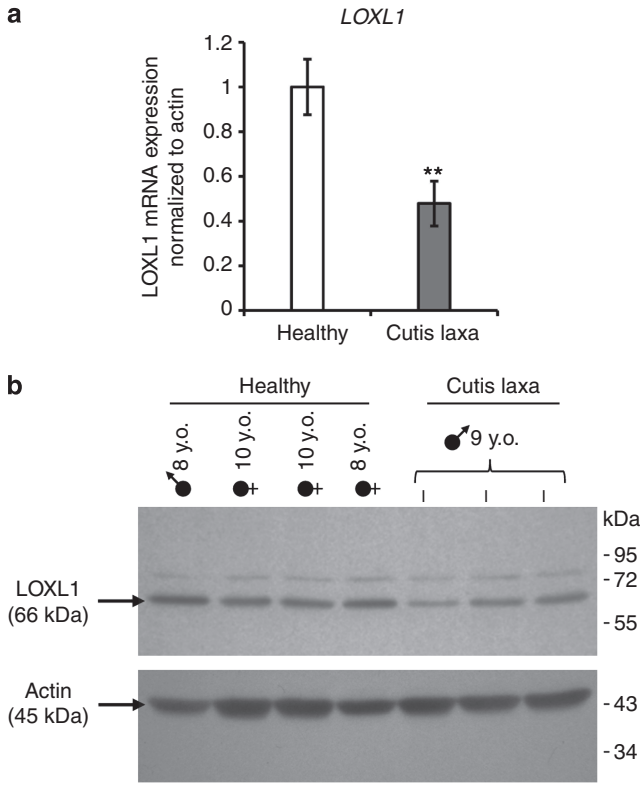
loose skin, especially evident on the face, the abdomen, and the dorsum of the hands and feet. We discovered a homozygous missense mutation (c.649T→C; p.C217R) in the *fibulin-5* gene (*FBLN5*). Fibroblast cultures obtained from one biopsy showed low expression levels of elastic fiber-related genes, particularly *LOXL1*, *fibrillin-1* (*FBN1*), and *elastin* (*ELN*).

In this study, we investigate the mechanisms leading to low *LOXL1* expression in fibulin-5 C217R cells. The identification of a molecular pathway occurring as a result of this mutation would offer a pharmacological approach for treating the consequences of this particular cutis laxa genotype.

RESULTS

LOXL1 is downregulated in FBLN5 (p. C217R) cutis laxa cells

To evaluate *LOXL1* steady-state mRNA expression level in cutis laxa cells, we performed real-time reverse transcription-PCR experiments on skin fibroblasts from a 9-year-old male patient with fibulin-5 mutation C217R (Claus *et al.*, 2008). A group of five skin fibroblast strains obtained from healthy children (3 girls and 2 boys, 8–10 years of age) served as the control. At 2 days after confluence, which is required for optimum *LOXL1* mRNA expression (Thomassin *et al.*, 2005), LOXL1 mRNA levels in cutis laxa cells were approximately half (52%) of that in the control healthy cells (Figure 1a). Western blotting (Figure 1b) of lysates from 8-day post-confluent cultures detected the 66 kDa LOXL1 proenzyme and a minor band with an apparent molecular weight of ~80 kDa. The nature of the larger band is unknown, but is likely a noncovalent complex containing LOXL1. Semiquantitative analysis of the 66 kDa LOXL1 band showed a trend toward lower levels (−42%, $P<0.057$; normalized to β-actin) in cutis laxa cells compared with the four healthy strains (Figure 1b). This correlation between transcript and protein expressions raises the possibility that *LOXL1* gene transcription is lower in cutis laxa cells.



	LOXL1/actin OD mean	SD	SEM	Wilcoxon test
Healthy	1	0.507	0.254	–
Cutis laxa	0.581	0.051	0.029	$P<0.057$

Figure 1. Decreased expression of *lysyl oxidase-like-1* (*LOXL1*) in cutis laxa. (**a**) At 2 days after confluence, monolayers of skin fibroblasts from cutis laxa patient (Cutis Laxa) or from five age-matched healthy children (Healthy) were analyzed by real-time PCR. β-Actin transcript was used for normalization. For the healthy group, results represent the average over the five strains ($n=6$ per strain), and cutis laxa results represent three independent experiments performed in triplicate. Results are expressed as mean ± SEM (**$P<0.01$, Wilcoxon test). (**b**) Skin fibroblasts were grown after confluence for 8 days before western blotting. Immunodetection of β-actin was performed to ensure equal protein loadings. The experiment was performed twice. Samples in the three cutis laxa lanes were obtained from three different extractions at three different passages.

Characterization of the human *LOXL1* promoter

To define whether the low expression level of LOXL1 mRNA and protein in cutis laxa could be explained by transcriptional mechanisms, we cloned and modified the human *LOXL1* promoter. The complete promoter sequence as well as deletion constructs described below were cloned inframe to a *luciferase* gene reporter to determine the DNA sequences responsible for its basal activity. For cloning and defining the initiation of translation (defined as the +1 nucleotide), the 5′ untranslated region sequence NM_005576 (GenBank, National Center for Biotechnology Information), was aligned with the NC_000015 sequence of human chromosome 15. The −2172/+1 region of *LOXL1* (called hPrLOXL) was amplified using specific primers from genomic DNA of human dermal fibroblasts (see Supplementary Table S1 online) and inserted in front of the *luciferase* gene reporter. In the absence of a TATA box, the presence of consensus sites for the binding of the transcription factor specific protein 1 (Sp-1) on GC boxes in its most proximal zone (−712/+1) suggests that Sp-1 could have a role in transcription initiation (Figure 2a). Several other putative regulatory sites were localized on the promoter sequence using the TRANSFAC software (Biobase, Germany, www.gene-regulation.com).

The luciferase activity of serially deleted hPrLOXL-carrying constructs was first defined in healthy dermal fibroblasts (Figure 3a). The −2172/+1 sequence was used as a reference. The −2172/−2151 and −712/−391 regions contain transcription activators, as their deletion resulted in a reduced activity of the corresponding constructs (−2151 and −391). In contrast, deletion of the −2002/−1438 region led to an increase in the hPrLOXL activity (−1438/+1 construct). The shorter constructs (−391/+1 and −81/+1) had almost no activity. Hence, the promoter −712 (−712/+1) was sufficient to maintain a strong transcriptional activity. This region contains most of the Sp-1 binding sites.

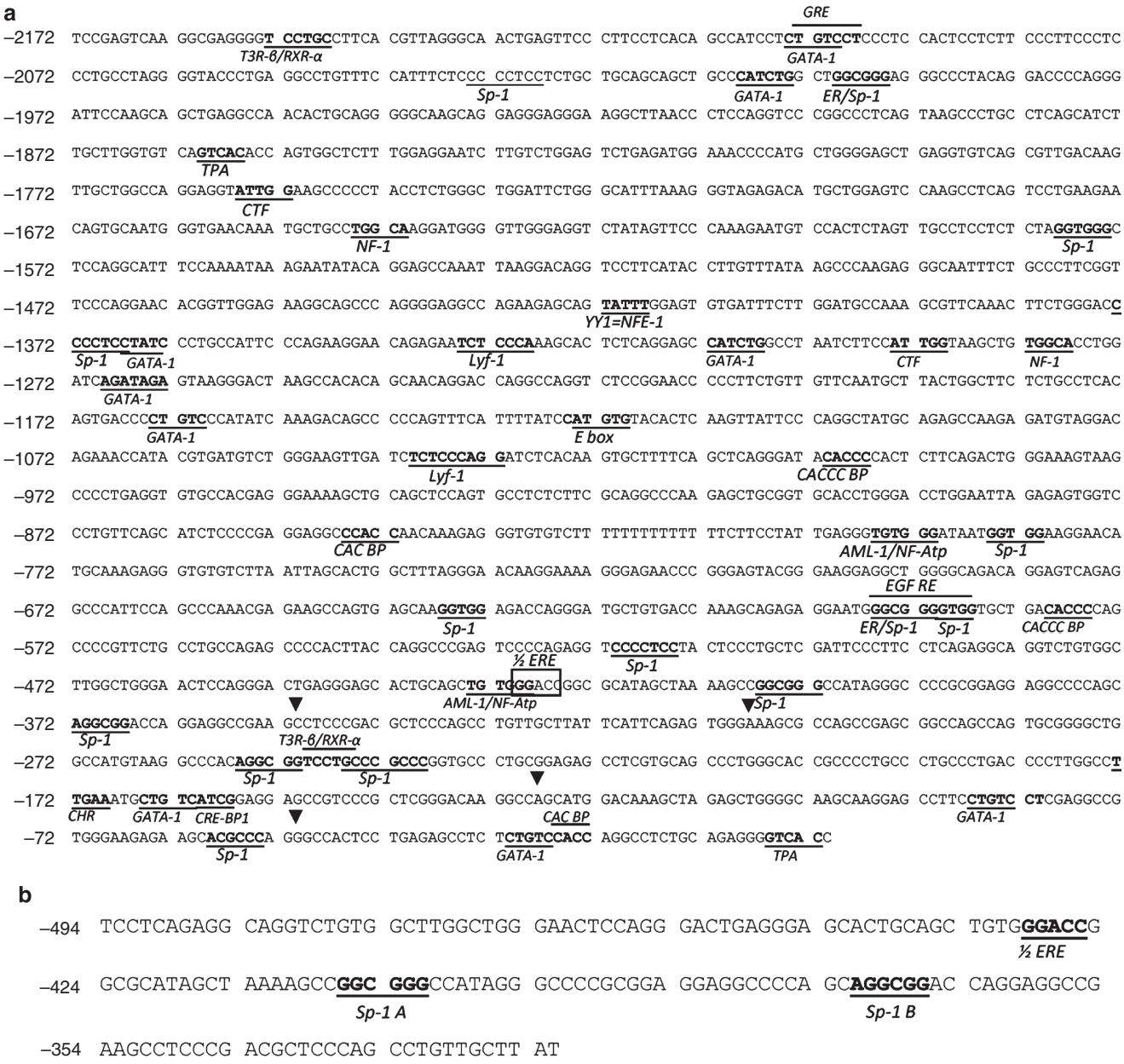


Figure 2. The human *lysyl oxidase-like-1* (*LOXL1*) promoter sequence shows GC-rich motifs in its proximal region but no TATA box. (**a**) The hPrLOXL sequence was obtained by pairwise alignment of LOXL complementary DNA (cDNA) with the human chromosome 15 sequence. The promoter contains numerous specific protein 1 (Sp-1) sites in its most proximal zone −712/−1. Other consensus sites recognized by transcription factors have been identified: GRE (glucocorticoid response element); T3R-β/RXR-α (heterodimer thyroid hormone receptor/retinoic acid receptor); CTF (CCAAT-binding factor); EGF response element; estrogen receptor/Sp-1; CACCC binding protein; TPA response element (activator protein-1 (AP-1) binding); CRE-BP1 (cAMP response element-binding protein-1); NF-1; YY1-NFE-1; Lyf-1; E box; AML1/NF-Atp; and GATA-1. Putative transcription initiation sites are indicated by arrowheads. (**b**) The −494/−323 promoter region containing two Sp-1 and one estrogen response element (ERE) half-site.

To clarify the importance of the −712/+1 region, we created deletions in the −712 construct by targeting the region close to the putative transcription initiation site (Figure 3b). The first deletion (−712Δ(−442/−418)) consisted of a putative estrogen response element site, whereas the two Sp-1 sites (Sp-1A and Sp-1B) were conserved. Using this construction, a slight decrease in the mean value (−25%) was observed when compared with the native −712 (−712/+1) construct. The second deletion of the −442/−342 region covered the putative estrogen response element site and the two putative Sp-1 binding sites. The resulting −712Δ(−442/−342) showed a strong and significant decrease in luciferase activity. Interestingly, the −391 (−391/+1) construct, containing the Sp-1B site only, resulted in luciferase activity similar to the −712Δ(−442/−342) construct. Competitive electrophoretic mobility shift assays were therefore performed to characterize Sp-1 binding activity onto the sequence −415/−353 (called phLL), which appeared

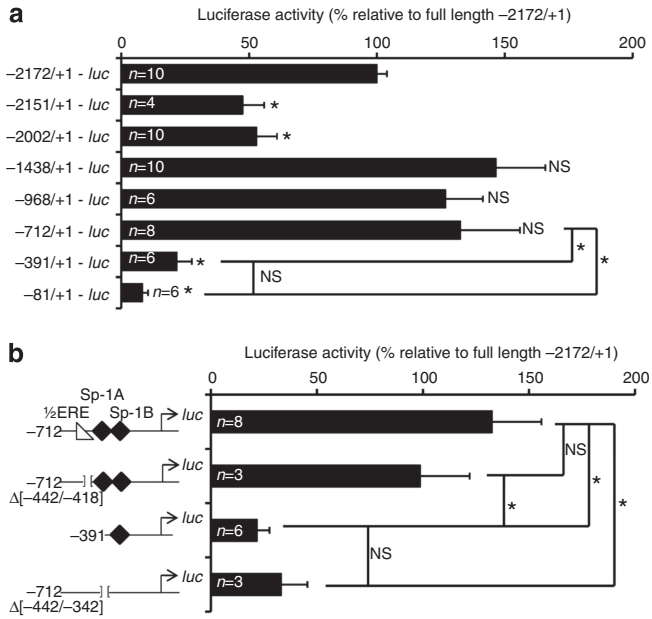


Figure 3. The −442/−342 region of the proximal *lysyl oxidase-like-1* (*LOXL1*) promoter is required for promoter activity. Luciferase activities at 48 hours after transfection were normalized to β-galactosidase activity reporter and compared with the longest promoter −2172. (**a**) Luciferase activity under the control of different promoter constructs was assessed in healthy fibroblasts. (**b**) Deletion effects on the −712 promoter activity in healthy fibroblasts. A 100-bp deletion of estrogen response (ER) and two specific protein 1 (Sp-1) binding sites (−712Δ(−442/−342)) resulted in a strong decrease in promoter activity. The 25-bp deletion of the ER binding site alone (−712Δ(−442/−418)) led to a moderate (25%) decrease in promoter activity. Statistical tests were performed using one-way analysis of variance (ANOVA). *Significant; $^1/_2$ERE, estrogen response element half-site; NS, not significant.

essential for the hPrLOXL activity. Nuclear protein extracts from healthy fibroblasts were subjected to analysis in the presence of the radiolabeled phLL probe alone (Figure 4b, lane 2). The most intense upper band and the third band disappeared after a specific competition with an unlabeled Sp-1 consensus probe (lane 3), whereas the shifted bands remained unchanged in the presence of an unlabeled activator protein-1 consensus probe used as a nonspecific competitor (lane 4). Identification of Sp-1 complexes was assessed by a supershift assay using a specific antibody raised against Sp-1. Our results show that Sp-1 DNA binding corresponds to the upper band that strongly decreased, whereas two slight supershifted bands appeared as indicated by asterisks (lane 5). The two supershifted complexes can be explained by the binding of one or two Sp-1 proteins simultaneously at two distinct sites. In contrast, the third protein–DNA complex inhibited by specific competition (sharp symbol) was not recognized by Sp-1 antibody, suggesting the binding of nonrelated factors. In conclusion, the −415/−353 phLL sequence can bind Sp-1 protein.

Sp-1 binding onto the essential region of the *LOXL1* promoter is not observed in cutis laxa cells

To establish whether modulation of *LOXL1* gene activation coincides with a Sp-1 binding defect in cutis laxa fibroblasts, we used the chromatin immunoprecipitation approach using an antibody raised against Sp-1, followed by a classical PCR amplification with primers specific for the −494/−323 sequence (see Supplementary Table S1 online). As shown in figure 4c, chromatin immunoprecipitation analyses of fibroblast DNA revealed that Sp-1 binding to the *LOXL1* promoter (−494/−323) region was not detectable in cutis laxa fibroblasts, whereas such an association was observed in fibroblasts from healthy children (Figure 4c). Western blotting experiments confirmed the presence of Sp-1 in nuclei of post-confluent cutis laxa and healthy fibroblasts strains (Figure 4d).

LOXL1 promoter undergoes hypermethylation in its proximal region

One hypothesis that could explain a change in Sp-1 binding activity concerns DNA modifications, such as cytosine methylation, resulting in a Sp-1 binding defect (Clark *et al.*, 1997). We therefore investigated the methylation status of cutis laxa DNA in the *LOXL1* promoter sequence. Computerized analysis of the −3500/+1 sequence of *LOXL1* promoter highlighted a small CpG island, spanning nucleotides −398 to −212, suggesting potential methylation site (Figure 5a). Bisulfite modification and PCR-based methylation assays on the (−506/−115) region were performed (Figure 5b). We observed that all of the cytosines within this region in healthy strains had been converted to uracil residues by the bisulfite agent, indicating the absence of methyl groups (open circles). In contrast, in cutis laxa fibroblasts, several cytosines were resistant to uracil conversion within the CpG island, showing that these cytosines were methylated in cutis laxa DNA (closed circles). These results indicate that methylation occurs in the CpG island of *LOXL1* promoter in cutis laxa cells at a higher rate than in fibroblasts from healthy donors. DNA methylation is generally associated with a transcriptional repression of the targeted gene. Hence, removing methylation from CpG sites would permit to restore the normal activity of the *LOXL1* promoter.

LOXL1 expression is restored by DNMT inhibition

In mammals, DNA methylation is mainly catalyzed by three DNA methyltransferases (DNMTs): DNMT1, DNMT3a, and DNMT3b. DNMT is a family of proteins responsible for the attachment of a methyl group to the 5-position of the cytosine ring when it is followed by a guanosine residue (Goll and Bestor, 2005). Subconfluent cutis laxa cells were treated for 5 days with the DNMT inhibitor 5-aza-2′-deoxycytidine (5aza-dC) and further grown until postconfluent for 2 days. This 5aza-dC treatment induced a 2-fold increase in the *LOXL1* mRNA level (Figure 6). No effect of 5aza-dC was observed on *LOXL1* mRNA expression levels in healthy cells (not shown). The 5aza-dC treatment also induced a 2.5-fold increase in *ELN* mRNA expression, whereas neither *FBN1* nor *LOX* mRNA expression levels were modified (Figure 6).

DISCUSSION

This work identifies cytosine methylation of the *LOXL1* promoter as a regulatory mechanism in skin fibroblasts from a cutis laxa patient but not healthy donors. Because cytosine

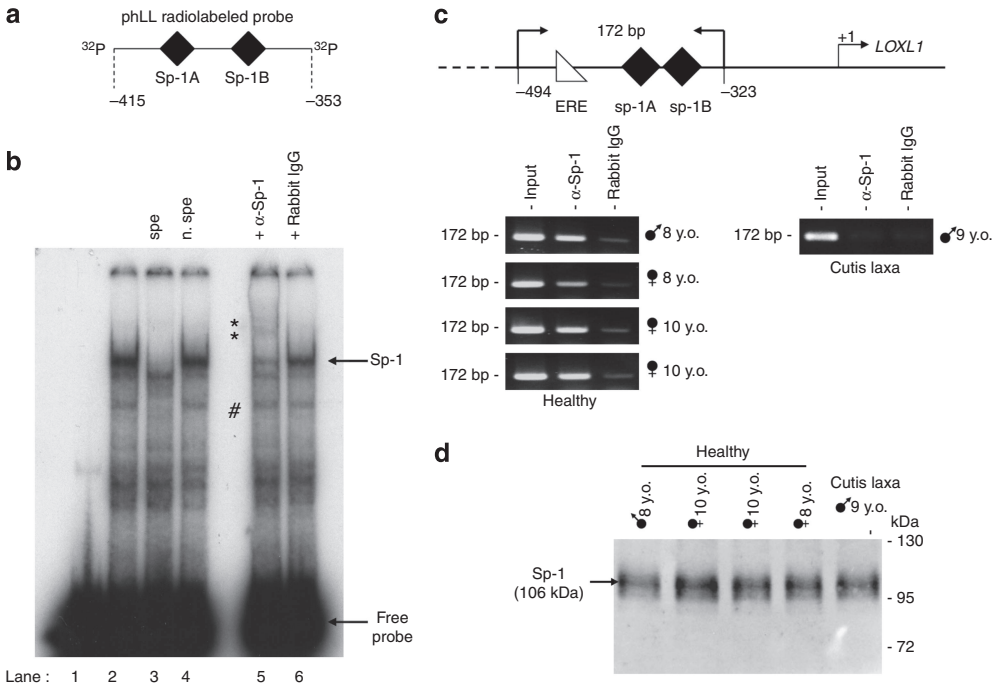


Figure 4. Binding of specific protein 1 (Sp-1) on the proximal *lysyl oxidase-like-1* (*LOXL1*) promoter region is impaired in cutis laxa. (**a**) The −415/−353 region of the *LOXL1* promoter used as radiolabeled probe in electrophoretic mobility shift assay (EMSA). (**b**) Unlabeled consensus Sp-1 (lane 3) or unlabeled consensus activator protein-1 (AP-1; lane 4) were used as specific and nonspecific competitors, respectively. Sp-1 complexes were identified by supershift using an anti-Sp-1 antibody (lane 5) or normal rabbit IgG as a negative control (lane 6). *Supershifted band; #nonspecific binding. (**c**) Chromatin extracted from healthy or cutis laxa cells was immunoprecipitated or not (inputs) and primers specific for the −494/−323 region were used for PCR. (**d**) Sp-1 content in nuclear extracts was assessed by western blotting. In **c** and **d**, one extraction from cutis laxa cells is representative of two independent extractions.

methylation can have a positive or negative effect on gene transcription (Jones and Takai, 2001), our observations suggest a mechanism responsible for *LOXL1* downregulation in this cutis laxa case.

No functional analysis of the *LOXL1* promoter has been undertaken until now, although potential GC boxes for Sp-1 protein binding have been described (Kim *et al.*, 1995). We therefore defined the main DNA sequences necessary for *LOXL1* promoter activity under normal conditions. We assigned Sp-1 as an activator of *LOXL1*, as the deletion of two consensus binding sequences (Sp-1A and Sp-1B) led to a dramatic decrease in promoter activity. In the absence of a functional TATA box, the binding of Sp-1 transcription factor to proximal GC-rich regions is considered as the main inducer to recruit the transcriptional machinery (Lania *et al.*, 1997; Li *et al.*, 2004). Interestingly, the *ELN* gene, which is also strongly downregulated in fibulin-5 C217R fibroblasts (Claus *et al.*, 2008), is similar to *LOXL1* in that its promoter does not contain TATA and CAAT boxes but does contain several Sp-1 binding sequences, which are involved in its positive response to insulin growth factor-1 (Jensen *et al.*, 1995; Conn *et al.*, 1996). In contrast, *LOX*, a gene less affected by the fibulin-5 C215R mutation, contains five Sp-1 binding sites in its 5′ flanking region and functional TATA and CAAT boxes (Hamalainen *et al.*, 1993). Altogether, our findings support the hypothesis that Sp-1 activity in fibulin-5 C217R fibroblasts influence *LOXL1* and eventually *ELN* expression.

No significant difference in Sp-1 protein content within fibroblast nuclei from healthy or pathologic cell strains was observed. However, we showed cytosine methylation sites within the *LOXL1* promoter in cutis laxa cells that were not methylated in their healthy counterpart. More precisely, CpG −369 in the Sp-1B site was methylated and two tandem Sp-1 sites (−245 and −256) are adjacent to several methylated CpG sequences. The role of cytosine methylation in gene regulation has been well documented. A decrease in Sp-1 transcription factor affinity and inhibition of Sp-1 binding to its recognition sequence through cytosine methylation has been reported (Clark *et al.*, 1997). Methylation of adjacent CpG motifs also contributes to Sp-1 binding inhibition *in vivo* (Zhu *et al.*, 2003). In both cases, one explanation could be that MeCP2, a repressor of transcription, recognizes methylated CpG motifs and counteracts Sp-1 binding by DNA accessibility competition (Kudo, 1998). Modification of the cytosine methylation status is mostly associated with a malignant transformation of cells (Robertson, 2005). It also contributes to organ or cell-specific regulation and differentiation during development and notably through inhibition of Sp-1 binding (Cao *et al.*, 2000; Butta *et al.*, 2006). A high DNA methylation status can also reflect a condensed state of chromatin that prevents the binding of transcription factors (D'Alessio and Szyf, 2006). We showed that deletion of the Sp-1A site induced the most important decrease in *LOXL1* promoter activity. However, this site was not methylated when analyzed by bisulfite modifications, but

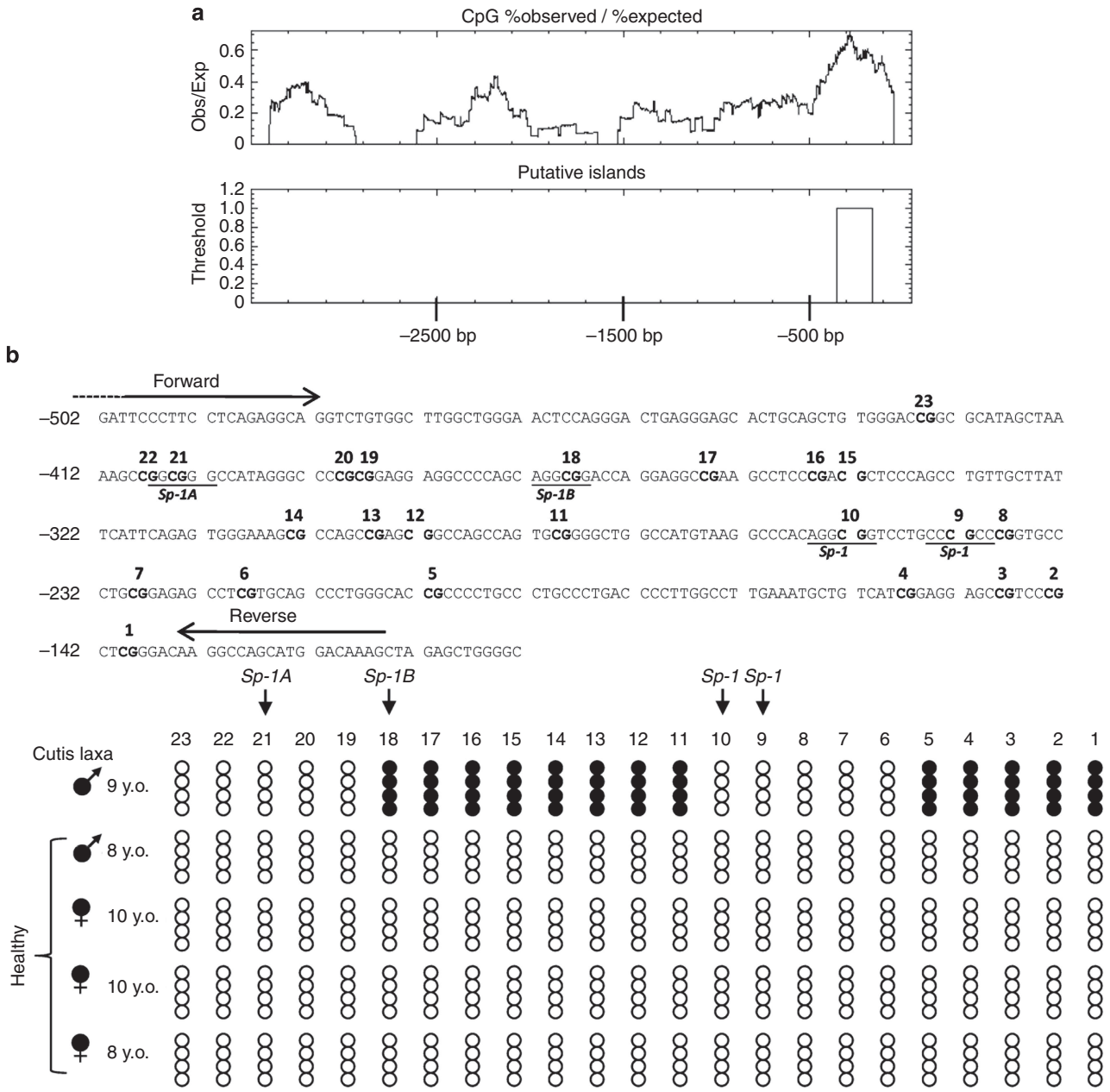


Figure 5. DNA methylation status of *lysyl oxidase-like-1* (*LOXL1*) promoter is increased in cutis laxa. (**a**) Distribution of the CpG sites in the −3500 promoter region from ATG of *LOXL1*. A graph of the percentage of CpG observed divided by the expected frequency and a graph of putative CpG islands. Dinucleotide frequencies were calculated in 200-nucleotide windows moved at 100-nucleotide intervals. The ratio (CpG-% observed/CpG-% expected) indicated that the −398/−212 region contains a CpG island encompassing the second specific protein 1 (Sp-1) binding site (Sp-1B, Figure 2b). (**b**) DNA sequences obtained after bisulfite modifications were amplified by high-fidelity PCR and cloned. Four clones for each strain were analyzed by sequencing. Open circle, unmethylated CpG site; closed circle, methylated CpG site.

many methylations were detected on adjacent CpG sequences, within the Sp-1B site. These observations support an inactivated state of chromatin in this region explaining why no binding of Sp-1 was detected by chromatin immunoprecipitation.

DNA hypermethylation can be reversed through the use of DNMT inhibitors such as 5aza-dC. When 5aza-dC was used on the fibulin-mutant cells, an increase in *LOXL1* and *ELN* expression to levels found in healthy cells was obtained; this implies that *LOXL1/ELN* gene downregulation in cutis laxa occurs through hypermethylation of promoter sequences. It is noteworthy that DNMT inhibitors have been clinically used in phase II in cancer chemotherapy (Goffin and Eisenhauer, 2002), in which they showed antitumoral activities on nonsolid tumors such as leukemia, and restored antioncogenes. But efficiency of DNMT inhibitors on tumors is also

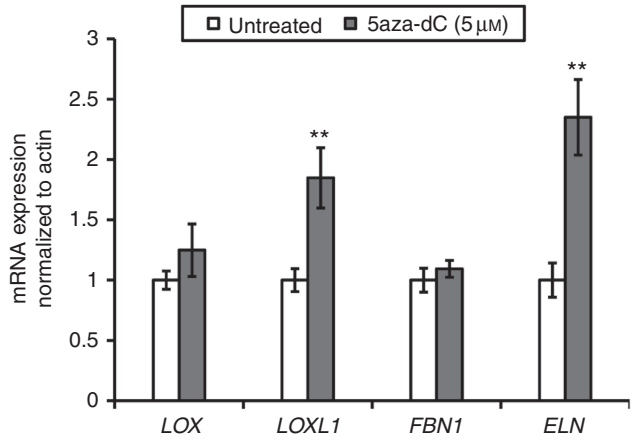


Figure 6. *Lysyl oxidase-like-1* (*LOXL1*) and *elastin* (*ELN*) expressions are restored in cutis laxa cells treated by 5-aza-2′-deoxycytidine (5aza-dC). Subconfluent skin fibroblasts from a cutis laxa patient were treated or not with 5aza-dC. *LOXL1*, *LOX*, *fibrillin-1* (*FBN1*), and *ELN* transcripts from cultures at 2 days after confluence were analyzed by real-time reverse transcription PCR (RT-PCR) and normalized to *ACTB*. Results represent the average of two independent experiments performed in triplicate and are expressed as mean ± SEM (**$P<0.01$, Student's *t*-test).

assigned to cytotoxicity through their contribution to cell growth inhibition. Consistent with these results, we observed that the 5aza-dC treatment inhibited cell proliferation of fibulin-5-mutated cells (not shown). In addition to an intrinsic toxicity of the 5aza-dC compound, its positive effect on elastin expression may also contribute to a reduced proliferation rate (Karnik *et al.*, 2003).

In the fibulin-5 C217R cutis laxa cells, *LOXL1* and *ELN* were both downregulated (Claus *et al.*, 2008), suggesting the existence of common regulation mechanisms for both genes. Similar examples of coordination have been described for other elastic fibers components, such as *FBLN5* and *ELN* (Tsuruga *et al.*, 2004). The identification of epigenetic mechanism leading to *LOXL1* downregulation offers a possible explanation for such coordination. Epigenetic mechanisms are known to be involved in the regulation of expression of extracellular matrix components (Chen *et al.*, 2009; Hardie *et al.*, 2009). However, attributing *LOXL1* and *ELN* downregulation to the fibulin-5 mutation is not so obvious. The underlying mechanism might be indirect, as it has been shown for the dysregulation induced by Marfan mutations. Indeed, the Marfan syndrome, which is caused by mutation in the *FBN1* gene, has been linked to abnormal transforming growth factor-β1 release due to poor retention of the latent transforming growth factor-β1 on the microfibril (Habashi *et al.*, 2006; Brooke *et al.*, 2008). To date, no such signaling pathway has been elucidated in the cutis laxa syndrome but this study shows that a gene reprogramming process should occur and should be carefully examined.

In conclusion, this study shows that downregulation of *LOXL1* and potentially *ELN* in fibroblasts from a young cutis laxa patient is a consequence of an abnormal DNMT activity. Inhibition of such epigenetic enzymes offers new strategies to counteract or attenuate part of the side defects of the fibulin-5 mutation in this case.

MATERIALS AND METHODS

Fibroblasts were isolated from skin biopsies (cutis laxa patient) or surgical explants (healthy children). In all, 5 healthy strains were used as controls: 2 male children (8 and 9 years of age) and 3 female children (8 and 10 years of age). The study was approved by the ethics committee of the Hopital La Pitie-Salpetriere (Paris, France) and all experiments were performed in accordance with the Declaration of Helsinki Principles. Informed consent was received. For all experiments, cells were used below 20 population doubling.

The human promoter of *LOXL1* consisted of the −2172/+1 region subcloned into the pGL3-basic vector upstream of the *luciferase* gene. The different constructs are listed in Supplementary Table S1 online. For promoter activity assessment, luciferase assays were conducted by transfection into dermal fibroblasts from a healthy child. Luciferase activity was expressed in relative light units after normalizing the values to the β-galactosidase activity of each sample.

Real-time reverse transcription-PCR experiments were performed as previously described (Claus *et al.*, 2008) using primer sequences listed in Supplementary Table S1 online. Relative gene expression was normalized with reference to the *ACTB* gene.

Electrophoretic mobility shift assay experiments were conducted with nuclear extracts prepared from fibroblasts of healthy children. The −415/−353 region of the *LOXL1* promoter encompassing the two Sp-1 sites was labeled with (α-^{32}P)adenosine 5′-triphosphate and used as probe. Specific and nonspecific competitions assays were set up with 50× excess of unlabeled Sp-1 consensus probe or activator protein-1 consensus probe, respectively, before incubation with radiolabeled probe. For supershift, anti-Sp-1 antibody (Millipore SAS, Molsheim, France) was added to the nuclear extract and radiolabeled probe for the last 10 minutes of the incubation period.

In chromatin immunoprecipitation experiments, chromatin was isolated and immunoprecipitated as described (Debret *et al.*, 2006). An anti-Sp-1 antibody (sc-14027X; Santa Cruz Biotechnology, Tebu-bio, Le Perray en Yvelines, France) or normal rabbit IgG (Sigma-Aldrich, Saint-Quentin-Fallavier, France) as negative control were used for immunoprecipitation. A positive control consisted of nonimmunoprecipitated chromatin (inputs). The purified DNA isolated by immunoprecipitation was analyzed by PCR using primers specific for the proximal *LOXL1* gene promoter (see Supplementary Table S1 online).

For LOXL1 immunodetection, 30 μg of protein extract (see Supplementary materials online for protein preparation) was separated by 8% SDS-PAGE. Electrophoretically resolved bands were then transferred to polyvinylidene difluoride membranes (Immobilon; Millipore SAS) by electroblotting. Sp-1 was detected from nuclear protein extracts (see electrophoretic mobility shift assay Supplementary Protocol online for details). A total of 10 μg of protein was separated by 10% SDS-PAGE and transferred on to nitrocellulose membranes. The blots were incubated with an antibody raised against Sp-1 (sc-14027; Santa Cruz Biotechnology) in a dilution of 1:5,000, or with an anti-LOXL1 antibody diluted 1:500 (Borel *et al.*, 2001) or an anti-β-actin polyclonal antibody (A5060, Sigma-Aldrich) diluted 1:7,500. Blots were then incubated with peroxidase-conjugated anti-rabbit IgG (Bio-Rad, Marnes-La-Coquette, France) before detection using an enhanced chemiluminescent kit (Perkin Elmer, Courtaboeuf, France).

Bisulfite modification was conducted based on the principle that sodium bisulfite treatment converts unmethylated cytosines to uracil by a deamination reaction, leaving methylated cytosines unchanged. Methylated and unmethylated DNA sequences thus become distinguishable after bisulfite conversion by sequence-specific primers (see Supplementary Table S1 online).

Detailed methodological information is available in Supplementary Materials and Methods online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Christine Bodemer, Smail Hadj-Rabia, Safa Saker, Elodie Kazmierczak, Judith Fischer, and Marie-Thérèse Zabot for their help and advice. We are especially grateful to Robert P Mecham for his help in editing the paper. This work was supported by the Centre National de la Recherche Scientifique, the University Lyon 1, the Hospices Civils de Lyon, the GIS-Maladies Rares, the Association Française contre les Myopathies, and the European Community (LSHM-CT-2005-018960).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Behmoaras J, Slove S, Seve S *et al.* (2008) Differential expression of lysyl oxidases LOXL1 and LOX during growth and aging suggests specific roles in elastin and collagen fiber remodeling in rat aorta. *Rejuvenation Res* 11:883–9

Borel A, Eichenberger D, Farjanel J *et al.* (2001) Lysyl oxidase-like protein from bovine aorta. Isolation and maturation to an active form by bone morphogenetic protein-1. *J Biol Chem* 276:48944–9

Brooke BS, Habashi JP, Judge DP *et al.* (2008) Angiotensin II blockade and aortic-root dilation in Marfan's syndrome. *N Engl J Med* 358:2787–95

Butta N, Larrucea S, Alonso S *et al.* (2006) Role of transcription factor Sp1 and CpG methylation on the regulation of the human podocalyxin gene promoter. *BMC Mol Biol* 7:17

Cao YX, Jean JC, Williams MC (2000) Cytosine methylation of an Sp1 site contributes to organ-specific and cell-specific regulation of expression of the lung epithelial gene t1alpha. *Biochem J* 350(Pt 3):883–90

Chen CZ, Peng YX, Wang ZB *et al.* (2009) The Scar-in-a-Jar: studying potential antifibrotic compounds from the epigenetic to extracellular level in a single well. *Br J Pharmacol* 158:1196–209

Clark SJ, Harrison J, Molloy PL (1997) Sp1 binding is inhibited by (m)Cp(m)CpG methylation. *Gene* 195:67–71

Claus S, Fischer J, Megarbane H *et al.* (2008) A p.C217R mutation in fibulin-5 from cutis laxa patients is associated with incomplete extracellular matrix formation in a skin equivalent model. *J Invest Dermatol* 128:1442–50

Conn KJ, Rich CB, Jensen DE *et al.* (1996) Insulin-like growth factor-I regulates transcription of the elastin gene through a putative retinoblastoma control element. A role for Sp3 acting as a repressor of elastin gene transcription. *J Biol Chem* 271:28853–60

D'Alessio AC, Szyf M (2006) Epigenetic tete-a-tete: the bilateral relationship between chromatin modifications and DNA methylation. *Biochem Cell Biol* 84:463–76

Debret R, Le Naour RR, Sallenave JM *et al.* (2006) Elastin fragments induce IL-1beta upregulation via NF-kappaB pathway in melanoma cells. *J Invest Dermatol* 126:1860–8

Goffin J, Eisenhauer E (2002) DNA methyltransferase inhibitors-state of the art. *Ann Oncol* 13:1699–716

Goll MG, Bestor TH (2005) Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* 74:481–514

Habashi JP, Judge DP, Holm TM *et al.* (2006) Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. *Science* 312:117–21

Hamalainen ER, Kemppainen R, Pihlajaniemi T *et al.* (1993) Structure of the human lysyl oxidase gene. *Genomics* 17:544–8

Hardie WD, Glasser SW, Hagood JS (2009) Emerging concepts in the pathogenesis of lung fibrosis. *Am J Pathol* 175:3–16

Hayashi K, Fong KS, Mercier F *et al.* (2004) Comparative immunocytochemical localization of lysyl oxidase (LOX) and the lysyl oxidase-like (LOXL) proteins: changes in the expression of LOXL during development and growth of mouse tissues. *J Mol Histol* 35:845–55

Hu Q, Loeys BL, Coucke PJ *et al.* (2006) Fibulin-5 mutations: mechanisms of impaired elastic fiber formation in recessive cutis laxa. *Hum Mol Genet* 15:3379–86

Jensen DE, Rich CB, Terpstra AJ *et al.* (1995) Transcriptional regulation of the elastin gene by insulin-like growth factor-I involves disruption of Sp1 binding. Evidence for the role of Rb in mediating Sp1 binding in aortic smooth muscle cells. *J Biol Chem* 270:6555–63

Jones PA, Takai D (2001) The role of DNA methylation in mammalian epigenetics. *Science* 293:1068–70

Kagan HM, Li W (2003) Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem* 88:660–72

Kagan HM, Trackman PC (1991) Properties and function of lysyl oxidase. *Am J Respir Cell Mol Biol* 5:206–10

Karnik SK, Brooke BS, Bayes-Genis A *et al.* (2003) A critical role for elastin signaling in vascular morphogenesis and disease. *Development* 130:411–23

Kielty CM (2006) Elastic fibres in health and disease. *Expert Rev Mol Med* 8:1–23

Kim Y, Boyd CD, Csiszar K (1995) A new gene with sequence and structural similarity to the gene encoding human lysyl oxidase. *J Biol Chem* 270:7176–82

Kudo S (1998) Methyl-CpG-binding protein MeCP2 represses Sp1-activated transcription of the human leukosialin gene when the promoter is methylated. *Mol Cell Biol* 18:5492–9

Lania L, Majello B, De Luca P (1997) Transcriptional regulation by the Sp family proteins. *Int J Biochem Cell Biol* 29:1313–23

Li L, He S, Sun JM *et al.* (2004) Gene regulation by Sp1 and Sp3. *Biochem Cell Biol* 82:460–71

Liu X, Zhao Y, Gao J *et al.* (2004) Elastic fiber homeostasis requires lysyl oxidase-like 1 protein. *Nat Genet* 36:178–82

Noblesse E, Cenizo V, Bouez C *et al.* (2004) Lysyl oxidase-like and lysyl oxidase are present in the dermis and epidermis of a skin equivalent and in human skin and are associated to elastic fibers. *J Invest Dermatol* 122:621–30

Robertson KD (2005) DNA methylation and human disease. *Nat Rev Genet* 6:597–610

Thomassin L, Werneck CC, Broekelmann TJ *et al.* (2005) The pro-regions of lysyl oxidase and lysyl oxidase-like-1 are required for deposition onto elastic fibers. *J Biol Chem* 280:42848–55

Tsuruga E, Yajima T, Irie K (2004) Induction of fibulin-5 gene is regulated by tropoelastin gene, and correlated with tropoelastin accumulation *in vitro*. *Int J Biochem Cell Biol* 36:395–400

Zhu WG, Srinivasan K, Dai Z *et al.* (2003) Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter. *Mol Cell Biol* 23:4056–65