Loss of DMD gene expression results in Duchenne-like molecular abnormalities across diverse tissues

Nancy Alnassar[1], Malgorzata Borczyk[2], Georgia Tsagkogeorga[3,4], Michal Korostynski[2], Namshik Han[3,5], Dariusz C Górecki[1*]

[1]School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, UK

[2]Laboratory of Pharmacogenomics, Maj Institute of Pharmacology PAS, Krakow, Poland

[3]Milner Therapeutics Institute, University of Cambridge, Cambridge, UK

[4]STORM Therapeutics Ltd, Babraham Research Campus, Cambridge, UK

[5]Cambridge Centre for AI in Medicine, University of Cambridge, Cambridge, UK

*to whom correspondence should be addressed

Abstract

While mutations in the *DMD* gene, encoding dystrophins, cause Duchenne muscular dystrophy (DMD), *DMD* expression occurs in a broader spectrum of tissues. Tumors were used as a model to investigate whether dystrophin loss across dissimilar tissues evokes related outcomes. Transcriptomic, proteomic, and mutation datasets from fifty healthy tissues and matching primary tumors (10,894 samples) and 140 corresponding tumor cell lines were analyzed. *DMD* expression was widespread across healthy tissues at levels comparable to housekeeping genes. In 80% of tumors, *DMD* expression was reduced due to transcriptional downregulation and not somatic mutations. The full-length transcript encoding Dp427 was decreased significantly in 68% of tumors, while Dp71 variants showed variability of expression but were upregulated in hematological malignancies. Hierarchical clustering analysis of *DMD* transcripts revealed six clusters distinguishing malignant from healthy tissues. Transcriptomes of primary tumors and tumor cell lines with low *DMD* expression showed enrichment of specific pathways in the differentially expressed genes, compared to tumors and cell lines with higher *DMD* expression. Pathways consistently identified: ECM-receptor interaction, calcium signaling and PI3K-Akt, are also altered in DMD muscle. Notably, low *DMD* expression correlated with reduced patients' survival across different tumors. Thus, *DMD* transcription takes place throughout a spectrum of healthy tissues. The molecular signature associated with its downregulation, frequently occurring in malignancies, is concordant with changes found in Duchenne muscles, even though these malignancies

originate from tissues never previously associated with dystrophin expression or function. Therefore, the importance of this largest known gene extends beyond its roles identified in DMD.

Introduction

Duchenne muscular dystrophy (DMD) is a debilitating and lethal neuromuscular disorder. Diagnosis is made between the age of two and five, but transcriptomes from pre-symptomatic DMD patients reveal typical dystrophic abnormalities(1). In fact, studies of human fetuses (2–4) and in various animal models (5–7) revealed that the pathology starts during prenatal development and continues into adulthood. The first DMD defects are detectable in developing cells even before their differentiation into muscle (8). Given that muscle regeneration replicates processes occurring in muscle development and that some developmental mechanisms are reactivated in tumors, it is intriguing that changes in *DMD* gene expression are increasingly being described in various malignancies (9). Moreover, *DMD* downregulation affects cell proliferation (10,11), adhesion, migration, and invasion (12) – abnormalities that are commonly associated with tumor development. Gaining some understanding of whether these alterations, in a range of diverse cells, have a common origin and lead to a related outcome would expand our knowledge of the role of the *DMD* gene and potentially open up new treatment avenues.

Such analysis must take into consideration the complexity of *DMD*, the largest human gene known, with 79 exons and eight independent tissue-specific promoters driving the expression of distinct dystrophin isoforms. Three promoters control the expression of 14-kb full-length transcripts encoding 427 kDa isoforms (Dp427). The Dp427m is expressed in myofibers and muscle stem (satellite) cells. In myofibers, it interacts with the dystrophin-associated protein complex (DAPC) (13) with structural and scaffolding roles and an involvement in the regulation of various signaling pathways (14). Yet, in satellite cells, Dp427m has a different interactome and it is essential for asymmetric cell divisions (11,15), and in myoblasts the loss of its expression results in abnormalities of cell proliferation and migration (10,16,17). Dp427c and Dp427p full-length dystrophins are expressed in various neurons (18,19), where their loss during development has been linked to the neuropsychological impairment in DMD.

Moreover, intragenic promoters give rise to transcripts encoding truncated isoforms: Dp260 in the retina, Dp140 in the CNS, Dp116 in Schwann glia (20), and Dp71, which is the most ubiquitous *DMD* product. Alternative splicing adds further structural and functional diversity (21,22). *DMD* transcripts are summarized in Supplementary Figure 1.

Importantly, loss of Dp427 expression, which is both necessary and sufficient for the occurrence of Duchenne MD, was also associated with increased metastasis of tumors with myogenic programs (17) and soft tissue sarcomas (23). In contrast to this potential tumor suppressor role of the full-length dystrophin, Dp71 expression was essential for myogenic tumor cells growth (17,23). Interestingly, a putative role for the *DMD* gene was also suggested in various non-myogenic cancers, including carcinomas (24–26), melanoma (27), leukemia (28), lymphoma (24), and CNS tumors (29–31).

Yet, many of the tumors showing phenotypic changes associated with the decreased *DMD* gene expression originate from healthy tissues that are not known to express the full-length dystrophin protein. Seemingly paradoxical, this observation is not limited to tumors: myoblasts, lymphocytes, and endotheliocytes express 14-kb dystrophin transcripts but are not known to synthesize detectable levels of dystrophin protein. Nevertheless, *DMD* downregulation leads to significant functional abnormalities in these cells (16,32,33).

We hypothesized that malignancy can be used as a model to investigate changes in *DMD* gene expression across healthy tissues and corresponding tumors and aid our understanding of the overall role of this gene, which clearly extends beyond Duchenne MD. Therefore, we studied *DMD* mRNA and protein expression across various healthy tissues and matching tumors and explored transcriptomic alterations in primary tumors and corresponding tumor cell lines with altered *DMD* expression to identify putative downstream molecular pathways that could be associated with *DMD* dysregulation across human tissues. We also examined the association of *DMD* gene expression with survival endpoints in cancer patients.

Methods

1. Gene, protein and clinical datasets

Dystrophin protein expression in normal tissues was examined using the Human Protein Atlas (https://www.proteinatlas.org/), which contains results from immunohistochemistry assays conducted in a variety of tissues and cell types (34).

The Proteomics DB data resource (https://www.proteomicsdb.org/vue/) was also used to examine the expression of the full-length dystrophin protein in normal tissues. This database contains quantitative proteomics data assembled from liquid chromatography tandem-mass-spectrometry (LC-MS/MS) experiments in a variety of normal tissues (35).

Tissue specificity (TS) scores (i.e. the enrichment) of dystrophin protein across a variety of normal tissues was visualized at (https://tsomics.shinyapps.io/RNA_vs_protein/), as well as the correlation between *DMD* gene and protein expression in 32 different tissue types covering all major organs including skeletal muscle (36).

The database "PaxDb" (Protein Abundances Across Organisms) was used to examine dystrophin protein abundance data across different normal tissues (https://pax-db.org). This database covers a large number of protein abundance datasets in a variety of tissues. For a specific protein of interest, it provides a table of abundances in all available datasets, along with the rank of this protein in the entire detectable proteome (37).

The RNA-seq data for tumor (n= 7,894) and healthy (n= 2,714) tissue samples was obtained from the UCSC Xena Functional Genomics Browser (http://xena.ucsc.edu). The "RSEM norm_count" and "RSEM expected_count" datasets of the TCGA TARGET GTEx cohort were used to examine expression data at the gene and transcript level, respectively, for a variety of cancers in The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/tcga), different non-diseased tissues in the Genotype-Tissue Expression (GTEx) project (https://gtexportal.org/), and two pediatric hematological malignancies in the TARGET (Therapeutically Applicable Research To Generate Effective Treatments) database (https://ocg.cancer.gov/programs/target). Samples from the TCGA, TARGET and GTEx have been re-analyzed and processed by the same bioinformatic pipeline to eliminate batch effects resulting from different computational processing (38). The expression level of *DMD* gene/ transcripts was compared between tumor samples and adjacent paired normal tissues from the TCGA (15 comparisons). When expression data for normal samples was not available in the TCGA, a comparison was made between *DMD* gene/ transcripts expression in tumor samples from the TCGA and normal tissue samples in the GTEx database (10 comparisons). Database sources in each comparison are stated in

Supplementary Table 1. The level of *DMD* transcripts was also compared between two TARGET pediatric malignancies and GTEx normal samples.

Mutation and copy number variation (CNV) data for primary tumor samples was derived from the datasets "somatic mutation (SNP and INDEL) – MC3 public version" and "gene-level copy number (gistic2_thresholded)" of the TCGA Pan-cancer (PANCAN) cohort available at the Xena Browser.

DMD gene expression and mutation data for tumor cell lines from the Cancer Cell Line Encyclopedia was obtained from cBioPortal website (https://www.cbioportal.org).

The normalized log-transformed RNA-seq data used to compare gene expression in primary tumor samples with low vs. high *DMD* expression in 15 TCGA primary tumors was obtained from the Xena Browser. Links to database sources can be found in Supplementary File 2.

Expression data for the *DMD* gene in tumor cell lines was obtained from the "Expression 21Q3 Public" dataset using the Cancer Dependency Map (DepMap) portal (https://depmap.org/portal), which includes RNA-seq expression data for 1,379 tumor cell lines from 37 lineages (39).

Gene expression microarray data for skeletal muscle samples from DMD patients (n= 12) and healthy donors (n= 11) was obtained from the dataset GSE1004 available at the Gene Expression Omnibus (GEO) from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/geo/).

Survival data was derived from the dataset "Phenotype – curated clinical data" of the TCGA Pan-cancer (PANCAN) cohort and the dataset "Phenotype – TARGET donor phenotype" of the TARGET Pan-cancer (PANCAN) cohort available at the Xena Browser.

The STRING database was used to construct dystrophin's protein-protein interaction (PPI) network (40).

2. *DMD* transcript identification and classification

The "RSEM expected_count" dataset of the TCGA TARGET GTEx cohort in the Xena Browser contains expression data for thirty *DMD* gene transcripts. The Ensembl database was used for transcript annotation and the identification of coding and non-coding transcripts, and protein products in the UniProt database. However, we note that estimates of transcript expression by RSEM may not be 100% accurate. Protein products were then aligned to known dystrophin isoforms in the UniProt database to identify the isoforms with the highest

percentage of sequence identity to these protein products. The HMMER web server (https://www.ebi.ac.uk/Tools/hmmer/) was used for the graphical representations of Pfam domains of different dystrophin isoforms.

3. Statistical analysis of differential *DMD* gene and transcript expression between tumor tissues and corresponding normal controls

Statistical analysis of the expression level of the *DMD* gene and transcripts was performed using the Xena Browser, using the two-tailed Welch's t-test. P-values were then adjusted for multiple testing using the Bonferroni correction; each of the obtained p-values was multiplied by the total numbers of tests, and when this adjustment gave a value above 1, the corrected p-values were set at 1. The Bonferroni-corrected p-values were then compared with the overall level of α (α= 0.05). Log-transformed expression data was downloaded from the Xena Browser and the mean of the *DMD* gene and transcripts expression level for tumor (A) and normal (B) samples was calculated. The following formula was then used to calculate Log Fold Change (LogFC): log (A/B) = log (A) – log (B). The Xena Browser's instructions can be found at (https://ucsc-xena.gitbook.io/project/faq/advanced-data-and-datasets#how-do-i-calculate-fold-change-fc).

4. Hierarchical clustering analysis of *DMD* transcripts

Thirty *DMD* gene transcripts were ranked based on their mean abundance levels in primary tumors and corresponding normal tissues, and the top ten highly expressed transcripts were identified. The expression values of the remaining twenty transcripts were collapsed into one value called "other transcripts". Expression values of the top ten highly expressed *DMD* transcripts as well as the "other transcripts" value were then standardized to a sum of 1 in order to compare the relative expression levels of these transcripts in different tumor and normal tissues. An UPGMA hierarchical clustering analysis was then performed using the R v4.0.4 on an Euclidean distance matrix with the hclust average method. The dendrogram was cut at different levels to yield 3-7 clusters (Supplementary Table 2). Six was chosen as the optimal number of clusters as it was the smallest number that provided more than two clusters with around 10 tissues, and higher numbers of clusters yielded additional smaller clusters with less than three tissues. The R script used to perform the analysis is available at (https://github.com/nancyalnassar/DMD-gene-in-cancer/commit/ca99cba54c769c5be760e1f04a09010685647d42). Next, the expression level of these ten transcripts were compared across three clusters that contained the majority of the analyzed tissues using Kruskal Wallis test and Dunn's multiple comparisons test.

5. **Association between *DMD* gene expression and mutation/ CNV status**

Tumor samples from 23 tumor types with available mutation and CNVs data (n= 6,751) and tumor cell lines with mutation data (n= 921) were ranked according to their level of *DMD* expression and those at the bottom 30% and top 30% were selected for further analysis (n= 2,025 in each group of tumor samples and n= 276 in each group of tumor cell lines). A Chi Square test was used to determine whether there is an association between *DMD* mutations/ CNVs and expression level in tumor samples and between *DMD* mutations and expression level in tumor cell lines.

6. **Identification of differentially expressed genes (DEGs) between primary tumor samples with low and high levels of *DMD* gene expression**

Gene expression in primary tumors samples with low vs. high levels of *DMD* gene expression from 15 different primary tumors was compared. For each tumor, samples were divided into three groups: Group A (samples at the bottom 33.3% of *DMD* expression), Group B (samples at the top 33.3% of *DMD* expression) and Group C (the remaining samples with medium level of *DMD* expression). Using the R v4.0.4, an Analysis of Variance (ANOVA) was preformed to identify the DEGs between the three groups, and p-values were adjusted using the False Discovery Rate (FDR) correction. Next, Post-Hoc Pairwise T tests were performed to identify the DEGs between Groups A and B, and p-values were adjusted using the Bonferroni correction. LogFC was calculated using the formula (LogFC): log (A/B) = log (A) – log (B). Genes with an FDR-corrected p-value < 0.1, a Bonferroni-corrected p-value < 0.05, and a |LogFC| value >= 0.7 were considered differentially expressed in Group A compared to Group B. The R script used to perform the analysis is available at (https://github.com/nancyalnassar/DMD-gene-in-cancer/blob/main/Differential%20gene%20expression%20in%20primary%20tumor%20samples).

7. **Identification of DEGs in tumor cell lines with low and high levels of *DMD* gene expression**

We performed two comparisons in tumor cell lines from two distinct categories, carcinoma, and sarcoma. A two-class comparison analysis was conducted using the DepMap custom analysis tool (https://depmap.org/portal/interactive/). The “Expression 21Q2 Public” was selected as the input dataset, and the cell lines with low *DMD* expression were used as the “in” group cell lines, while cell lines with high *DMD* expression were used as the “out” group cell lines. The analysis compared gene expression between the selected groups and generated

estimates of effect size and corresponding q-values. The DEGs in each of these two comparisons (q-value < 0.05) were identified.

8. **Identification of DEGs in skeletal muscle samples from DMD patients in comparison to healthy skeletal muscle**

The GEO2R tool at the NCBI website (http://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to identify the DEGs between 12 skeletal muscle samples collected from DMD patients and 11 samples collected from healthy donors. The default parameters of the GEO2R tool were used, and the NCBI-generated annotations were used to display the list of DEGs. P-values were adjusted using the FDR correction. Genes with an FDR-adjusted p value < 0.1 and a |LogFC| value >= 0.7 were considered differentially expressed between DMD and normal skeletal muscle samples. The R script generated by the GEO2R tool is available at (https://github.com/nancyalnassar/DMD-gene-in-cancer/blob/main/GEO2R%20Script).

9. **Identification of DEGs between hematological malignancies samples with low and high levels of Dp71 expression**

We compared gene expression in hematological malignancies samples with low vs. high levels of Dp71 expression from two TCGA and two TARGET studies. Samples (n= 286) were ranked based on the sum expression level of Dp71, Dp71b and Dp71ab. Samples were then divided into three groups: Group A (samples at the bottom 33.3% of Dp71 expression), Group B (samples at the top 33.3% of Dp71 expression) and Group C (the remaining samples with medium level of Dp71 expression). Using the R v4.0.4, an ANOVA was preformed to identify the DEGs between the three groups, and p-values were adjusted using the FDR correction. Next, Post-Hoc Pairwise T tests were performed to identify the DEGs between Groups A and B, and p-values were adjusted using the Bonferroni correction. Genes with an FDR-corrected p-value < 0.1, a Bonferroni-corrected p-value < 0.05, and a |LogFC| value >= 0.7 were considered differentially expressed in Group A compared to Group B.

10. **Functional enrichment analysis**

The EnrichR tool (41) was used to identify the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) Biological Process terms enriched in the DEGs in the previously mentioned comparisons. In comparisons where the number of DEGs was higher than 1,000, only the top 1,000 genes with the largest |LogFC| were used in the pathway and GO term enrichment analysis.

11. **Survival analysis**

Samples (n= 6,931) from 15 different TCGA primary tumors were ranked based on their level of *DMD* gene expression. Next, the following survival endpoints were compared between patients at the bottom 25% of *DMD* expression and those at the top 25% of *DMD* expression: overall survival (OS), progression-free interval (PFI), disease-specific survival (DSS) and disease-free interval (DFI).

Samples (n= 271) from two TCGA and two TARGET studies of hematological malignancies were ranked based on the sum expression level of Dp71, Dp71b and Dp71ab. Next, OS was compared between patients at the bottom 25% of Dp71 expression and those at the top 25% of Dp71 expression. Kaplan-Meier curves were generated in GraphPad and analyzed using the log-rank test.

Results

1. Significant expression of dystrophin transcript and protein in a range of healthy tissues

Expression data for 24 different types of healthy tissues was examined. *DMD* expression levels across this spectrum of normal tissues averaged 66% of *GAPDH* (range between 27 and 82%) and 108% (52 to 132%) of *HPRT1* housekeeping genes levels with the lowest expression found in whole blood (Supplementary Table 3). This range was comparable to skeletal muscle, where *DMD* expression was 72% of *GAPDH* and 146% of *HPRT1*. Given this widespread presence of considerable *DMD* transcript levels, we investigated whether it is accompanied by protein expression. Analysis of dystrophin protein across healthy tissue samples in the Human Protein Atlas gave confusing results. The antibody HPA002725, directed against amino acids 186-333 of the full-length dystrophin, detected moderate cytoplasmic and/or membranous staining in a range of tissues in addition to the expected staining in skeletal and cardiac muscle and the CNS. However, the antibody HPA023885, raised against amino acids 2843-2992 and therefore supposed to detect all dystrophin isoforms upstream of Dp71, showed staining in skeletal and cardiac muscle, while other tissues were negative. This staining pattern disagrees with the established expression of Dp260, Dp140 and Dp116, which is broader than that of Dp427 isoforms, and so it could not be accurate.

Therefore, we have interrogated mass spectrometry (MS) protein expression datasets available at Proteomics DB. MS identified the full-length dystrophin protein in a variety of

normal tissues (Supplementary Figure 2), which agreed with the Human Protein Atlas data obtained with the antibody HPA002725 detecting the full-length isoform.

Moreover, quantitative profiling of the proteome of 201 samples from 32 tissues in the GTEx database identified dystrophin as a housekeeping protein, as it was present in all the 32 tissues analyzed (36). Dystrophin protein expression was statistically significantly and positively correlated with *DMD* gene expression in those samples (Spearman correlation = 0.67).

Finally, in the Protein Abundance Database (PAXdb), dystrophin expression was ranked in the top 25% of MS-quantified proteins in a range of tissues, such as fallopian tubes, esophagus, uterus, bladder, colon, prostate, rectum, in addition to the heart. These data from three databases demonstrated significant expression of dystrophin protein in a range of healthy tissues. Therefore, we investigated whether alterations in *DMD* expression might occur in tumors that originate from tissues not commonly associated with dystrophin protein expression or function.

2. Downregulation of *DMD* gene expression across malignancies

We investigated RNA-seq expression data for 25 different types of primary tumors from the TCGA database (carcinomas, melanoma, lymphoma, and leukemia) and their corresponding healthy tissues. The analyzed primary tumors were acute myeloid leukemia (LAML), adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical and endocervical cancer (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), diffuse large B-cell lymphoma (DLBC), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney chromophobe cell carcinoma (KICH), kidney clear cell carcinoma (KIRC), kidney papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), rectal adenocarcinoma (READ), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), uterine carcinosarcoma (UCS) and uterine corpus endometrioid carcinoma (UCEC).

Global *DMD* gene expression was reduced in 20 out of 25 primary tumors in comparison to their corresponding healthy tissues (Figure 1; Supplementary Table 1). The largest difference in expression was found in primary breast invasive carcinoma (LogFC = -3.7), and the

smallest in primary kidney papillary cell carcinoma (LogFC = -1). *DMD* gene expression was increased in primary tumors in two out of 25 comparisons, namely in primary thyroid carcinoma and diffuse large B-cell lymphoma, with LogFC values of 0.9 and 4.8, respectively. No significant expression changes were found in three comparisons (acute myeloid leukemia, kidney clear cell, and chromophobe cell carcinomas).

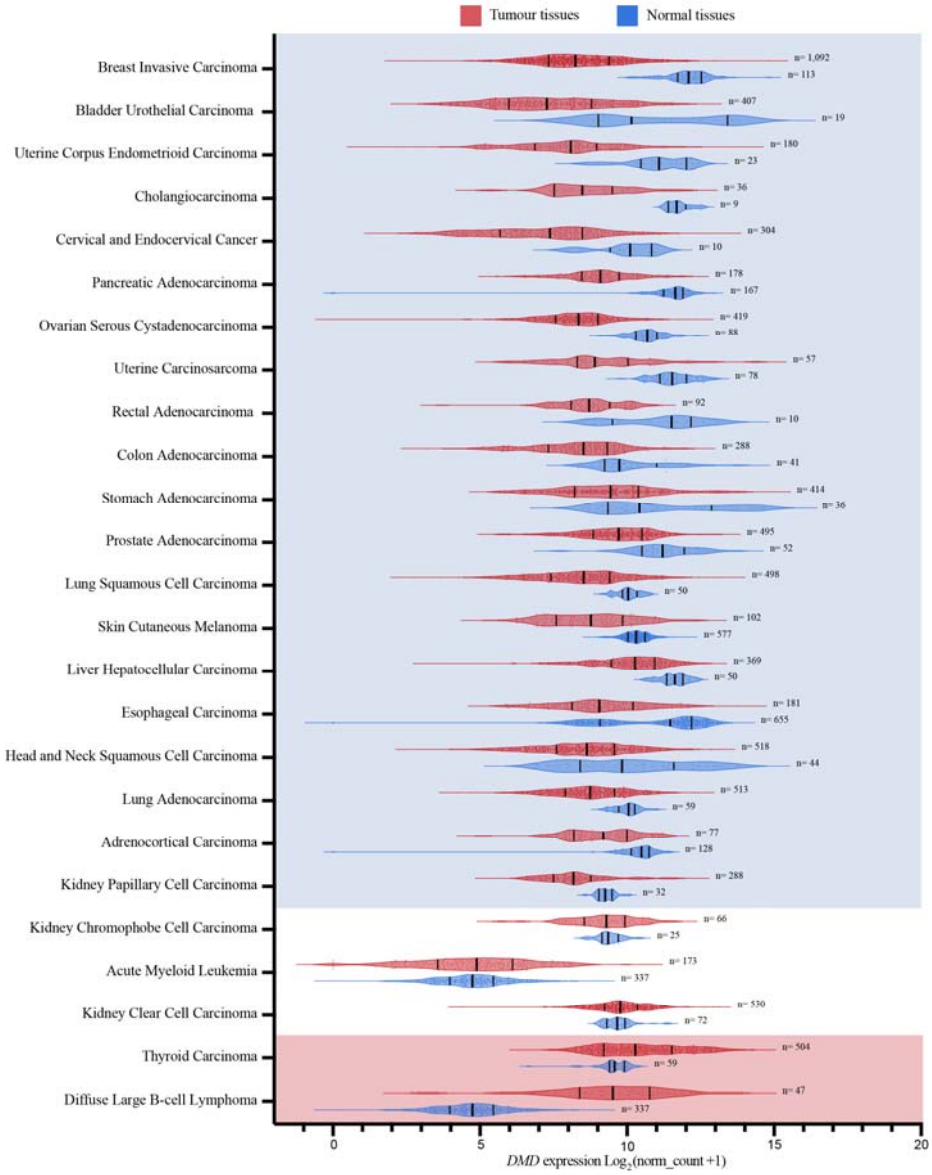

Figure 1. *DMD* gene expression level in primary tumors and corresponding healthy tissues. Red and blue violin blots represent tumor and healthy tissues, respectively. Vertical black lines represent the median and quartiles. Comparisons (n= 20) with a statistically significant downregulation of *DMD* expression in tumor samples compared to healthy tissues are highlighted in blue, and comparisons (n= 2) with a statistically significant upregulation of *DMD* expression in tumor samples compared to healthy tissues are highlighted in red (corrected

$p < 0.05$, Supplementary Table 1). P-values were calculated using the Xena Browser using a two-tailed Welch's t test and adjusted for multiple testing using the Bonferroni correction.

Given the well-known multiplicity of transcripts originating from the *DMD* gene and the variability in their expression patterns, a hierarchical clustering analysis was performed to identify changes in *DMD* expression profile at the transcript level across various healthy tissues and corresponding tumors.

3. Hierarchical clustering analysis of the relative expression of *DMD* transcripts distinguishes healthy from tumor tissues

Of the top ten highly expressed transcripts (Table 1), nine were predicted to be protein-coding (Supplementary Figure 3). Three of these mRNAs were not canonical *DMD* gene transcripts. Of those, ENST00000358062.6 encoding H0Y304 protein is a poorly annotated transcript (Ensemble). The start of its CDS is unknown due to a 5' truncation of the available sequence. The 5' sequence upstream of the first exon (equivalent to exon 48 of the full-length transcript) is composed of the last 50 base pairs of the intron located between exons 47-48. Therefore, it is not clear whether this is a pre-mRNA sequence undergoing co-transcriptional splicing or a mature mRNA with this part of the intron spliced in. If the latter, the predicted protein encoded by such a transcript would have an N-terminus longer than Dp140 with valine as the putative initiation codon (UniProt). The transcript encoding H0Y864 appears to be a partial 4-exon transcript, whose predicted protein sequence does not encode any functional domains (Supplementary Figure 3). ENST00000475732.1 is a two-exon sequence not predicted to encode a protein.

Alternative splicing is a discernible feature of *DMD* transcripts found in the analyzed samples, with the splice variant Dp71b lacking exon 78 being the predominant one. The mRNA encoding Dp140c differs from the canonical Dp140 transcript as it lacks exons 28 to 31 (equivalent to exons 71 to 74 of the full-length transcript) (Supplementary Figure 3).

Table 1. The top ten highly expressed *DMD* transcripts in the analyzed tumor and healthy tissues and their predicted protein products. The identity percentage of unknown vs. known dystrophin isoforms is shown. Source: Ensemble and UniProt.

Transcript ID	Number of exons	Protein size (aa)	UniProt match

ENST00000357033.8	79	3,685	A0A075B6G3 (99.973 % sequence identity to **Dp427m**)
ENST00000619831.4	51	2,341	A0A087WV90 (99.957 % sequence identity to **Dp260-2**)
ENST00000358062.6	30	1,386	H0Y304
ENST00000541735.5	32	1,115	A0A5H1ZRR9 (99.91 % sequence identity to **Dp140c**)
ENST00000343523.6	25	970	A0A5H1ZRP7 (98.454 % sequence identity to **Dp116**)
ENST00000378702.8	18	617	P11532-7 (**Dp71**)
ENST00000378723.7	17	635	P11532-6 (**Dp71b**)
ENST00000361471.8	16	622	P11532-5 (**Dp71ab**)
ENST00000493412.1	4	134	H0Y864
ENST00000475732.1	2	None	

Hierarchical clustering analysis of these ten highly expressed transcripts in healthy and tumor tissues yielded six main clusters (Figure 2). Supplementary Table 2 reports other dendrogram cut-offs. The relative expression of the transcript encoding Dp427m was significantly lower in the clusters composed mainly of tumors but not in clusters with healthy tissues, while the relative expression of Dp71 variants was higher in clusters with tumors (Supplementary Figure 4). Transcripts ENST00000493412.1 and ENST00000475732.1 did not show significantly different levels of expression between any of the clusters (Supplementary Figure 4) and the differential expression of ENST00000358062.6 was not followed due to its uncertain annotation.

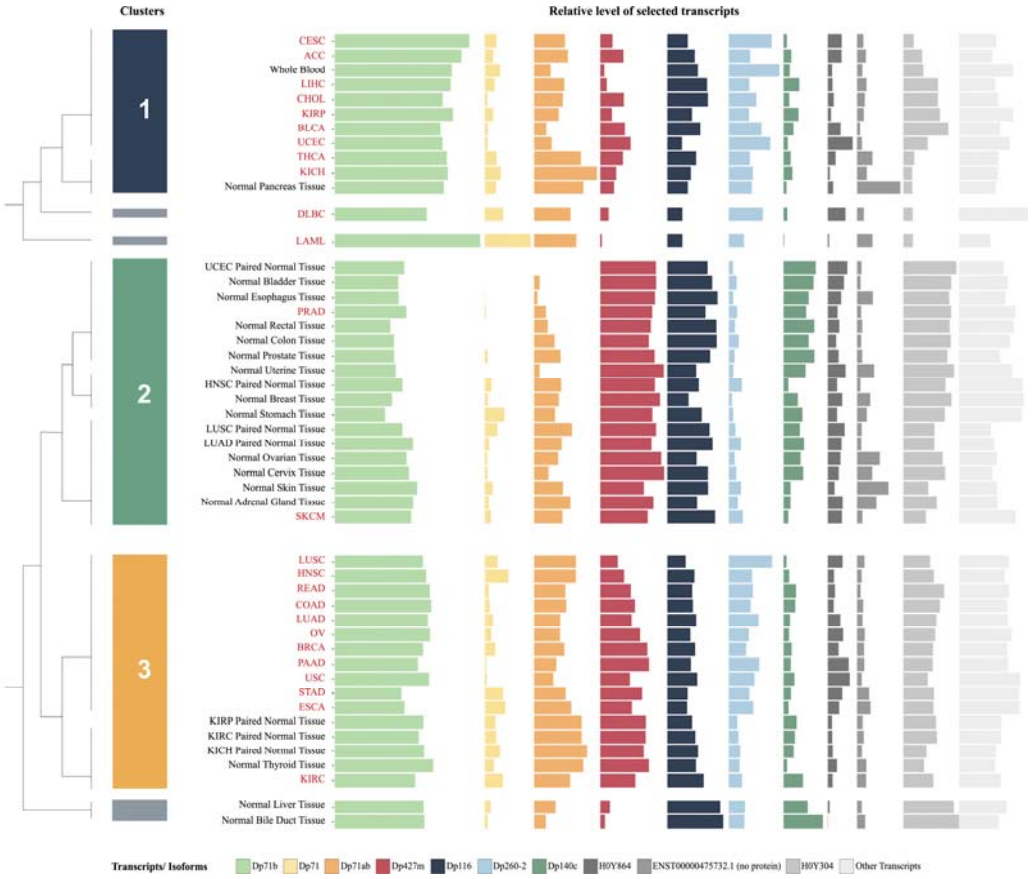

Figure 2. Bar plots representing the relative expression levels of the top 10 highly expressed *DMD* transcripts in tumor and healthy tissues and a dendrogram displaying the clusters identified by the hierarchical clustering analysis. Abbreviations: CESC (cervical and endocervical cancer), ACC (adrenocortical carcinoma), LIHC (liver hepatocellular carcinoma), CHOL (cholangiocarcinoma), KIRP (kidney papillary cell carcinoma), BLCA (bladder urothelial carcinoma), UCEC (uterine corpus endometrioid carcinoma), THCA (thyroid carcinoma), KICH (kidney chromophobe cell carcinoma), DLBC (diffuse large B-cell lymphoma), LAML (acute myeloid leukemia), PRAD (prostate adenocarcinoma), HNSC (head and neck squamous cell carcinoma), LUSC (lung squamous cell carcinoma), LUAD (lung adenocarcinoma), SKCM (skin cutaneous melanoma), READ (rectal adenocarcinoma), COAD (colon adenocarcinoma), OV (ovarian serous cystadenocarcinoma), BRCA (breast invasive carcinoma), PAAD (pancreatic adenocarcinoma), USC (uterine carcinosarcoma), STAD (stomach adenocarcinoma), ESCA (esophageal carcinoma), KIRC (kidney clear cell carcinoma).

4. Changes in the expression of Dp427m and Dp71 transcripts across malignancies

Given the importance of the full-length dystrophin Dp427m, whose loss is responsible for Duchenne muscular dystrophy, and also the predominance of the transcript encoding Dp427m (Table 1), its expression patterns were analyzed in more detail. As Dp71 is the isoform most widely expressed across the body and its splice variants were among the top highly expressed *DMD* transcripts in tumor and healthy tissues (Table 1), we also compared

expression patterns of this transcript. (Supplementary Table 4). Dp427m expression was decreased in primary tumors compared to control tissues in 17 out of 25 comparisons with LogFC values ranging from -7.2 to -2.7 in primary uterine carcinosarcoma and primary thyroid carcinoma, respectively (Figure 3). There was a statistically significant change in the expression of transcripts encoding Dp71 in 10 out of 25, Dp71ab in 13 out of 25, while Dp71b was altered in 20 out of 25 comparisons (a decrease in 17 and an increase in three) (Supplementary Table 4).

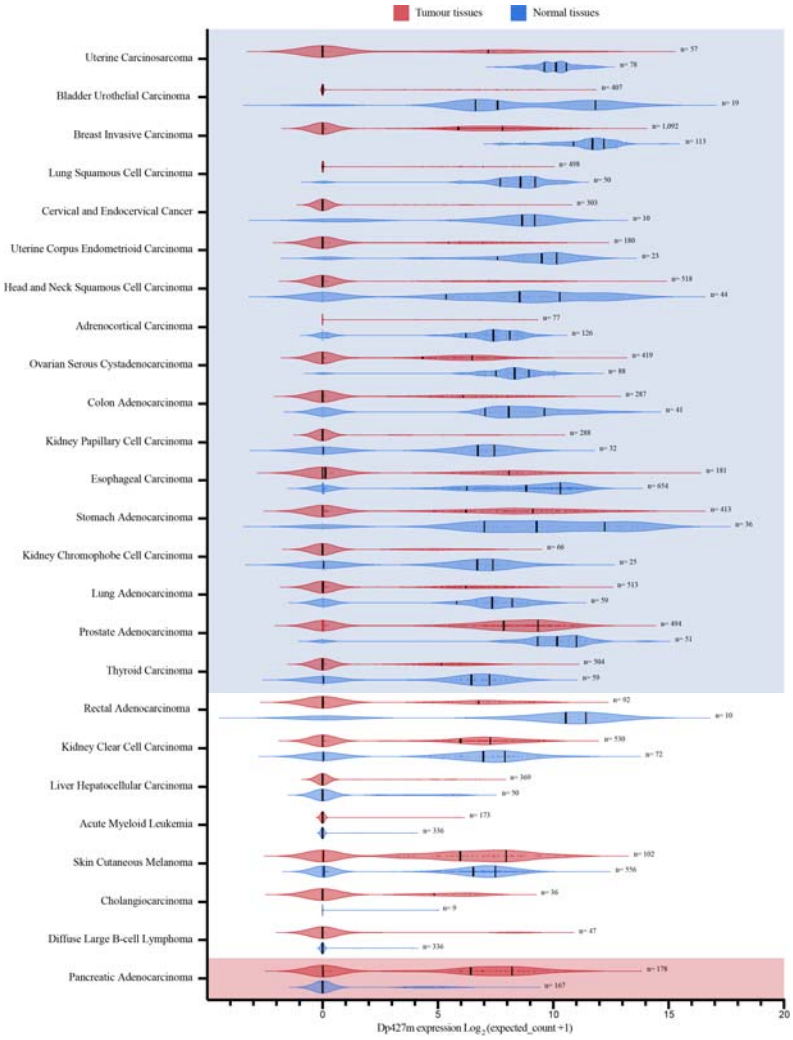

Figure 3. Dp427m transcript expression level in primary tumors and corresponding healthy tissues. Red and blue violin blots represent tumor and healthy tissues, respectively. Vertical black lines represent the median and quartiles. Comparisons (n= 17) with a statistically significant downregulation of Dp427m expression in tumor samples compared to controls are highlighted in blue, and one comparison with a statistically significant upregulation of Dp427m expression in tumor samples compared to controls is highlighted in red (corrected $p < 0.05$, Supplementary Table 4). P-values were calculated using the Xena Browser using a two-tailed Welch's t test and adjusted for multiple testing using the Bonferroni correction.

Further analysis of these *DMD* transcripts showed that changes in overall *DMD* expression levels in two tumor categories were confounded by the opposing dysregulation of Dp427m and Dp71 variants. Specifically, while *DMD* expression was higher in primary thyroid carcinoma compared to normal thyroid tissue, Dp427m expression there was lower compared to healthy tissue and the increase in the overall *DMD* gene expression resulted from the elevated expression of Dp71b (Figure 4.A). In contrast, in primary pancreatic adenocarcinoma, total *DMD* expression was lower compared to the normal pancreas, but this decrease resulted from the lower expression of transcripts encoding Dp71 variants, while Dp427m expression was higher in this tumor type (LogFC = 3.7 compared to healthy tissue) (Figure 4.B).

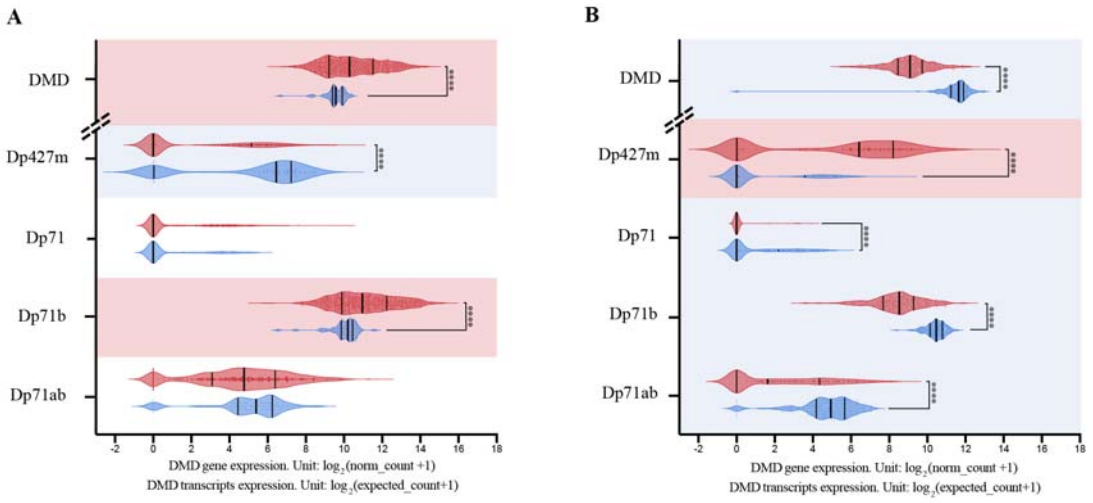

Figure 4. Total *DMD* gene expression and expression of transcripts encoding Dp427m, Dp71, Dp71b and Dp71ab in (A) primary thyroid carcinoma samples (n= 504) compared to adjacent normal thyroid (n= 59) and (B) primary pancreatic adenocarcinoma samples (n= 178) and the normal pancreas (n= 167). Red and blue violin blots represent tumor and healthy samples, respectively. Vertical black lines represent the median and quartiles. Comparisons where there is a statistically significant decrease in *DMD* gene/ transcript expression in tumor tissue compared to healthy controls are highlighted in blue, while comparisons where there is a statistically significant increase in *DMD* gene/ transcript expression in tumor tissue compared to healthy controls are highlighted in red (****corrected $p < 0.0001$). Expression values for the *DMD* gene were derived from the "RSEM norm_count" dataset, while expression values for *DMD* transcripts were derived from the "RSEM expected_count" dataset of the TCGA TARGET GTEx cohort at the Xena Browser. P-values were calculated using the Xena Browser using a two-tailed Welch's t test and adjusted for multiple testing using the Bonferroni correction.

5. *DMD* expression downregulation occurs irrespective of somatic mutations within the *DMD* locus

Next, using datasets for samples from 23 of the previously analyzed TCGA tumors (LAML and SKCM samples were excluded as their mutation and CNV details are not available) we investigated the association between *DMD* expression and exonic and non-exonic mutations as well as CNVs in the *DMD* gene. With regard to exonic mutations, the majority of tumor samples had no identified *DMD* mutations (n= 6,114), while the remaining had missense (n= 334), silent (n= 86), nonsense (n= 41), splice site (n= 17), frame shift insertion/ deletion (n= 22) and multiple mutations (n= 137).

As for non-exonic mutations, 40 samples had mutations in intronic regions, while 3' UTR and 5' UTR mutations were identified in 26 and 19 samples respectively. Multiple non-exonic mutations we found in 34 samples, and the remaining 6,632 samples had no mutations in the non-exonic regions of the *DMD* gene.

Regarding CNVs, the majority of samples were diploid for the *DMD* gene (n= 4,503), 234 had a homozygous deletion, 1,197 had a heterozygous deletion, 766 had a low-level copy number amplification and 51 had a high-level copy number amplification.

As expected, tumor samples with *DMD* mutations and CNVs had a significantly lower level of *DMD* expression compared to those with no alterations (Supplementary Figure 6.A). Tumor samples diploid for *DMD* had a higher level of expression compared to those with homozygous and heterozygous deletions and those with a low-level copy number amplification (Supplementary Figure 6.B).

Tumor samples were then ranked based on their level of *DMD* expression and samples at the bottom 30% (n= 2,025) and top 30% (n= 2,025) were then used to determine whether there is an association between *DMD* exonic and non-exonic mutations/ CNVs and expression level in tumor samples using a Chi Square test. This revealed that there is an overrepresentation of *DMD* mutations and CNVs in tumor samples with low level of *DMD* expression ($X^2(1) = 135.0$, $p < 0.0001$). 51.2% of tumor samples with low *DMD* gene expression (1,037 out of 2,025) had *DMD* mutations or CNVs. However, 48.8% of tumor samples (988 out of 2,025) had low levels of *DMD* expression and no detectable mutations or CNVs.

We further investigated the association between *DMD* expression and mutations using data from 921 tumor cell lines (cBioPortal). The majority of tumor cell lines had no identified

mutations within the *DMD* gene region (n= 773), while the remaining had missense (n= 120), truncating (n= 11), splice (n= 4), and multiple mutations (n= 13). Cell lines were then ranked based on their level of *DMD* expression and those at the bottom 30% (n= 276) and top 30% (n= 276) were selected to determine whether there is an association between *DMD* mutations and expression level in tumor cell lines using a Chi Square test. There was an overrepresentation of *DMD* mutations in tumor cell lines with low levels of *DMD* expression ($X^2(1) = 9.727$, $p = 0.0018$). However, only 23% of tumor cell lines with low *DMD* gene expression (63 out of 276) had *DMD* mutations but the majority of cell lines (213 out of 276) had low levels of *DMD* expression without any detectable mutations.

Therefore, downregulation of *DMD* expression in tumors cannot be simply attributed to somatic mutations within the *DMD* locus but rather involves a regulatory mechanism.

6. Decreased *DMD* gene expression in primary tumors is associated with specific transcriptomic alterations

Expression of the *DMD* gene is significantly altered in tumors, with the majority having lower expression levels compared to their respective healthy tissues. This downregulation predominantly affects the transcript encoding the full-length dystrophin and it is a regulatory alteration. To identify downstream molecular pathways that could be associated with such *DMD* downregulation (analogous to the impact of full-length dystrophin loss in muscle), we compared transcriptomes of primary tumor samples with low vs. high *DMD* gene expression from 15 different tumor types: BRCA, BLCA, UCEC, CESC, OV, COAD, STAD, PRAD, LUSC, ESCA, HNSC, LUAD, KIRP and THCA, where Dp427m was found to be downregulated compared to healthy tissues. In addition, we included sarcoma (SARC) in this comparison because sarcomas originate from tissues (muscle and bone) known to express the *DMD* gene. For clarification, RNA-seq data for control tissues for sarcomas was not available in the TCGA TARGET GTEx cohort, and therefore *DMD* gene expression in sarcomas vs. healthy tissues could not be compared. In each tumor type, the DEGs between samples at the bottom 33.3% and top 33.3% of *DMD* expression were identified and used to perform a pathway enrichment analysis. Figure 5 shows the combined score values (-Log (p value) * odds ratio) for the KEGG pathways that were found to be enriched in the DEGs in more than 50% of the analyzed primary tumors (adjusted p-value < 0.05).

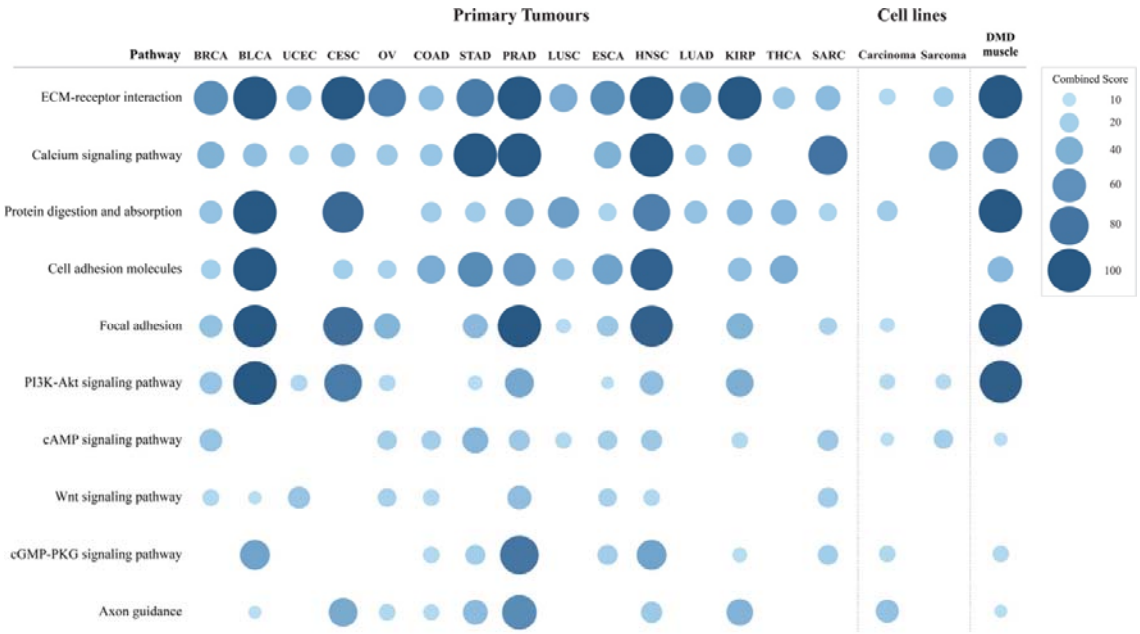

Figure 5. KEGG pathways enriched in the DEGs in primary tumor samples and tumor cell lines with low vs. high *DMD* gene expression as well as in DMD skeletal muscle. Only pathways that were identified in more than 50% of primary tumor samples with low *DMD* expression are displayed (adjusted p value < 0.05). Data is represented as the combined score for the enrichment (-Log (p value) * odds ratio). Combined score values greater than 100 were given a value of 100.

The differentially expressed transcripts in tumors with low *DMD* expression showed enrichment in the ECM-receptor interaction pathway in all 15 primary tumors analyzed. Calcium signaling and protein digestion and absorption were enriched in 13 tumors, cell adhesion molecules in 12, focal adhesion in 11, PI3K and cAMP signaling in 10, Wnt signaling in 9, and cGMP-PKG signaling, and axon guidance were enriched in 8 comparisons.

The specific GO Biological Process terms enriched in the DEGs in primary tumors with low vs. high *DMD* expression were also identified (Figure 6). The GO terms enriched in more than 50% of tumors were: extracellular matrix organization (14 out of 15 tumors), axonogenesis (12), regulation of cell migration, synapse organization and nervous system development (11), skeletal system development and regulation of ERK1 and ERK2 cascade (10) and calcium ion transmembrane import into cytosol (8). Lists of the DEGs in each comparison and results of the pathway and GO term analysis can be found in Supplementary File 2.

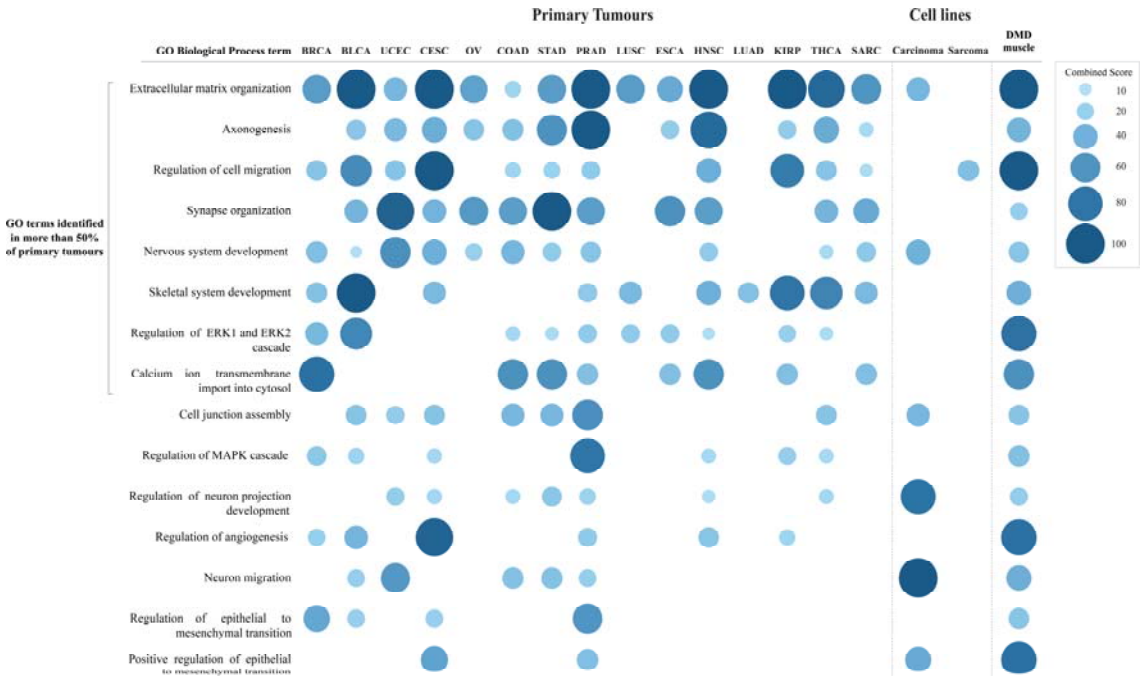

Figure 6. GO Biological Process terms enriched in the DEGs in primary tumor samples and tumor cell lines with low vs. high *DMD* gene expression as well as in DMD skeletal muscle (adjusted p value < 0.05). Data is represented as the combined score for the enrichment (-Log (p value) * odds ratio). Combined score values greater than 100 were given a value of 100.

In order to confirm that these transcriptomic changes between primary tumor samples with low vs. high *DMD* expression are associated with *DMD* downregulation and not a result of other factors, we repeated the analysis for each tumor type using three groups of tumor samples identical in size to the groups used in the previous analysis, but that were chosen at random. *DMD* gene expression was not statistically significantly different between the three random groups in all the 15 tumors, and no statistically significant differences were found in the transcriptomes between these groups, confirming the specific gene expression alterations to be associated with *DMD* downregulation (Supplementary File 2).

7. Transcriptomic alterations associated with decreased *DMD* gene expression in tumor cell lines

To further confirm that these transcriptomic changes are evoked by *DMD* downregulation specifically in cancer cells rather than originate from *DMD* expression in the stromal or infiltrating immune cells present in tumor tissue samples, we conducted a two-class comparison analysis using the DepMap portal to identify DEGs between cell lines of the same tumor origin (carcinoma and sarcoma) but with low vs. high level of *DMD* gene

expression. While the *DMD* gene was not found to be essential for tumor cell lines survival (42,43), its altered expression may have an important common effect across malignancies.

One-hundred and forty tumor cell lines were grouped into four groups based on their level of *DMD* expression (low or high) and their origin (carcinoma or sarcoma) (Supplementary File 2). The ranges for *DMD* expression for these cell lines were as follows (Unit: $\log_2$ (TPM+1)): carcinoma cell lines with high (3.3 – 7.4) and low (0 – 0.01) *DMD* expression, sarcoma cell lines with high (4.07 – 7.6) and low (0 – 0.06) *DMD* expression. The carcinoma cell lines used in this analysis originated from tissues where primary tumors were found to have a lower level of Dp427m transcript compared to healthy tissues. The two-class comparison analysis between cell lines with low vs. high *DMD* expression identified 998 DEGs for carcinoma, and 543 DEGs for sarcoma cell lines. Interestingly, in carcinoma cell lines with low *DMD* expression, the majority of DEGs were downregulated (n= 976 out of 998). Figure 5 shows the combined score values (-Log (p value) * odds ratio) for the KEGG pathways that were found to be enriched in DEGs in these two comparisons (adjusted p-value < 0.05).

Pathway enrichment for the DEGs in carcinoma cell lines with low *DMD* expression suggested that *DMD* downregulation may be associated with changes in the following KEGG pathways: ECM-receptor interaction (p= 0.037), protein digestion and absorption (p= 0.015), focal adhesion (p=0.029), PI3K-Akt signaling (p= 0.021), cAMP signaling (p= 0.044), cGMP-PKG signaling (p= 0.024) and axon guidance (p= 0.002). These pathways were also enriched in the DEGs in more than 50% of primary tumors with low *DMD* expression.
Similar to carcinoma cell lines, the following KEGG pathways were enriched in the DEGs in sarcoma cell lines with low *DMD* expression: ECM-receptor interaction (p= 0.048), PI3K-Akt signaling (p= 0.041) and cAMP signaling (p= 0.034). The calcium signaling pathway, which was enriched in the DEGs in more than 50% of primary tumors with low *DMD* expression was also enriched in the DEGs in the comparison of sarcoma cell lines (p= $7.24e^{-04}$).

Moreover, GO Biological Process term analysis for DEGs in carcinoma cell lines with low vs. high *DMD* expression (Figure 6 and Supplementary File 2) identified the following terms: extracellular matrix organization (p= $5.22e^{-04}$), cell junction assembly (p= 0.012) and positive regulation of epithelial to mesenchymal transition (p= 0.038). Terms related to the development of the CNS and synaptic transmission: nervous system development (p= $8.39e^{-05}$), neuron migration (p= $8.39e^{-05}$) and regulation of neuron projection development (p= $1.04e^{-05}$) were also found.

The GO Biological Process term regulation of cell migration was enriched (p= 0.02) in the DEGs in sarcoma cell lines with low vs. high *DMD* expression (Figure 6 and Supplementary File 2). Lists of the DEGs in each comparison and results of the pathway and GO term analysis can be found in Supplementary File 2.

8. Transcriptomic alterations in Duchenne skeletal muscle compared to healthy muscle samples

Next, we investigated whether the transcriptomic changes resulting from *DMD* downregulation in primary tumors and tumor cell lines are similar to those observed in skeletal muscles of DMD patients. We compared gene expression data from 12 DMD skeletal muscle and 11 healthy muscle samples. The GEO2R tool identified 1,160 gene to be differentially expressed between DMD and healthy skeletal muscle. Figure 5 shows the combined score values (-Log (p value) * odds ratio) for the KEGG pathways that were found to be enriched in the top 1,000 DEGs with the highest |LogFC| values in this comparison (adjusted p-value < 0.05).

Pathway enrichment analysis suggested that *DMD* downregulation may be associated with changes in the following KEGG pathways: ECM receptor interaction (p= 7.82e^{-11}), calcium signaling pathway (p= 8.80e^{-08}), protein digestion and absorption (p= 3.67e^{-08}), cell adhesion molecules (p= 1.55e^{-04}), focal adhesion (p= 2.87e^{-12}), PI3K-Akt signaling (p= 7.82e^{-11}), cAMP signaling (p= 0.020), cGMP-PKG signaling (p= 0.007) and axon guidance pathways (p= 0.030). These very pathways were enriched in the DEGs in more than 50% of primary tumors with low *DMD* expression, and some were also enriched in the DEGs in carcinoma and sarcoma cell lines with low vs. high *DMD* expression.

GO Biological Process term analysis for DEGs in in DMD skeletal muscle compared to healthy muscle (Figure 6) identified the following terms: extracellular matrix organization (p= 6.25e^{-17}), axonogenesis (p= 9.35e^{-5}), regulation of cell migration (p= 3.39e^{-12}), synapse organization (p= 0.009), nervous system development (p= 1.87e^{-4}), skeletal system development (p= 2.22e^{-4}), regulation of ERK1 and ERK2 cascade (p= 5.15e^{-8}), calcium ion transmembrane import into cytosol (p= 0.001). These GO terms were enriched in the DEGs in more than 50% of primary tumors with low *DMD* expression. GO terms that were found to be in common with carcinoma cell lines with low *DMD* expression were: extracellular matrix organization, nervous system development, cell junction assembly (p= 0.004), regulation of neuron projection development (p= 0.005), neuron migration (p= 0.004) and positive regulation of epithelial to mesenchymal transition (p= 2.52e^{-4}). Finally, the GO term,

regulation of cell migration, was found to be in common with sarcoma cell lines with low *DMD* expression.

Furthermore, functional enrichment tests of the dystrophin protein-protein interaction network constructed using high confidence PPI information demonstrated that the enriched functional terms were consistent with the pathways found to be significantly enriched in the aforementioned comparisons of primary tumors, tumor cell lines and DMD skeletal muscle: extracellular matrix organization (p= 1.10e^{-18}), axon guidance (p= 9.45e^{-10}), focal adhesion (p= 3.27e^{-14}) and PI3K-Akt signaling pathways (p= 9.27e^{-09}) (Supplementary Figure 5).

9. Low *DMD* gene expression is associated with poor survival in patients with 15 different types of primary tumors

Given these similarities between pathways altered in tumors and in the lethal muscle disease, we examined the association between *DMD* gene expression and patients' survival in the following tumor types: BRCA, BLCA, UCEC, CESC, OV, COAD, STAD, PRAD, LUSC, ESCA, HNSC, LUAD, KIRP, THCA and SARC. We compared overall survival (OS), progression-free interval (PFI), disease-specific survival (DSS) and disease-free interval (DFI) endpoints between patients at the bottom 25% of *DMD* expression and those at the top 25%. OS was lower in the low *DMD* group (HR 1.33; 95% 1.17, 1.51; p< 0.0001) with 2,417 days compared to 3,253 for the high *DMD* group. PFI was also decreased in the low *DMD* group (HR 1.28; 95% 1.14, 1.45; p< 0.0001) with 2,472 days compared to 3,669 days, respectively. Finally, the low *DMD* group had lower DSS (HR 1.46; 95% 1.24, 1.72; p< 0.0001) and DFI (HR1.30; 95% 1.06, 1.59; p= 0.012) compared to the high *DMD* group.

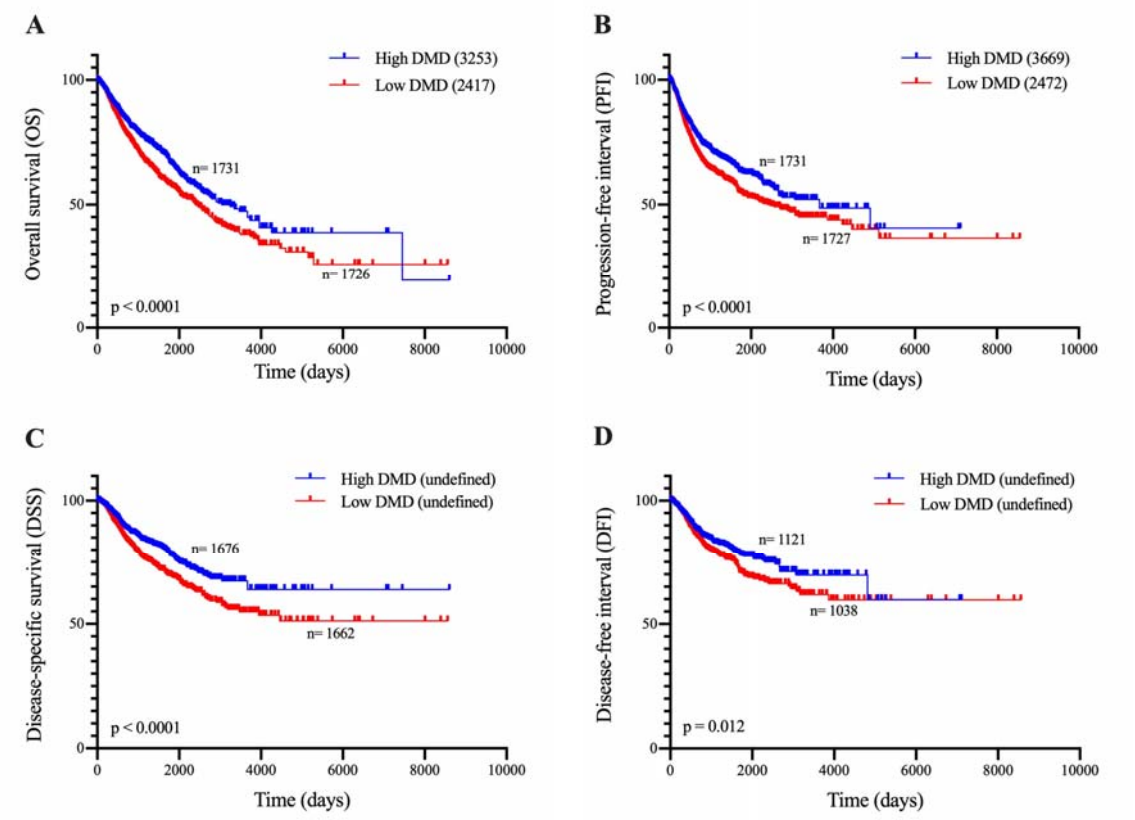

Figure 7. Kaplan-Meier curves of (A) overall survival (B) progression-free interval (C) disease-specific survival and (D) disease-free interval for patients with low or high levels of *DMD* gene expression. Numbers in brackets are overall survival times in days. Numbers of patients in each group and p-values for the log-rank test are displayed in the figure.

In order to confirm that these changes in survival outcomes between patients with low vs. high *DMD* expression in their tumors are associated with *DMD* downregulation and not a result of other factors, we repeated the survival analysis using two groups of patients chosen at random, and no statistically significant differences in survival endpoints were found between the two.

10. Transcriptomic alterations in hematological malignancies samples with low Dp71 compared to samples with high Dp71 expression

The hierarchical clustering analysis revealed that blood malignancies had a unique pattern of *DMD* transcripts to the point that these two malignancies were classified as a cluster of their own. While no changes in Dp427m expression were observed in both TCGA datasets for acute myeloid leukemia (LAML) and diffuse large B-cell lymphoma (DLBC) compared to healthy blood, Dp71 levels including its splice variants Dp71b and Dp71ab were higher in these tumors (Figure 1, Supplementary Table 2). Levels of Dp71 and its splice variants were

also found higher in two TARGET datasets (acute myeloid leukemia and acute lymphoblastic leukemia) compared to healthy whole blood (Supplementary Table 5).

Therefore, we compared gene expression between samples from the previously mentioned TCGA and TARGET datasets at the bottom 33.3% and top 33.3% of Dp71 expression. The pathways enriched in the top 1,000 DEGs with the largest |LogFC| were: protein digestion and absorption (p= 8.59e^{-05}), ECM-receptor interaction (p= 3.87e^{-04}), focal adhesion (p=0.003) and PI3K-Akt signaling pathway (p= 0.02). The identified GO Biological Process terms for these genes were extracellular matrix organization (p= 1.57e^{-10}), regulation of angiogenesis (p= 7.94e^{-04}), skeletal muscle development (p= 8.38e^{-04}), positive regulation of MAPK cascade (p= 0.005), regulation of ERK1 and ERK2 cascade (p= 0.006), regulation of cell migration (p= 0.01) and positive regulation of calcium ion import (p= 0.02) (Supplementary File 2). Thus, these pathways and processes were identical with those found in comparisons with low vs. high expression of Dp427 in completely unrelated malignancies. A comparison between random groups of samples from these hematological tumors did not reveal any statistically significant changes in gene expression.

11. Low expression of Dp71 is associated with poor survival in patients with hematological malignancies

We compared OS between hematological malignancies patients with low and high expression of Dp71 and its splice variants. OS was lower in the low Dp71 group (HR 2.39; 95% 1.44, 3.99; p= 0.0003) with 792 days compared to 1,992 for the high Dp71 group (Figure 8). For specificity testing, the survival analysis was conducted using two random groups of patients with these hematological malignancies and no statistically significant difference in overall survival was identified between them.

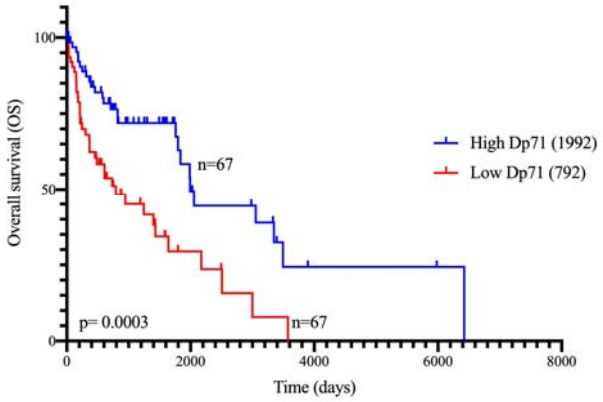

Figure 8. Kaplan-Meier curve of overall survival for hematological malignancies patients with low or high levels of Dp71 expression. Numbers in brackets denote overall survival times in days. Numbers of patients in each group and p-values for the log-rank test are displayed in the figure.

Thus, while specific dystrophins are differentially regulated across various tumors, the low expression of both full-length dystrophin and the Dp71 variant in the analyzed tumors is associated with analogous molecular alterations and significantly decreased patients' survival.

Discussion

The *DMD* gene encodes a spectrum of dystrophin isoforms, but significant expression of the majority of these appeared to be restricted to specific tissues. Loss of the full-length dystrophin results in Duchenne muscular dystrophy, while mutations additionally disrupting other isoforms result in exacerbated phenotypes (36,37). In skeletal myofibers, cardiomyocytes and neurons, which express the highest levels of the full-length dystrophin, this protein has been described to serve as a structural scaffold for proteins engaged in ECM and cell-cell interactions, and in intracellular signaling. However, more recent data demonstrate that the loss of *DMD* expression impacts a broader spectrum of cells than those affected in DMD. In myoblasts (10,16,45,46), lymphocytes (33), endotheliocytes (32,47,48), mesodermal (8), and myogenic cells (11,15), loss of *DMD* expression leads to significant abnormalities. Moreover, the same abnormalities can occur in very distinct cells, e.g., calcium dys-homeostasis was found across multiple cells (49), and the damaging purinergic phenotype affects myoblasts and lymphocytes (33,45). Some old findings, such as platelets abnormalities (50), have recently been vindicated (51). Yet, these defects cannot be clearly attributed to the loss of interaction between dystrophin and the known dystrophin-associated proteins. Indeed, these cell-autonomous defects appear to affect dystrophic cells, which, when healthy, express the 14-kb *DMD* transcript, but were not shown to produce detectable levels of full-length dystrophins. This phenomenon, where expression of the 14-kb *DMD* transcript in cells such as myoblasts and lymphocytes does not correlate with detectable dystrophin protein, was known for decades but just disregarded as an "illegitimate transcription" (52).

Moreover, changes in *DMD* gene expression appear to affect progression in various malignancies (9), many of which originate from tissues not commonly associated with dystrophin expression or function, in which *DMD* transcription would thus be described as "illegitimate". Yet, we found *DMD* transcript levels in these tissues to be comparable to the levels of common housekeeping genes. Therefore, we investigated a spectrum of tumors of

different origins with the expectation that *DMD* alterations in a range of diverse tissues and cell lines may have a common origin and lead to a related outcome. Indeed, data presented here indicate that the current view on the role of the *DMD* gene in health and disease requires significant re-evaluation.

Firstly, although many tumors with downregulated *DMD* expression originated from healthy tissues previously not known for the expression of the full-length dystrophin, interrogation of proteomics datasets demonstrated a much wider distribution profile for the full-length dystrophin isoform than indicated by immunodetection (Supplementary Figure 2). Given the ambiguous immunodetection data and the high sensitivity and specificity of the mass-spec (53,54), identification of the full-length dystrophin in a wide spectrum of normal tissues using this latter method is likely to represent the true expression status. Thus, dystrophin may be present in many tissues at low levels and/or in a tightly controlled spatiotemporal manner, which might be missed using standard detection methods.

Moreover, dystrophin deficiency leads to a variety of cell-specific phenotypes, resulting from the loss of function of specific isoforms in a particular cell. This variability is reflected in the unique subcellular localization of dystrophins in different cell types, from subsarcolemmal distribution in muscle through postsynaptic puncta in neurons (55) to nuclear localization (22). Therefore, we compared expression of specific *DMD* transcripts that showed differential expression in tumors and respective healthy tissues.

We found a significant downregulation of *DMD* gene expression across diverse primary tumors. Both full-length and truncated dystrophin variants were differentially expressed, and hierarchical clustering of the top highly expressed transcripts distinguished tumors from matched healthy tissue samples. A similar trend for *DMD* downregulation across carcinomas has been described previously (24) and our analysis discriminating specific *DMD* transcripts showed that levels of Dp427m mRNA were statistically significantly decreased in the clusters composed mainly of tumor tissues (Supplementary Figure 4), suggesting a specific impact of the loss of the full-length transcript. Indeed, pancreatic adenocarcinoma was the only primary tumor with higher levels of Dp427m transcript compared to healthy tissue. In contrast, the relative expression of Dp71 splice variants was increased in tumor clusters.

In contrast to primary carcinoma samples, in hematological malignancies, total *DMD* expression and the expression of the full-length and Dp71 transcripts was higher or unchanged when compared to normal blood, which also showed the lowest *DMD* expression relative to housekeeping genes of all tissues analyzed (Supplementary Table 3). In contrast,

human and mouse hematopoietic stem cells were found to express Dp71, but its expression was decreasing with cell differentiation, which agrees with whole blood showing the low *DMD* expression. Interestingly, while Dp71 expression is found upregulated in hematological malignancies, its low levels were found associated with the very same dystrophic molecular alterations in cancer cells and also the loss of Dp71 expression was shown to disturb the functional development of macrophages (Chira et al., submitted).

Given some evidence of a causative link between *DMD* downregulation and phenotypic changes in tumor cells (17,23) and that alterations in Duchenne, such as increased cell proliferation, abnormalities in adhesion, migration, and invasion (11,12) are commonly associated with malignancy, transcriptomes of primary tumor samples as well as tumor cell lines with low vs. high level of *DMD* gene expression were compared.

While no causation can be confirmed at this stage, it is important to note that *DMD* dysregulation was associated with specific transcriptomic changes across 15 primary tumors and 140 various tumor cell lines. Functional enrichment analysis showed that the pathways and GO Biological Process terms significantly enriched in DEGs in primary tumors and cell lines with low vs. high *DMD* expression were consistent with the pathways and GO terms enriched in DEGs in DMD skeletal muscle as well as the functional dystrophin PPI network (Supplementary Figure 5). Key pathways altered, including cell adhesion, ECM interactions and PI3K-Akt signaling, correspond with alterations found in Duchenne patients' cells (56,57). The calcium signaling pathway enriched in DEGs in tumors samples with low *DMD* expression from 13 out of the 15 analyzed primary tumors, and well as in sarcoma cell lines with low *DMD* expression agrees with the dysregulation of calcium signaling across a whole spectrum of dystrophic cells (reviewed in (49)), as do GO terms related to the regulation of the developmental mechanisms (8). Thus, the *DMD* gene may play similar roles in cancer and development, two processes showing biological and molecular similarities (58).

The importance of the *DMD* gene in tumorigenesis is supported by the finding that low *DMD* expression was associated with poor survival outcomes in patients with 15 different types of tumors (14 carcinomas and sarcoma). The overall survival of cancer patients with decreased *DMD* expression in tumors was 27 months lower than that of patients with high *DMD* expression. Meanwhile, in low-grade glioma (59) and B-cell chronic lymphocytic leukemia (28), increased *DMD* gene expression was found to worsen survival outcomes for patients. Mutations in the *DMD* gene were associated with poor overall survival of patients of two out of 11 analyzed tumors, namely uterine corpus endometrioid carcinoma and breast invasive

carcinoma (24). Moreover, dystrophin protein was identified as a survival biomarker in upper gastrointestinal cancer patients as poorer survival was observed in patients with low compared to high levels of dystrophin protein (60).

The overall survival of hematological malignancies patients with decreased Dp71 expression was about 39 months lower than that of patients with high Dp71 expression. In contrast, in low-grade glioma, high Dp71 expression was associated with poor patients' survival (59)

A further indirect indication of the functional significance of the *DMD* gene in this paradigm is that *DMD* downregulation across various malignancies involves regulatory changes, not just results from somatic gene mutations. Although, as expected, the presence of somatic mutations or CNVs was associated with significantly lower levels of expression, in about 50% of tumors and 77% of cell lines, *DMD* downregulation could not be linked to such events. While deletions were described as a causative factor for the downregulation of *DMD* gene expression in some tumors (17,23,30), significantly reduced expression was also found in the absence of deletions or nonsense mutations (27), in agreement with our comprehensive analysis across different malignancies. Moreover, in primary pancreatic adenocarcinoma, Dp427m transcript was increased while Dp71 expression was reduced (Figure 4.B). Given the *DMD* gene structure, such an expression pattern can only be explained by differential regulation. Targeted degradation of dystrophin transcripts was suggested before (61) and recently, an epigenetic mechanism responsible for reduced *DMD* transcript levels has been described (62). These data indicate that, rather than simply being an effect of random mutations, quite likely to occur in this large gene, *DMD* alterations in tumor cells may have a complex regulatory nature involving mechanisms such as transcriptional regulation, chromatin remodeling or transcript degradation.

In conclusion, specific alterations in *DMD* gene expression are a common feature across a spectrum of malignancies including those originating from tissues with very low levels or previously not known to express the full-length dystrophin protein. The molecular signature associated with decreased *DMD* expression in tumors and corresponding tumor cell lines is concordant with that found in Duchenne muscular dystrophy. Therefore, more attention should be given to the subtler *DMD* gene functions, beyond those causing the main symptoms of DMD. Such studies have a potential to identify new therapeutic targets for the treatment of this debilitating and still incurable disease. Moreover, given the poor survival

rate of patients with tumors downregulating dystrophin, *DMD* gene may be important in oncology.

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Author contributions

NA carried out all the analyses with support from MB, GT, MK and NH and wrote the initial manuscript. DCG designed the project and wrote the final version of the manuscript with assistance from all co-authors.

Competing interest declaration

Authors declare no competing interests.

Additional information

All data are presented in the manuscript and the Supplementary information file.

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