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Citation for published version:

Digital Object Identifier (DOI):

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Molecular Genetics and Metabolism

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Transcriptional profiling of the human fibrillin/LTBP gene family, key regulators of mesenchymal cell functions

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Abstract

The fibrillins and latent transforming growth factor binding proteins (LTBPs) form a superfamily of extracellular matrix (ECM) proteins characterized by the presence of a unique domain, the 8-cysteine transforming growth factor beta (TGF-β) binding domain. These proteins are involved in the structure of the extracellular matrix and controlling the bioavailability of TGF-β family members. Genes encoding these proteins show differential expression in mesenchymal cell types which synthesize the extracellular matrix. We have investigated the promoter regions of the seven gene family members using the FANTOM5 CAGE database for human. While the protein and nucleotide sequences show considerable sequence similarity, the promoter regions were quite diverse. Most genes had a single predominant transcription start site region but LTBP1 and LTBP4 had two regions initiating different transcripts. Most of the family members were expressed in a range of mesenchymal and other cell types, often associated with use of alternative promoters or transcription start sites within a promoter in different cell types. FBX was the lowest expressed gene, and was found only in embryonic and fetal tissues. The different promoters for one gene were more similar to each other in expression than to promoters of the other family members. Notably expression of all 22 LTBP2 promoters was tightly correlated and quite distinct from all other family members. We located candidate enhancer regions likely to be involved in expression of the genes. Each gene was associated with a unique subset of transcription factors across multiple promoters although several motifs including MAZ, SP1, GTF2I and KLF4 showed overrepresentation across the gene family. FBX1 and FBX2, which had similar expression patterns, were regulated by different transcription factors. This study highlights the role of alternative transcription start sites in regulating the tissue specificity of closely related genes and suggests that this important class of extracellular matrix proteins is subject to subtle regulatory variations that explain the differential roles of members of this gene family.

1. Introduction

Vertebrate genomes contain many gene families, consisting of genes with related sequence that arose through a range of duplication events (reviewed by [1]). The existence of gene families can provide redundancy and resilience to the genome, since one family member may substitute (at least in part) for another which has been mutated. This has been shown in knockout mouse models where a severe phenotype may only be found when several family members are inactivated. For example, several members of the SRC gene family had to be deleted to produce a myeloid phenotype (reviewed by [2]). The presence of multiple ion channel genes in the human genome led to the concept of repolarization...
reserve: since there are multiple channels capable of transporting potassium, sodium and calcium ions in cardiac myocytes, the heart beat can be maintained even in the presence of one non-functional protein [3,4]. Thus mice homozygous for a deletion of the potassium channel gene Kcnq2 had no physiologial or clinical phenotype, attributed to upregulation of potassium ion channels encoded by the Kcnq4 and Kcnq5 genes [5]. The possibility that members of a gene family may be able to substitute for each other has implications for genetically determined clinical conditions. To assess overlapping roles it is important to understand the structural and functional relationships between gene family members. In this study we have used the FANTOM5 promoter-based expression atlas encompassing the large majority of human cell types to examine promoter architecture and expression of members of the human fibrillin/LTBP gene family.

The fibrillins and latent transforming growth factor binding proteins (LTBPs) form a small family of extracellular matrix (ECM) proteins characterized by the presence of a unique domain, the transforming growth factor beta (TGFβ) binding domain (TB domain) [6]. These proteins consist primarily of repeated epidermal growth factor (EGF) domains, most with the ability to bind calcium (Ca-EGF domains), interspersed with TB domains (reviewed by [7]; see Fig. 1 of that paper). The family members are important to both the structural integrity of the ECM and the regulation of bioavailability of members of the TGFβ family of growth factors. As well as being structurally similar, fibrillins and LTBPs appear to interact functionally in the sequestering and hence inactivation of TGFβ family members [8].

In vertebrates, including eutharian, marsupial and monotreme mammals, birds, reptiles and fish, fibrillins are encoded by three genes, FBN1, FBN2 and FBN3. In rats and mice the FBN3 gene appears to have degenerated and does not produce a functional mRNA [9], but in most mammals FBN3 is likely to be active since transcripts have been detected (data from http://www.ensembl.org). There are a variable number of annotated LTBPs across species, from two in fish to four in mammals: LTBP1, LTBP2, LTBP3 and LTBP4. It is possible that one or more of the gene family members take over the role of FBN3 in rats and mice.

Expression of fibrillin/LTBP family members is principally found in cells and tissues of mesenchymal origin. In mouse, Fbn1 mRNA is ubiquitous in mesenchymal cell types [10], whereas Fbn2 appears more restricted in expression [7]; see http://biogps.org, data for Fbn2). A similar pattern was reported for human FBN2 [7]. Human FBN3 expression is restricted to embryonic/fetal tissues [9]. The LTBPs are also expressed primarily in cell types of mesenchymal origin, particularly osteoblasts and chondrocytes (http://biogps.org; [7]). This restricted expression suggests that there may be common regulatory elements, permissive for expression in mesenchymal cells, in the promoter regions of the seven genes, with specific elements determining the precise cell types in which the gene is expressed.

Consistent with their function in mesenchymal cell types, mutations in members of this gene superfamily result in phenotypes that primarily affect connective tissue types (reviewed in [7]). Although some aspects of the phenotypes overlap, each gene is associated with a unique spectrum of anomalies, reflecting the cell/tissue specific expression pattern [7]. Understanding the relationships between the family members and their differential regulation may lead to novel therapies in which alternative genes are upregulated to compensate for the mutated gene (as has been suggested for treatment of Duchenne muscular dystrophy by upregulating the dystrophin paralogon utrophin [11]).

The FANTOM (Functional Annotation of Mammals) projects coordinated by the RIKEN Institute in Japan have provided extensive information on gene expression in human and mouse and allowed the identification and characterization of a large number of gene promoter regions [12,13], using cap-selected 5’ end sequencing of mRNAs (cap analysis of gene expression; CAGE). Promoters were described as being sharp (with transcription initiating on a single base, usually associated with a conventional TATA box) or broad (with transcription initiating over a range of bases, usually associated with a CpG island). Analysis of transcription start sites also showed that genes often have alternative promoters which may have different expression profiles, and may change the protein product of the gene by skipping initial exons [14]. We previously used the results from the FANTOM3 project to identify the promoter region of human and mouse FBN1 [15] and FBN2 (unpublished). However those data included few tissues showing expression of FBN3 in humans. The current FANTOM5 project involves many more tissue and cell types likely to express members of the fibrillin and LTBPs families [16]. We have now taken advantage of the extensive information on gene expression, promoter usage and regulation available through FANTOM5 to identify the promoter regions, possible enhancer sequences and potential regulatory motifs for each of these genes in human. We show that different promoters and enhancers are used in a tissue-specific manner under differential regulation, with predominant expression in a subset of mesenchymally derived cells but additional expression in cells of neural, embryonic and other origins, highlighting the varied roles of this gene family. This information may lead to strategies for treatment of diseases that result from mutation of these genes, such as Marfan syndrome (OMIM: 154700; FBN1 OMIM: 134797), congenital contractual arachnodactyly (OMIM: 121050; FBN2 OMIM: 612570), primary congenital glaucoma (OMIM: 613086; LTBP2 OMIM: 602091) and cutis laxa with severe systemic abnormalities (OMIM: 613177; LTBP4 OMIM: 604710) [7].

This work is part of the FANTOM5 project. Data downloads, genomic tools and co-published manuscripts have been summarized at http://fantom.gsc.riken.jp/5/.

2. Methods

2.1. Cell lines and cell culture

Five human cell lines and a primary adult derived mesenchymal stem cell culture were used to analyze expression patterns of fibrillin/LTBP gene family members. SAOS-2 (sarcoma osteogenic 2) is a hypotriolipid line derived from the osteosarcoma of an 11-year-old Caucasian female and has been in culture since 1973 [17,18]. It retains many of the characteristics of osteoblasts including the ability to mineralize in the presence of phosphate molecules [18]. MG63 is a hypotriolipid line derived from the osteosarcoma of a 14-year-old Caucasian male and has been in culture since 1977 [19,20]. This line fails to mineralize under experimental conditions and may represent a more immature, less osteoblastic cell line when compared to Saos-2 [21,22]. Saos-2 and MG63 cells were cultured in McCoy’s5A medium (Gibco) with 10% heat inactivated fetal bovine serum, antibiotics and antifungals. Normal human dermal fibroblast (NHDF) cells, grown from human circumcision material, were obtained from Dr Finn Grey, Roslin Institute. The cells were cultured in DMEM with 10% non-hea inactivated fetal bovine serum, antibiotics and antifungals. The cells were removed from the flasks with 1× Trypsin–EDTA solution (Sigma). All cell lines were seeded at approximately 25% of confluence and processed for RNA extraction at two days after plating for the osteosarcoma cell lines and seven days after plating for NHDF.

2.2. Analysis of RNA and protein expression

Expression of fibrillin/LTBP family members was initially examined in cell lines using quantitative reverse transcriptase PCR from RNA extracted from the cell lines described above. In addition RNA was extracted from cell pellets from Day 2 of embryoid body formation from two human ES cell lines, H1 [23] and RHI [24] (provided respectively by Professor Lesley Forster and Dr Paul De Sousa, Scottish Centre for Regenerative Medicine, University of Edinburgh), and Day 2 of culture of primary adipose derived mesenchymal stem cells (ADMSC) (provided by Dr. Paul DeSousa). Total RNA was extracted from cell lines using RNA-Bee (Amsbio, Abingdon, UK; http://www.amsbio.com/
RNA was quantified using a Nanodrop spectrophotometer and cDNA was synthesized using MMLV reverse transcriptase (Promega Corporation, Madison WI, USA) and an annealing temperature of 60 °C [25]. Primers for quantitative PCR were designed using Roche primer design (Roche Diagnostics Ltd., Burgess Hill, UK; http://www.roche-applied-science.com, Supplementary Table 1). Human GAPDH was used as the reference gene (Qiagen Ltd., Crawley, UK; Hs_GAPDH_2_SG).

Quantitative PCR was performed in triplicate using LightCycler 480 SYBR Green 1 Master Mix (Roche) on the LightCycler 480 machine (Roche). Products were quantified using software supplied by the manufacturer.

The presence of the encoded proteins was ascertained using immunofluorescence with a range of antibodies. 20,000 NHDF cells per well were seeded into an eight well chamber slide (NUNC). After 7 days, the cells were fixed with 95% ethanol and 5% acetic acid for 20 min and washed with PBS. The samples were blocked with 1% BSA in PBS for 1.5 h prior to addition of a 1:100 dilution of the primary antibody and incubated over night at 4 °C. Following a wash with PBS, the secondary antibody was added at 1:100 dilution, for 1 h at room temperature. The cells were then washed with PBS, and mounted with ProGold + DAPI (Invitrogen). Samples were viewed using Zeiss LSM 710 confocal microscope and analyzed with ZEN software at standard settings (Zeiss). Details of antibodies used are supplied in Supplementary Table 1.

2.3. Identification of promoter and enhancer sequences

The tissues and cells used for the FANTOM5 analysis of transcription initiation sites have been described in a parallel publication [16]. Briefly, promoters were identified using CAGE (cap analysis of gene expression) followed by Helicos high throughput sequencing [12,16,26]. Decomposition-based peak identification (DPF [16]) was used to identify a set of transcription start sites (TSS), at permissive and robust levels (containing at least one nucleotide position with >2 or >10 independent single molecule observations respectively in a single library) [16]. Expression levels were determined using RLE normalized counts of CAGE tags mapping to each promoter region [16]. Promoters were then numbered by expression rank at the permissive level [16], so p1@GENE had the highest expression of all promoters associated with that gene, followed by p2@GENE etc. At the more stringent robust level some of these promoters were lost and this accounts for gaps in the numbering of robust promoters. The full permissive set can be seen on the ZENBU browser [27] (http://fantom.gsc.riken.jp/zenbu/). Composite promoters are those where two or more robust peaks occur within 100 bp of each other [16]. Identification of putative enhancers was based on the presence of bidirectional CAGE tag clusters within a 400 bp window [28]. We linked enhancers and nearby promoters (within 500 bp) with correlation tests using their expression levels across all FANTOM5 samples, requiring a Benjamini–Hochberg false discovery rate of less than 10⁻⁵ [28]. We used this resource to identify the total set of robust promoters annotated to the seven fibroblast/LETB family gene members.

2.4. Analysis of gene co-expression clusters

All 81 DPI-detected robust promoters for the seven genes were clustered using Biolayout Express [29,30], based on CAGE-determined expression. To enter all promoters into the analysis a low cut off pair-wise Pearson correlation coefficient of 0.17 was used. Additional analysis at higher Pearson thresholds (as outlined in the Results) was performed to discover tightly coregulated promoters. The MCL inflation value was 2.2 for all analyses.

The parallel paper [16] presents clustering of 124,090 human robust promoters over all samples and in three subsets (primary cells, tissues and cell lines) based on CAGE-determined expression patterns, with Pearson correlation coefficient >0.75 and MCL inflation value of 2.2. These data were reanalyzed for the present paper, looking for coexpression of the promoters for the seven fibroblast/LETB genes with promoters for other genes.

2.5. Identification of transcription factor binding motifs and transcription factor activity

We downloaded the whole-genome alignment of the human genome with 45 other vertebrate genomes from the UCSC Genome Browser database [31]. We retained the alignments between the human, macaque, mouse, rat, cow, horse, dog, opossum, and chicken genomes, and used the T-Coffee alignment tool [32] on 1000 bp segments of the genome to optimize the alignment for the nine selected genomes. We then ran MotEvo [33] on these whole-genome alignments to predict the occurrence of transcription factor binding sites (TFBSs) in the human genome, using a background prior probability of 0.98, a prior for the UFE (unidentified functional element) [33] model of 200 relative to the weight matrices, a UFE motif length of 8 bp, and a uniform background sequence probability. Motif Activity Response Analysis (MARA) [34] was applied on these TFBS predictions to infer the regulatory network.

3. Results

3.1. Gene and protein expression of fibroblast/LETB family members

We used quantitative reverse transcriptase PCR (qRT-PCR) to examine expression of gene family members in cell lines including H1 and RH1 embryonic stem cells, ADMSC mesenchymal stem cells from adipose tissue, SAOS2 and MG63 osteosarcoma cell lines and NHDF fibroblasts. (Sections 2.1 and 2.2). The results are presented in Fig. 1.

FBN1 expression was consistently high in ADMSC, NHDF and MG63 cell types but minimal in early ES cells (Fig. 1A). It was lower in SAOS2 cells. Immunocytochemistry using monoclonal antibodies for fibroblast-1 on human fibroblast cell line NHDF showed the formation of a rich extracellular fibrillar matrix (Fig. 1B). FB2N was highly expressed early in embryoid body formation from H1 and RH1 embryonic stem cells and in SAOS2 and NHDF cells (Fig. 1B). Immunocytochemistry using anti-fibrillin-2 antibodies showed the formation a fibrillar matrix similar to fibrillin-1 (Fig. 1D). Low but detectable expression of FB3N was present in both ES cell lines (H1 and RH1), with no expression in the remaining cell types (Fig. 1E), consistent with a role restricted to early development. qPCR for LTBMP1 and LTBPP4 RNA revealed some expression in cells types tested (Fig. 1F and I) while LTBPP2 was high in ADMSC and NHDF with low expression in all other cell types (Fig. 1G). LTBP3 showed similar expression patterns except that mRNA was detectable in MG63 (Fig. 1H).

These results suggested a mesenchymal distribution of most fibrillin/LETB gene expression. To confirm this we also carried out qPCR and immunocytochemistry on the same cell lines for COL1A1, COL1A2, BGN and ACTA2 (Fig. 2), genes previously shown to be highly expressed in mesenchymal cells [10]. qPCR-RT studies showed similar expression of COL1A1 with FB1N and LTBPP2 while COL1A2 and BGN were expressed in all mesenchymal cells examined except SAOS2 (Fig. 2). This was similar to LTBPP1 except that the latter was also highly expressed in SAOS2. The smooth muscle actin gene ACTA2, was highly expressed in NHDF and SAOS2 (Fig. 2D). We have previously shown high expression of this gene in a range of mesenchymal cell types [10,35]. Immunocytochemistry showed positive staining of the fibroblast cell line for collagen type1, although the protein remained intracellular (Fig. 2C), which was also seen previously for collagen 1 staining of osteosarcoma cell line MG63 [22]. Consistent with the RNA expression pattern, immunocytochemistry revealed strong staining for biglycan in NHDF cells (Fig. 2E). Positive intracellular staining of NHDF cells for smooth muscle actin was seen (Fig. 2G). These results confirm the coexpression of fibrillin/LETB family members with mesenchymal cell markers.
**Fig. 1.** Expression of fibrillin/LTBP gene family members. qPCR was performed on RNA from cell lines SAOS2, MG63, H1 human embryonic stem cells and RH1 human embryonic stem cells and from primary cells ADMSC and NHDF. Results were normalized using GAPDH. FBN3 was detected solely in H1 and RH1 embryonic stem cells. See Methods for more details. (A. FBN1; C. FBN2; E. FBN3; F. LTBP1; G. LTBP2; H. LTBP3; I. LTBP4). Fluorescent immunocytochemistry was performed on Day 7 cultured human fibroblasts, NHDF. Antibody information is available in Supplementary Table 1. (B. fibrillin-1; D. fibrillin-2).

**Fig. 2.** Expression of mesenchymal marker genes. qPCR was performed on RNA from cell lines SAOS2, MG63, H1 human embryonic stem cells and RH1 human embryonic stem cells and from primary cells ADMSC and NHDF. Results were normalized using GAPDH. See methods for more details. (A. COL1A1; B. COL1A2; D. BGN; F. ACTA2). Fluorescent immunocytochemistry was performed on Day 7 cultured human fibroblasts, NHDF. Antibody information is available in Supplementary Table 1. (C. collagen type 1; E. biglycan; G. smooth muscle actin).
3.2. Identification of promoters of the fibrillin/LTBP gene family

To assess further the tissue distribution and regulation of gene family members, we used FANTOM5 CAGE-based data for promoter expression. There were between 1 (FBN3) and 21 (LTBP2) robust promoters associated with members of the gene family. Fig. 3 shows the location and architecture of the promoters for FBN1 along with regulatory elements detected by the ENCODE project [36]: trimethylation of the lysine at position 4 of histone 3 (H3K4Me3), DNase I hypersensitivity sites and transcription factor binding sites detected by chromatin immunoprecipitation and sequencing. The FBN1 gene is aligned to the image in A. Enlargement of the region containing the promoters for FBN1, showing the consensus gene start site (Entrez gene), the start sites of known transcripts, the CpG island and the promoters in this region.

Fig. 3. Architecture of the human FBN1 gene. Promoters were numbered using the permissive [16] set, based on expression, with p16@FBN1 as the highest expressing promoter. Images derived from the ZENBU browser (http://fantom.gsc.riken.jp/zenbu/) and from the USCS Genome Browser (http://genome.ucsc.edu/). Images for the other gene family members are available in the Supplementary Figure or by accessing the ZENBU browser. A. The whole of the FBN1 gene, showing the physical location, exon/intron structure (introns — horizontal lines; exons — vertical lines), robust promoters, CpG islands, and enhancers. Boxed area shows the region enlarged in C. B. Regulatory elements detected for FBN1 by the ENCODE Project [36], showing trimethylation of lysine 4 in Histone 3 (H3K4Me3), DNase I hypersensitivity sites and transcription factor binding sites detected by chromatin immunoprecipitation and sequencing. The FBN1 gene is aligned to the image in A. C. Enlargement of the region containing the promoters for FBN1, showing the consensus gene start site (Entrez gene), the start sites of known transcripts, the CpG island and the promoters in this region.

Almost all the promoters were broad promoters in CpG islands and there were no TATA box promoters (Supplementary Figure and Table 2). The majority of robust peaks were within 100 bp of another peak, forming 18 composite promoters [16] across the gene family (Supplementary Table 2). The frequency of singleton peaks was significantly lower than the 42% seen across the whole human genome [16] (18/81; $X^2_1 = 13.0; p = 0.0003$) and this was attributable to LTBP1 (0/11 singletons; $X^2_1 = 7.9; p = 0.005$), LTBP2 (2/21 singletons; $X^2_1 = 9.1; p = 0.0025$) and FBN2 (1/10 singletons; $X^2_1 = 4.2; p = 0.04$).

There were known transcripts (RefSeq, Ensembl ENST number etc.) starting in the region of the majority of the detected promoters, and most known alternative transcripts were supported by this analysis (Supplementary Table 2). Notably, promoters for the three alternative first exons of FBN1 [37,38] were detected (Fig. 3). The vast majority of FBN1 CAGE tags localized to the main composite promoter region containing p1@FBN1 (Exon A), but p11@FBN1 is probably associated with the rarely used Exon B of previous reports [37,38], while promoters p7@FBN1 and p25@FBN1 may be associated with the previously described Exon C [38] (Fig. 3B). p14@FBN2 and p18@FBN2, found in intron 25 of FBN2 (Supplementary Figure: FBN2 A. — red box and D.), were associated with the short transcript ENST00000507835.1. Two promoters were identified in the 3′ noncoding region of the LTBP2 gene (p12@LTBP2 and p27@LTBP2; Supplementary Figure: LTBP2 A. — red box). They may be associated with a short overlapping transcript from LOC730019.

Some published transcripts were not supported by CAGE tags. For example, there was no support at the robust level for a promoter for the long FBN2 transcript ENST00000507835.1 which has an additional six 5′ non-coding exons (data not shown; available at http://fantom.gsc.riken.jp/zenbu/), nor were there promoters for two long transcripts (AY203940 and AK022050) which overlap FBN3. Similarly there was no evidence for a promoter for the longest reported LTBP4 transcript, NM_003573 (Supplementary Figure: LTBP4 A. — ENST00000545697 and ENST00000204005).

Transcription start sites were also detected where there was no identified transcript. For instance, at the 5′ end of LTBP1, alternate transcripts with a long or short 5′ UTR have been reported (Supplementary...
3.4. Tissue specificity of promoters of fibrillin/LTBP family members

The promoters all showed the characteristics of tissue restricted expression, as defined in the parallel paper [16]. Specifically, 76% of FBN promoters and 50% of LTBP promoters had a median expression level across the 889 cell and tissue types of 0 and for all promoters the maximum expression was substantially greater than 10 times the median (Supplementary Table 2).

Table 1

<table>
<thead>
<tr>
<th>Promoter name</th>
<th>RLE normalized expression value</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1@FBN1</td>
<td>2562</td>
<td>Smooth muscle cells (aorta)</td>
</tr>
<tr>
<td>2496</td>
<td>Fibroblast (aortic adventitia)</td>
<td></td>
</tr>
<tr>
<td>1437</td>
<td>Fibroblast (skin)</td>
<td></td>
</tr>
<tr>
<td>1407</td>
<td>Sertoli cells</td>
<td></td>
</tr>
<tr>
<td>1358</td>
<td>Preadipocyte (breast)</td>
<td></td>
</tr>
<tr>
<td>p1@FBN2</td>
<td>1547</td>
<td>Cell line: HEPM (derived from normal human embryonic palatal mesenchyme)</td>
</tr>
<tr>
<td>1002</td>
<td>Fibroblast (skin)</td>
<td></td>
</tr>
<tr>
<td>994</td>
<td>Cell line: HTST (derived from a human sacrococcygeal teratoma)</td>
<td></td>
</tr>
<tr>
<td>768</td>
<td>Hair follicle outer root sheath cells</td>
<td></td>
</tr>
<tr>
<td>753</td>
<td>Cell line: H-EMS-SS (derived from a human myxoid chondrosarcoma)</td>
<td></td>
</tr>
<tr>
<td>p2@FBN3</td>
<td>6</td>
<td>Occipital lobe (fetal)</td>
</tr>
<tr>
<td>5</td>
<td>Cell line: SK-N-MC (derived from a human neuroepithelioma)</td>
<td></td>
</tr>
<tr>
<td>p1@LTBP1</td>
<td>1903</td>
<td>Chondrocyte, redifferentiated</td>
</tr>
<tr>
<td>1365</td>
<td>Chondrocyte, redifferentiated</td>
<td></td>
</tr>
<tr>
<td>1278</td>
<td>Mesenchymal precursor cell from bone marrow</td>
<td></td>
</tr>
<tr>
<td>1051</td>
<td>Smooth muscle cells (aorta)</td>
<td></td>
</tr>
<tr>
<td>186</td>
<td>Dura mater (adult)</td>
<td></td>
</tr>
<tr>
<td>152</td>
<td>Preadipocyte (breast)</td>
<td></td>
</tr>
<tr>
<td>143</td>
<td>Chondrocyte, redifferentiated</td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>Cell line: Hs 132 (derived from a human spindle cell sarcoma)</td>
<td></td>
</tr>
<tr>
<td>p1@LTBP3</td>
<td>391</td>
<td>Fibroblast (aortic adventitial)</td>
</tr>
<tr>
<td>332</td>
<td>Mesenchymal stem cells (adipose)</td>
<td></td>
</tr>
<tr>
<td>295</td>
<td>Smooth muscle cells (aorta)</td>
<td></td>
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<tr>
<td>246</td>
<td>Cardiac myocyte</td>
<td></td>
</tr>
<tr>
<td>244</td>
<td>Aorta (adult pooled)</td>
<td></td>
</tr>
<tr>
<td>391</td>
<td>Aorta (adult pooled)</td>
<td></td>
</tr>
<tr>
<td>382</td>
<td>Mitral valve (adult heart)</td>
<td></td>
</tr>
<tr>
<td>380</td>
<td>Cervix, adult</td>
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</tr>
<tr>
<td>260</td>
<td>Prostate (adult)</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>Tricuspid valve (adult heart)</td>
<td></td>
</tr>
</tbody>
</table>

Expression values are RLE normalized tags per million [16].

To investigate this tissue restricted expression further, we used a number of ontologies [16] to classify the tissues and cell types with maximum expression of the promoters (FANTOM5 SSTAR http://fantom.gsc.riken.jp/5/stsar/ and Supplementary Table 4). Promoters in one composite cluster within a gene tended to be expressed in tissues associated with the same ontology terms (homogeneous composite promoters [16]), and promoters in separate regions of the same gene were often associated with different ontology terms, indicating promoter switches associated with cell-type specificity.

In general, the major promoters of all gene family members other than FBN3 were strongly expressed in cells associated with ontology terms indicating mesenchymal origin (Table 1, Supplementary Table 4). The strongest promoters of FBN1, p1@FBN1 and p2@FBN1, were highly expressed in cells of the same ontology terms (including fibroblast, mesodermal cell, dense mesenchyme tissue and a number of muscle-related terms) (Supplementary Table 4). Similar ontology terms were found for the main promoters of FBN2, which was expressed in a range of cells from fetal and embryonic tissues, fibroblasts, osteoblasts, placenta, hair follicle and lens epithelial cells (Table 1). p2@LTBP4 shared many terms with FBN1 and FBN2 (for example, fibroblast, muscle cell, myoblast) (Supplementary Table 4). The main composite promoter region of LTBP1 (containing p1@LTBP1; Supplementary Figure: LTBP1 C.) was highly expressed in adult heart samples and also expressed in tissue and cells types of mesenchymal origin. This composite promoter was associated with mesenchyme ontology terms including fibroblast but also with many muscle related terms.

Although most promoters were located in CpG islands, there was little conservation of sequence around the promoters. Many of the promoter regions themselves were pyrimidine-rich (on the coding strand). The coding strand of the main FBN1 promoter region contained a characteristic pyrimidine-rich (T-Rich) stretch, about 70 nucleotides upstream of the transcription start site associated with p1@FBN1 [15,37,39]. The main FBN2 promoter was in a C-Rich region but the promoter region of the alternative transcript (p14@FBN2 and p18@FBN2) was not in a CpG island and was AT rich. The FBN3 promoter was also pyrimidine rich but shared no sequence homology with the other genes. The region around p1@LTBP1 contained alternating strings of purines and of pyrimidines.

The major promoters were all supported by regulatory elements detected in the ENCODE project [36] (Fig. 3, Supplementary Figure: panel B for each gene). Where regulatory elements were not detected this may result from lack of expression in the cell types used in the ENCODE project. For example, there are no ENCODE elements for the composite promoter containing p14@FBN2 and p18@FBN2 (Supplementary Figure: FBN2 A. — red box compared with B.), which is almost exclusively expressed in testis, a tissue type not represented in the cell lines examined in the ENCODE Project.

3.3. Enhancers correlated with promoters of the fibrillin/LTBP gene family

Expression correlation tests, using pairs of expression peaks within 500 kb of each other (Section 2.3), predicted between four and 10 enhancers for the genes, with one to seven predicted enhancers per promoter (Supplementary Table 3). Putative enhancers were located as close as 2 kb up- or downstream of the promoter region. There was no significantly associated possible enhancer activity for the most 5' promoters of LTBP1 (p3@LTBP1, p7@LTBP1, p8@LTBP1, p9@LTBP1, p10@LTBP1 and p15@LTBP1). Promoters within a compound promoter were associated with the same predicted enhancer(s) (Table 1, Supplementary Tables 2 and 3). All promoters for FBN1, LTBP2 and the single FBN3 promoter had enhancers which could be putatively linked with the promoter by means of expression correlations (see Section 2.3), while about half of the promoters for the other genes lacked any significant association with enhancer expression.

p7@FBN1 and p25@FBN1, initiating the alternative first exon, Exon C [38], were associated with different putative enhancers from p1@FBN1 and associated promoters. p14@FBN2 and p18@FBN2, in intron 25 of FBN2, were associated with a putative enhancer expression peak about 460 kb downstream of the promoter (Supplementary Table 3). This enhancer was not correlated with the other FBN2 promoters. p12@LTBP2 was uniquely associated with a bidirectional expression peak 50 kb upstream (Supplementary Table 3). In contrast, p10@LTBP3, p12@LTBP3 and p21@LTBP3 were located within the second and third exons of the major LTBP3 transcript were correlated with one of the same predicted enhancers as the major promoter region containing p1@LTBP3. For LTBP4, each promoter tended to be associated with a different enhancer.

Figure: LTBP1 D.). However, there was no CAGE support for the transcript with the shorter UTR (ENST00000354476.3). Instead, a small TSS cluster was detected midway between the main (long) start site about 200 bp upstream of the annotated beginning of the putative shorter transcript, which may indicate that the shorter transcript is incomplete. Similarly a number of identified TSS in LTBP3 did not correlate with known transcripts (for example, see Supplementary Figure: LTBP3 D. and E.).
Although the promoter regions identified for LTBP2 were associated with some generic mesenchymal ontology terms (such as fibroblast and myoblast), the high expression levels in cardiovascular cell types also led to a preponderance of terms associated with the vasculature (vascular associated smooth muscle cell, vascular system, blood vessel, vascular cord) (Supplementary Table 4).

A number of promoters were expressed in tissues of other origins. Thus p7@FBN1 and p10@LTBP3 were enriched in ontology terms reflecting development of the nervous system (presumptive neural plate, neural tube), and terms relating to mesenchyme (mesenchyme/mesoderm expression) (Supplementary Table 4). p3@FBN2 peaked in hematopoietic cell types, and was associated with ontology terms such as classical monocyte as well as terms reflecting mesenchyme/mesoderm expression (Supplementary Table 4). p2@LTBP2 was also expressed in some hematopoietic cells while p3@LTBP4 was associated with lymphocytes of the immune system (ontology terms mature alpha-beta T cell, immature T cell) but also with terms associated with other organs (cariocervical region, skin of body, surface structure). p7@LTBP2 demonstrated significant expression levels in both hematopoietic and epithelial samples and was enriched for terms reflecting this expression pattern (endoepithelial cell, epithelial cell) (Supplementary Table 4). The high expression promoter of LTBP4, p1@LTBP4, was enriched in ontology terms reflecting an endodermal origin. p14@FBN2 and p18@FBN2, located in intron 25, (Supplementary Figure) displayed highest expression in testis and both promoters were associated with ontology terms such as testis, male reproductive organ, gonad and external genitalia/reproductive organ (Supplementary Table 4).

Some promoters were associated with early development. The sole robust promoter for FBn3, p2@FBn3, was almost exclusively expressed in cells of fetal and embryonic origin. This expression pattern was associated with ontology terms such as embryonic stem cell, neuronal stem cell, neuroectodermal cell as well as some terms relating to pigmentaton (melanocyte, melanoblast and pigment cell) (Supplementary Table 4). Promoters of LTBP1 leading to the longer transcript (Supplementary Figure) were highly expressed in embryonic and extraembryonic cell/tissue types including chorionic and amniotic membrane cells, fetal heart, epithelial and endothelial tissue samples. Within this region, p3@LTBP1 had the highest activity, and transcription from this region was found primarily in fetal heart. It was most strongly associated with ontology terms such as extraembryonic cell/structure, mesenchyme, compound organ and membranous layer (Supplementary Table 4).

Variability of promoter use in different cell types was supported by the H3K4Me3 track from the ENCODE data (Supplementary Figure), with different colored peaks (representing histone modifications) in different cell types) can be seen for different promoters. For example, the composite LTBP1 promoter containing p1@LTBP1 showed strong trimethylation of H3K4 in human lung fibroblasts (NHLF), while the composite promoter containing p3@LTBP1 was strongly trimethylated in most of the cell lines, except embryonic stem cells and a human lymphoblastoid cell line (Gm12878) (Supplementary Table 2). A. Clustering of expression pattern at low correlation coefficient (0.17) using Biolayout Express3D. All 81 promoters were included. Genes whose promoters are within the different colored clusters are shown on the figure. Promoters for most genes formed separate clusters, except that p6@LTBP1 (circled in red) grouped with FBn1 promoters. Two LTBP3 clusters and three LTBP4 clusters formed separate closely related groups (circled in black), although there were also other distinct clusters containing promoters for these genes. C. Clustering at high correlation coefficient threshold (0.75). Twenty-five promoters were included. Genes whose promoters are within the different colored clusters are shown on the figure. Promoters for all genes formed separate clusters with four distinct but closely related clusters (circled in black) for LTBP2.

### 3.5. Coexpression of fibrillin/LTBP family members

To explore further the similarities and differences in promoter expression across the gene family, we used RLE-normalized tag counts [16] to analyze the level of expression of promoters for all seven gene family members across the 889 RNA samples from human tissues, primary cells and cell lines previously described [16] (data available in Supplementary Table 2). CAGE-determined expression levels for each of the 81 promoters were entered into Biolayout Express3D. Biolayout Express3D employs a statistical approach to look at transcript-to-transcript similarities in expression patterns across the samples analyzed, by calculation of a Pearson pairwise correlation matrix [29,30]. To enter all promoters into the analysis, a very low Pearson correlation coefficient threshold of 0.17 was used initially. Fig. 4A shows each promoter as a node (sphere) and the edges (lines) between them represent pairwise Pearson correlation coefficients of ≥ 0.17. All LTBP2 promoters formed a separate cluster (Cluster 1) with no edges to promoters for any of the other genes, even at this low correlation level. This indicates that the LTBP2 promoters are more similar in expression pattern to each other than to any other promoters. FBn2 promoters formed two separate clusters (Clusters 3 and 11) and LTBP1...
promoters were also in two clusters (Clusters 4 and 8) suggesting two patterns of expression for these genes. In contrast, at this low level of correlation LTBP3 and LTBP4 promoters were frequently in the same cluster (Clusters 5 and 7), or associated with the single FBN3 promoter (Cluster 6), showing that these promoters share some similarity of expression pattern. Some LTBP3 and LTBP4 promoters formed separate distinct clusters (Cluster 12 and Clusters 9 and 10 respectively). One LTBP1 and six LTBP3 promoters also clustered with the FBN1 promoters (Cluster 2).

At a Pearson correlation coefficient threshold of 0.5, 11 promoters were excluded from the analysis indicating that their expression pattern was sufficiently different from any other promoter that they did not correlate at this level. The sole FBN3 promoter, p2@FBN3, was in this excluded group as were a promoter for FBN2 and LTBP1, seven for LTBP3 and two for LTBP4. Nine of these 11 were singleton promoters while two were within a composite promoter. The network of associations at P ≥ 0.5 is shown in Fig. 4B. All LTBP2 promoters continued to cluster together (Cluster 1) while FBN1 (Clusters 2 and 12), FBN2 (Clusters 3 and 11) and LTBP1 (Clusters 4 and 6) formed two clusters each. p6@LTBP1 clustered with the majority of the FBN1 promoters, indicating an expression pattern more similar to FBN1 than to the other LTBP1 promoters. p7@FBN1 and p25@FBN1, initiating the alternative first exon, Exon C [38] clustered separately from the other FBN1 promoters. LTBP3 (Clusters 5, 9, 13 and 15) and LTBP4 (Clusters 7, 8, 10 and 14) formed four clusters each. These results suggest that the most diverse expression patterns were found for promoters of LTBP3 and LTBP4, while LTBP2 had the most similar expression pattern across all promoters.

The grouping of the promoters was also assessed at a Pearson correlation threshold of 0.75. At this level, only 25 promoters were included in the analysis, indicating that the expression of the majority did not correlate with any other at a correlation coefficient of ≥ 0.75. The remaining tight clusters consisted of four clusters of LTBP2 promoters and single clusters for FBN1, LTBP1 and LTBP4 (Fig. 4C).

3.6 Identification of genes co-expressed with promoters for fibrillin/LTBP family members

The results in Sections 3.1, 3.4 and 3.5 established that the fibrillin/LTBP gene family members tend to be expressed in cells of mesenchymal origin, although some promoters were associated with expression in diverse other cell types. To obtain further insight into the variability of expression patterns, we analyzed coexpression with the full set of promoters determined by CAGE, performed for the previous study [16], which presented a network of 4664 coexpression groups derived from expression profiles of 182,000 promoters across 887 primary cell types, tissues and cancer cell lines (Fig. 7 in [16]). The analysis was performed both for the entire sample and for the subsets of tissues, cell lines and primary cells. This enabled us to identify promoters that were co-expressed with members of the fibrillin/LTBP gene family (data shown in Supplementary Table 2).

Overall, promoters for the gene family members tended to cluster with promoters for genes such as ACTA2, BGN, CALU, CCDC80, COL1A2, COL1A2, COL3A1 COL4A1, COL5A1, COL7A1, COL17A1, COL21A1, DCN, Eln, FBN2, FBN5, F1N, LOXL3, PCOLCE, POSTN, SOST, SPARC, TGB1, TGFBR3 and THBS1 which have previously been identified to be expressed in mesenchymal cell types [10]. However, many promoters failed to cluster, or clustered only with other promoters for the same gene, indicating distinctive expression patterns that were not shared by other genes.

p1@FBN1, p2@FBN1, p3@FBN1 and p9@FBN1 tended to cluster together with a small number of other promoters such as promoters for CALU and CCDC80. In contrast, the promoters associated with the alternative exon C, p7@FBN1 and p25@FBN1 clustered with promoters for neurally expressed genes. Similarly the promoters for the short tests specific FBN2 transcript, p14@FBN2 and p18@FBN2, clustered together with testis specific and connective tissue genes; the remaining FBN2 promoters frequently failed to cluster. p2@FBN3 was found in large clusters of promoters for genes involved in regulating development including NANOG, FOXG1, FOXO6, FOXP1, PRICKLE1 and SOX14 as well as neural genes such as potassium channels and MAPT and connective tissue genes including COL5A1, COL5A3, ACTA1 and ACTN3.

Promoters for LTBP genes tended to fail to cluster or form clusters with other promoters for the same gene, particularly in the primary cell analysis. p1@LTBP1, p2@LTBP1, p4@LTBP1 and p5@LTBP1 formed a small cluster when all samples were analyzed and p3@LTBP1, p7@LTBP1 and p8@LTBP1 formed a cluster when primary cells were analyzed (Supplementary Table 2). However some LTBP promoters clustered with promoters for mesenchymal genes and occasionally were found with promoters for other members of the fibrillin/LTBP gene family. LTBP3 promoters usually failed to cluster, even with each other. The coexpression analysis for LTBP4 promoters consistently grouped p2@LTBP4, p6@LTBP4 and p7@LTBP4 in clusters containing only these promoters (Supplementary Table 2).

3.7. Transcription factors regulating fibrillin/LTBP gene family members

We used Motif Activity Response Analysis (MARA) [34] to infer the regulatory network of transcription factor motifs in 47 promoters of the fibrillin/LTBP gene family (Supplementary Table 2). Motifs associated with the MAZ, SP1, GTF2I and KLF4 transcription factors were found to regulate 16 to 20 promoters. For FBN1 (seven regulated promoters in total), the motifs for GTF2I, NKX3-1, TBX4-5 and TFAP4 regulated five promoters, while for FBN2 (6 promoters) HIF1A was found to regulate four promoters and TFDP1, KLF4, TFAP2B and TOPO3B regulated three promoters. There were no significant regulatory edges for FBN3 (Supplementary Table 2). However, binding motifs for the transcription factors MAZ, PATZ1, RREB1, SP1 and TFAP4 were detected in the promoter region of FBN3.

LTBP1 (regulated at nine promoters in total) and LTBP2 (regulated at 16 promoters) showed significant regulation by the MAZ motif in seven and eight promoters respectively. Five promoters of LTBP1 were also regulated by HIC1 and NFY while four promoters were regulated by PRDM1, RKR and SOX 17. LTBP2 was predicted to be regulated by KLF4 (at 10 promoters), SP1 (at eight promoters), GATA4 and TEAD (five promoters) and XCP1 (at four promoters) was associated with LTBP2. Regulation at seven LTBP3 promoters was detected, through motifs for GTF2I and SP1 (at six promoters), MED-1 (at five promoters) and TFAP2 and ZFP161 (at four promoters). Only two LTBP4 promoters were significantly associated with motifs, with TRAF2B found for both (Supplementary Table 2).

4. Discussion

Members of the fibrillin/LTBP gene family appear to play two roles in the extracellular matrix. Firstly, they contribute structurally to the formation of the matrix. In particular, fibrillin-1 and fibrillin-2 are integral components of the 10 nm microfibrils which provide strength and elasticity to the matrix [40,41]. Secondly, they are instrumental in regulating the bioavailability of members of the TGFβ superfamily of growth factors [6]. While some fibrillin/LTBP family members (fibrillin-1, fibrillin-2 and LTBP2) do not appear to bind TGFβ molecules directly, they interact with other members of the family that do bind to these growth factors. The TGFβ superfamily contains 33 proteins [42,43] and it is possible that fibrillin/LTBP family members bind directly to other TGFβ-like molecules such as bone morphogenetic proteins (BMPs) and activins/inhibins. TGFβ activation requires the action of force on the TGFβ prodomain, and this may be exerted via fibrillins reacting to perturbations of the ECM [44]. Therefore these proteins are an important component of the extracellular matrix both in maintaining the structural integrity of the matrix and in controlling the growth and differentiation of the cells they surround. Their overlapping roles raise the possibility
that they can substitute for each other in some circumstances, providing some resilience to inactivating mutations in family members.

In this study we have examined gene expression and regulation of the seven fibrillin/LTBPs family members from the FANTOM5 CAGE analysis, a genome wide analysis of transcription start sites and bidirectional putative enhancers [16,28]. We have demonstrated the predominant expression of this gene family in mesenchyme-derived cells and tissues. It is also clear that some gene family members are required in early development, in neural tissue and the cardiovascular system. Expression derived from CAGE tag analysis was confirmed for a number of mesenchymal and embryonic stem cell lines using quantitative reverse transcriptase PCR and immunocytochemistry.

There were multiple examples of differential use of promoters in different tissue types (promoter switching) and this was frequently associated with differences in ENCODE regulatory elements and distinctive predicted enhancer activity. The main promoter region of FBN1 was highly expressed in a range of connective tissue cells, consistent with previously published transcription factor activity [10,15,37] but p7@FBN1 expression was predominantly found in neural cell types enriched in ontology terms related to neural development, clustered with other neural-associated genes and was associated with a unique enhancer. This promoter showed regulation by FOXD3 (Supplementary Table 2) a transcriptional regulator essential for progenitor cells and found mainly within the embryonic neural crest [45].

Promoter switching with a change in tissue specificity was also seen for FBN2, where p1@FBN2 and p18@FBN2 were expressed in reproductive cell types. The transcript associated with these promoters, ENST00000507625.1, covers coding exons 26 to 34 of the full length FBN2 and the polypeptide would consist of eight cbEGF domains with functional cell types. The transcript associated with these promoters showed highest expression in aortic smooth muscle cells, and various other mesenchyme specific tissue and cell types.

Because its expression is restricted to fetal and embryonic tissue types [9], the promoter of FBN3 could not be determined from the earlier FANTOM3 data which lacked samples from relevant human cells. FANTOM5 has a much greater representation of human cells and tissues from early developmental stages and we have now identified the promoter region and the most common starting nucleotide for human FBN3. We have confirmed that fibrillin-3 is expressed in early development and neural tissues of humans [9]. Since Fbn3 is inactive in mouse and rats [9], it is possible that one of the other gene family members substitutes for its function. Since transcription factor binding motifs found in the promoter region of FBN3 overlap with those of LTBPs but not fibrillin genes, it is conceivable that its role is taken by one of the LTBPs. From our analysis possible candidates based on expression pattern in human embryonic cell types would be Ltbp1 (through p3@LTBP1) or Ltbp4 (through p5@LTBP4). Preliminary analysis of FANTOM5 data for mouse indicates that the equivalent of p3@LTBP1 is strongly expressed in embryonic tissue, especially neuron derived neurospheres, while a region equivalent to p5@LTBP4 is expressed in embryonic stem cells (data not shown; available at FANTOM5 website http://fantom.gsc.riken.jp/5/), supporting this possibility.

Mutations in human FBN1, FBN2, LTBPs, LTPs and LTBPs have been associated with connective tissue disease in humans. Transgenic and natural mutation mouse models, available for Fbn1, Fbn2, Ltbp1, Ltbp2, Ltbp3 and Ltbp4 also show phenotypic abnormalities of connective tissue (reviewed in [71]), consistent with the predominant expression in mesenchymal cells of these genes. Further analysis of gene family members for genetic variation which affects the different promoter regions may reveal a role in more subtle connective tissue phenotypes. In addition, examination of promoter usage in relevant tissues in the presence of these mutations may reveal whether the severity of the phenotype is modified by changes in expression of gene family members, indicating a degree of redundancy. Because of similarities in structure and expression patterns, upregulation of fibrillin/LTBPs family members may provide a possible therapeutic approach for these connective tissue conditions.

5. Conclusions

This study supports the differential roles of members of the fibrillin/LTBPs gene family. We have shown the strong mesenchymal expression of most family members and most promoters and highlighted promoter and enhancer switching associated with changes in tissue specificity. We were unable to find evidence for a single “master mesenchyme regulator” but there was considerable overlap in the transcription factor activity associated with these genes. These data aid in better understanding the overall function and activity of the members of the fibrillin/LTBPs family.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgme.2013.12.006.
Authors’ contributions

MRD carried out bioinformatic and molecular genetic analysis, participated in the design of the study and helped to draft the manuscript. RA provided the analysis handling. ARRF was responsible for FANTOM5 management and concept. KMS conceived this study, participated in its design and coordination, carried out much of the analysis and interpretation, performed the clustering analysis of the promoters and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments

FANTOM5 is supported by a research grant to the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) through RIKEN Preventive Medicine and Diagnosis Innovation Program to Yoshiole Hayashizaki, by a research grant for RIKEN Omics Science Center from MEXT to YH, by a Grant of the Innovative Cell Biology by Innovative Technology (Cell Innovation Program) from MEXT to YH, and by a research grant from MEXT to the RIKEN Center for Life Science Technologies. The Roslin Institute is supported by an Institute Strategic Programme Grant from the Biotechnology and Biological Resources Council of the United Kingdom. Participation by members of the Roslin Institute in FANTOM5 is supported by a Japan Partnering Grant from the Japanese Ministry of Education, Culture, Sports, Science and Technology. The Roslin Institute is supported by an Institute Strategic Grant from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

JKB was supported by a Wellcome Trust Clinical Fellowship (090385/Z/09/Z) through the Edinburgh Clinical Academic Track. RA and AS were supported by funds from FP7/2007-2013/ERC grant agreement 204135, the Novo Nordisk Foundation and the Lundbeck Foundation. We thank Alisa J Carlisle for technical assistance. We would like to thank all members of the FANTOM5 consortium for contributing to the generation of samples and analysis of the data-set and we thank GenAS for data production.

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