DNA Damage, Somatic Aneuploidy, and Malignant Sarcoma Susceptibility in Muscular Dystrophies

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Abstract

Albeit genetically highly heterogeneous, muscular dystrophies (MDs) share a convergent pathology leading to muscle wasting accompanied by proliferation of fibrous and fatty tissue, suggesting a common MD–pathomechanism. Here we show that mutations in muscular dystrophy genes (Dmd, Dysf, Capn3, Large) lead to the spontaneous formation of skeletal muscle-derived malignant tumors in mice, presenting as mixed rhabdomyo-, fibro-, and liposarcomas. Primary MD–gene defects and strain background strongly influence sarcoma incidence, latency, localization, and gender prevalence. Combined loss of dystrophin and dysferlin, as well as dystrophin and calpain-3, leads to accelerated tumor formation. Irrespective of the primary gene defects, all MD sarcomas share non-random genomic alterations including frequent losses of tumor suppressors (Cdkn2a, Nf1), amplification of oncogenes (Met, Jun), recurrent duplications of whole chromosomes 8 and 15, and DNA damage. Remarkably, these sarcoma-specific genetic lesions are already regularly present in skeletal muscles in aged MD mice even prior to sarcoma development. Accordingly, we show also that skeletal muscle from human muscular dystrophy patients is affected by gross genomic instability, represented by DNA double-strand breaks and age-related accumulation of aneuploidies. These novel aspects of molecular pathologies common to muscular dystrophies and tumor biology will potentially influence the strategies to combat these diseases.

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Introduction

“…fibres were found to be completely destroyed, the sarcomous element being diffused, and in many places converted into oil globules and granular matter…”

(Edward Meryon, 1852)

Muscular dystrophies (MDs) comprise a group of inherited disorders, characterized by progressive muscle wasting and weakness, frequently causing premature death due to lack of effective therapies. More than 150 years ago, Edward Meryon was the first to characterize the detrimental “fatty degeneration of the voluntary muscles” in Duchenne MD (Meryon E. Lancet 2:588, 1851). Today it is well accepted that the progressive loss of functional muscle tissue and its replacement by adipose and fibrous tissue represent a pathology common to all MDs despite their heterogeneous genetic etiology. Most MDs are caused by gene mutations that lead to absence or dysfunction of structurally and/or functionally important molecules of the muscle fiber [1,2]. The sarcoplasmic spectrin-related protein dystrophin is thought to structurally stabilize the muscle fiber sarcolemma by linking the actin-based cytoskeleton to the extracellular matrix via interaction with the dystroglycan (DG)-complex. Lack or vast reduction of dystrophin causes severe Duchenne muscular dystrophy (DMD) [3] in humans and myopathy in corresponding mouse models, such as the mdx [4] or mdx-3Cv [5] mouse. Mutations in several glycosyltransferase-encoding genes, such as the fukutin related protein (FKRP) or LARGE lead to defective glycosylation of the α-subunit of DG. This molecular defect underlies the second most common group of MDs, the so-called “secondary dystroglycanopathies”. Numerous other MD-related molecules are not known to directly interact with the DG complex, such as dysferlin or calpain-3. Defective expression of dysferlin, a ubiquitously expressed 230-kDa transmembrane protein that has been shown to be involved in resealing muscle fiber membranes, causes limb-girdle muscular dystrophy type 2B (LGMD2B) or Miyoshi-myopathy in humans [6,7]. An inbred mutation in the murine dysferlin (Dysf) gene makes the SJL-mouse a naturally occurring animal model for the human dysferlinopathies [8]. Mutations of the C4PN3 gene encoding the muscle-specific calcium-activated neutral protease calpain-3, a proteolytic switch in muscle remodeling [9], cause LGMD2A, a MD with a wide clinical spectrum [10]. Again, the corresponding animal model, the Capn3-
Author Summary

All kinds of muscular dystrophies (MDs) are characterized by progressive muscle wasting due to life-long proliferation of precursor cells of myo- (muscle), fibro- (connective tissue), and lipogenic (fat) origin. Despite discovery of many MD genes over the past 25 years, MDs still represent debilitating, incurable diseases, which frequently lead to premature death. Thus, it is imperative to gain novel insights into the underlying MD pathomechanisms. Here, we show that different mouse models for the most common human MDs frequently develop skeletal muscle-associated tumors, presenting as complex sarcomas, consisting of myo-, lipo-, and fibrogenic compartments. Collectively, these tumors are characterized by profound genomic instability such as DNA damage, recurring mutations in cancer genes, and aberrant chromosome copy numbers. We also demonstrate the presence of these cancer-related aberrations in dystrophic muscles from MD mice prior to formation of visible sarcomas. Moreover, we discovered corresponding genomic lesions also in skeletal muscles from human MD patients, as well as stem cells cultured thereof, and show that genomic instability precedes muscle degeneration in MDs. We thus propose that cancer-like genomic instability represents a novel, unifying pathomechanism underlying the entire group of genetically distinct MDs, which will hopefully open new therapeutic avenues.

deficient mouse is only affected by a mild progressive muscular dystrophy [11]. Given the diverse and obviously unrelated functions of these proteins, whose absence or dysfunction causes MDs, a common pathomechanism driving the complex events of parallel muscle regeneration and degeneration and progressive proliferation of fibrous and fatty tissue seen in all MDs is likely but still remains elusive. In the light of the fact that nearly 25 years ago the DMD gene was identified as the molecular basis for Duchenne MD, the lack of causative therapies has dampened earlier therapeutic promises based on the discovery of molecular defects underlying several MDs and underscores the imperative need for a comprehensive understanding of pathology involved in these rare but lethal diseases.

When starting to study age-related phenotypes of murine MDs, we have observed the frequent and spontaneous occurrence of skeletal muscle-derived tumors in our colony of C57BL/10 mdx mice, suggesting a tumor-suppressive role of dystrophin in mice. Therefore we extended our studies to other dystrophin mutations, mouse strains, and even to other MD-mouse models for the most frequent MDs in humans, like dysferlin, calpain-3 and Large, respectively. We show that all of these MD-mouse lines are prone to develop mixed soft-tissue sarcomas containing tumor elements displaying histological and molecular characteristics of rhabdomyo-, fibro-, and liposarcoma. These MD-associated tumors share complex, non-random genomic alterations affecting well-known tumor suppressor as well as oncogenes and these cancer signatures are already detectable in dystrophic muscle tissue, independent of the underlying mutation. Consequently, we show that genomic instability and DNA damage are present also in muscle of human MD patients. Collectively, these data strongly support an unprecedented general link between muscular dystrophy and cancer, driven by the accumulation of DNA damage, chromosome copy number aberrations, and finally the origin of cell clones harboring cancer-like mutations in dystrophic muscle tissue. We propose that - similar to pre-neoplastic lesions - the dystrophic muscle is characterized by genomic instability, which contributes to a common hyperproliferative pathomechanism promoting the degenerative process in human MDs and favoring age-related tumorigenesis in the respective mouse models.

Results

Spontaneous occurrence of skeletal muscle-derived tumors in various dystrophin-deficient mouse lines

During the last two decades we have observed the spontaneous occurrence of soft tissue tumors arising from various skeletal limb and trunk muscles in our dystrophin-deficient C57BL/10 mdx-mouse [4] cohort. These tumors arose in aged mdx mice (mean age-of-onset: ~540 d) with an incidence of almost 40%, whereas we never observed the occurrence of such tumors in our C57BL/10 wild-type mice. In our colony of another dystrophin-deficient mouse line, mdx-3Cv, which lacks both the muscle 427 kDa and non-muscle 71 kDa dystrophin isoforms due to a mutation at the intron-exon 66 junction [5], we observed the spontaneous occurrence of skeletal muscle-derived tumors indistinguishable from those observed in C57BL/10-mdx mice. However, mdx-3Cv developed skeletal muscle-tumors at a significant older age (~660 d) and a decreased incidence of only 5% as opposed to our mdx colony. Because we could not figure out if these differences were due to the different genetic backgrounds (mdx: C57BL/10, mdx-3Cv: C57BL/6 x B6C3Fe) or due to the different dystrophin-mutations, we generated two novel mdx-inbred strains, i.e. BALB/c-mdx, and C3H-mdx, respectively, and further studied ndx-mice on mixed C57BL/6 x BALB/c and C57BL/10 x B6C3Fe backgrounds. Indeed we observed the spontaneous occurrence of skeletal muscle-tumors also in these ndx-mice, underlining a strain-independent tumor-suppressor role of dystrophin. Mean ages-of-onset, incidences and gender distributions of tumor-formation were strongly strain-dependent, whereby the C57BL/10 background was most tumor-susceptible (Table 1). The spontaneous occurrence of skeletal muscle-associated tumors in different dystrophin-deficient mouse lines independent of the underlying dystrophin gene mutation supported a candidate tumor suppressor role of dystrophin.

Mice mutated in dysferlin, calpain-3, and Large are also prone to develop skeletal muscle-derived sarcomas

In order to learn whether other MD-genes, which are not directly related to dystrophin, might also suppress tumor formation, we studied mice lacking dysferlin (Dysf<sup>312</sup> mutation [8]; Dysf<sup>-/-</sup>), calpain-3 (Capn3<sup>-/-</sup>; knockout [11]), or Large (Large<sup>md</sup> mutation [12]; Large<sup>-/-</sup>). In a colony of Dysf<sup>-/-</sup> mice inbred onto C57BL/10 (n = 151), we also observed high incidence (23%; male-to-female ratio ~3:1) of age-related sarcomas (~640 d), which mainly arose from proximal hind limb muscles. Also for Dysf<sup>-/-</sup> mice a strain-dependent effect with respect to mean age of sarcoma-onset was detected, which was more than 100 days later (~755 d) when the mutation was bred on a mixed C57BL/10 x B6C3Fe background, whereas the sarcoma incidence remained unchanged (22%). Notably, dysferlin-deficiency on the mixed C57BL/10 x B6C3Fe background resulted in a predominant abdominal wall location of sarcomas (Figure 1A, Table 1).

Based on the spontaneous occurrence of skeletal muscle-tumors in mice deficient for the so far molecularly unrelated genes dystrophin and dysferlin, we hypothesized that MD-genes in more general might act as tumor suppressors. To this end, we conducted a life-span study with mice lacking calpain-3, the animal model for LGMD2A in humans. Indeed, also Capn3<sup>-/-</sup> mice developed skeletal muscle-derived sarcomas at an incidence of 5%. Finally,
we also observed the rare occurrence of sarcoma formation even in mdx mice (representing a model for a severe congenital MD in humans), in spite of their considerably short lifespan (Table 1).

### Combined defects in MD genes accelerate sarcomagenesis in mice

In order to test if dystrophin, dysferlin and calpain-3 have tumor-suppressor effects in vivo, we generated double-mutant mouse lines, i.e. dystrophin-deficient (mdx) mice with additional lack of either dysferlin (Dmd/−/− Dyf/−/−) or calpain-3 (Dmd/−/− Capn3/−/−). Dmd/−/− Dyf/−/− mice (C57BL/10) clinically presented with significant weakness characterized by severe dystrophic signs in the skeletal muscle (R.B., manuscript in preparation), and had a severely reduced life-span of ~13 months. Remarkably, malignant skeletal muscle-derived sarcomas (Figure 1B) constituted the main cause of premature death in this condition. While penetrance was sharply increased in male mice, 63% of which developed sarcomas, a dramatic decrease in tumor latency was observed in both genders, with the mean age-of-onset reduced to ~390 d (compared to 540 d in Dmd/−/− and 640 d in Dyf/−/−).

The combined effect of Dmd/−/− Capn3/−/− in double-knockout mice, which also presented with a severe MD-phenotype leading to a shortened life-span of ~13 months (R.B., manuscript in preparation), resulted in spontaneous sarcoma-formation in 44% of the animals with a mean-age of onset of ~390 days (Figure 1C). Thus, additional loss of both dysferlin and calpain-3 in dystrophin-deficient mdx mice dramatically reduced sarcoma latency (Figure 1D).

### The skeletal muscle-derived tumors in MD mice present as mixed rhabdo-, fibro-, and liposarcomas

Because the macroscopic appearances of the skeletal muscle-derived tumors showed areas of different colors and varying consistencies (Figure 1E), we speculated that this might be due to a mixed composition of diversely differentiated tumor-cell lineages. Indeed, careful histopathological examinations revealed that all tumors independent from the underlying MD mutation(s) resembled mixed sarcomas, comprising variably sized coexisting compartments of rhabdomyosarcoma (RMS), fibrosarcoma (FS) and liposarcoma (LS), respectively (Figure 1F–1K). Histopathology of RMS mainly presented as embryonic (ERMS) or spindle-cell tumors independent from the underlying MD mutation(s).

| Genotype      | Strain background | Cohort | Incidence a | Mean age | Female/male ratio | Tumor site predilection |
|---------------|-------------------|--------|-------------|----------|-------------------|------------------------|
| **Dystrophin**|                   |        |             |          |                   |                        |
| Dmd/−/− (mdx) | C57BL/10          | n = 122| 48          | 39%      | 539 d             | [516–562]              | 1: 1.1 proximal hind limb, trunk, head & neck |
|               | C57BL/10 × B6C3Fb | n = 46 | 8           | 17%      | 580 d             | [460–700]              | 1.7: 1 proximal hind limb, trunk, head & neck |
|               | C57BL/6 × BALB/c  | n = 99 | 10          | 10%      | 651 d             | [567–735]              | 1: 1.4 proximal hind limb, fore limb, head & neck |
|               | BALB/c            | n = 82 | 7           | 9%       | 515 d             | [413–607]              | 1: 1.3 proximal hind limb, head & neck |
|               | C3H               | n = 60 | 9           | 15%      | 523 d             | [411–635]              | 2.5: 1 proximal hind limb, trunk, head & neck |
| Dmd/−/− (mdx-3Cv) | C57BL/6 × B6C3Fb | n = 55 | 3           | 5%       | 663 d             | n.a.                   | n.a. n.a. n.a. |
| **Dysferlin** |                   |        |             |          |                   |                        |
| Dysf/−/−       | C57BL/10          | n = 151| 35          | 23%      | 640 d             | [601–679]              | 1: 2.9 proximal hind limb and trunk |
|               | C57BL/10 × B6C3Fb | n = 78 | 17          | 22%      | 755 d             | [668–804]              | 1: 2.9 trunk |
| **Large**     |                   |        |             |          |                   |                        |
| Large/−/− (myd) | B6C3Fb           | n = 71 | 1           | 1%       | 278 d             | n.a.                   | n.a. n.a. n.a. |
| **Calpain**   |                   |        |             |          |                   |                        |
| Capn3/−/−     | C57BL/6 × [129/Sv: C57BL/6] | n = 19 | 1 | 5% | 716 d | n.a. | n.a. n.a. n.a. |
| **Dystrophin-Dysferlin** |                   |        |             |          |                   |                        |
| Dmd/−/− Dysf/−/− | C57BL/10       | n = 89 | 42          | 47%      | 389 d             | [369–409]              | 1: 2.4 proximal hind limb, fore limb, trunk, head & neck |
| **Dystrophin-Calpain** |                   |        |             |          |                   |                        |
| Dmd/−/− Capn3/−/− | C57BL/10 × [129/Sv: C57BL/6] | n = 55 | 24 | 44% | 391 d | [360–422] | 1: 1.6 proximal hind limb, trunk |

a Incidences of clinically overt sarcomas. Different C57BL/6 proportions in mixed backgrounds are indicated: b 25%, c 50%, and d 75% C57BL/6.

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Incidence and age-of-onset of malignant sarcomas in MD mouse models.
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Figure 1. MD mice are prone to develop skeletal muscle-related malignant mixed mesenchymal tumors. (A) Dysferlin-deficient mouse (male, 878 d) with sarcoma located in the caudal abdominal wall (arrow; weight of excised tumor: 4.2 g). (B) Dystrophin-dysferlin double-mutant (male, 250 d) with large sarcoma (9.1 g), which arose in proximal and distal skeletal muscles of the hindlimb (arrow). (C) Dystrophin-calpain 3 double-mutant (male, 474 d) with sarcoma (2.3 g) located in ischiocural muscles of the right hindlimb (arrow). (D) Graphical representation of sarcoma incidence and age-of-onset from life-span studies of mice lacking dystrophin (n = 48/122; C57BL/10), dysferlin (n = 35/151; C57BL/10), or calpain 3 (n = 1/19; 129/Sv x C57BL/6) and of dystrophin-dysferlin (n = 42/89) and dystrophin-calpain 3 (n = 24/55) double-mutant mice. Error bars indicate confidence interval of the mean (95%). (E) Representative examples of excised tumors (from dystrophin-dysferlin double-mutant mice) with myo- (⁎), lipo- (♦), and fibrosarcomatous (▲) compartments macroscopically recognizable as different parts of the tumor mass. (F–H) Histological examination of the tumor shown in E (upper panel) revealed a sarcoma with mixed mesenchymal differentiation characterized by rhabdomyo- (⁎), lipoblastic (♦), and fibrosarcomatous (▲) components. Serial sections used for H&E staining (F), myogenin staining (G), and double staining with myogenin and dystrophin (H). (I) Morphological analysis of a sarcoma that arose in a 486 d-old C57BL/10 mdx mouse revealing a prominent fibrosarcoma area characterized by the typical collagen fibres bundles arranged in “herringbone pattern” (H&E). (J,K) Well-differentiated liposarcoma area within a mixed sarcoma from a Dmd−/− Capn3−/− double-mutant mouse (J, H&E), with intense Cdk4-positivity (K). (L) Electron micrograph showing a rhabdomyosarcoma cell displaying myofibril structures. (M) Electron micrograph from the same tumor showing a liposarcoma cell densely packed with large lipid vesicles and exhibiting a highly aberrantly shaped nucleus. (N) Photomicrograph showing cultured tumor cells propagated from a typical mixed sarcoma (from a dysferlin-deficient mouse), indicating the presence of different types of tumor cells (round-shaped, spindle cell-like, and myotube-like elongated rhabdomyoblasts). (O) Photograph of two tumor cells grown in vitro from the same explanted tumor. Double-staining for myogenin and Sudan Black depicted a myogenin-positive myogenic tumor cell next to a myogenin-negative but lipogenic tumor cell containing Sudan Black-positive droplets.

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fibroblasts, which were arranged in a typical herringbone pattern, hereby recapitulating the histopathological hallmark of human FS (Figure 1F, H). The third identifiable compartment was LS consisting of lipocytes, which showed both well-differentiated and de-differentiated morphologies. The unifying characteristics of all LS-cells were positivity for lipid staining by Sudan Black (Figure 1H) and, moreover, immunoreactivity for Cdk4 [a human LS biomarker] (Figure 1K). By electron microscopy, LS-cells characteristically contained numerous fat droplets (Figure 1M). In line with the histological findings, propagated tumor cell cultures also revealed co-existence of different cell types (Figure 1N), most prominently myogenin-positive cells and lipogenic but myogenin-negative cells (Figure 1O), providing further support that tumors arising in MD-mice are mixed-type sarcomas. Because these findings disclosed all MD-tumors as complex, mixed sarcomas, we next studied the expression of select human sarcoma-related genes in mice (Figure 1O), most prominently myogenin-positive cells and lipogenic but myogenin-negative cells (Figure 1O), providing further support that tumors arising in MD-mice are mixed-type sarcomas. Because these findings disclosed all MD-tumors as complex, mixed sarcomas, we next studied the expression of select human sarcoma-related genes ([13]). Indeed we found increased expression levels for RMS-related markers (Myo1e, Myl4, Plau, Myo5a) and CMS-related genes (Dysf, Met, Nf1, and Cdkn2a) in MD-tumors, which further established the MD-tumors as mixed sarcoma consisting of RMS, LS and FS compartments (Figure S1).

Genomic hallmarks of murine MD sarcomas: amplification of Met and Jun, loss of Cdkn2a and Nf1, and whole chromosome 8 and 15 gains

In order to characterize the emerging link between MD and sarcoma susceptibility, we investigated genetic lesions in tumors originating in our MD-mouse strains. DNA extracted from solid tumors from 2-month-old mice was subjected to an arrayCGH-based screen (n = 8), which revealed that the majority of Dmd−/− tumors were characterized by multiple segmental chromosomal changes, chromosome number aberrations, and amplification of loci harboring the Met (encoding the Met proto oncogene hepatocyte growth factor receptor) or Jun oncogene, while tumors from Dysf−/− mice typically displayed less genomic instability (Figure 2A). Frequent disruption of the tumor suppressor loci Cdkn2a, encoding p16INK4a and p19ARF, Nf1, encoding neurofibromin 1, and Trp53, together with whole chromosome 8 and/or 15 gains represented key non-random alterations of sarcomas in both MD models. Quantitative PCR (qPCR) experiments (Figure 2B and 2C) of DNA extracted from tumors of Dmd−/−, Dysf−/−, Dmd−/− Dysf−/−, and Dmd−/− Capn3−/− mice (n = 98) revealed that these genetic lesions were common but occurred at different degrees, depending on the specific gene defect(s). Frequent amplification of Met or Jun oncogenes was observed in Dmd−/− (41%) and Dmd−/− Dysf sarcomas (44%). In contrast, amplifications of Cdkn2a and/or Cdk4 (which were additionally tested because of their frequent amplification in human sarcomas, most prominently liposarcomas [14]) were rare (<5%; not shown).

Lesions of the Nf1 gene (exons 23 and/or 56) were more frequently found in Dmd−/− (34%) and Dmd−/− Dysf−/− sarcomas (31%) as opposed to Dysf−/− (14%) or Dmd−/− Capn3−/− (18%). Conversely, exon 2 of the Cdkn2a tumor suppressor gene, which encodes parts of both p16INK4a and p19ARF, was reduced in 73% of Dysf−/− tumors whereas ~50% of Dmd−/− Dysf−/− and Dmd−/− Capn3−/− tumors carried this deletion. Notably, many of the qPCR-ratios obtained for Cdkn2a and Nf1 were consistent with losses throughout the tumor. In 25% of DNA samples from sarcomas with qPCR values indicating Cdkn2a loss, exon 2 copy numbers were <0.2, which suggested the presence of a homozygous deletion in ~80% of tumor cells, compatible with an early event in tumorigensis.

Based on the arrayCGH-findings we screened a large cohort of tumors also for chromosome 8 and 15 copy number aberrations. We found gains of either or both chromosomes in the vast majority (80%) of sarcomas. While ~40–60% of tumors from all MD models displayed gains of both chromosomes, chromosome 8 alone was preferably gained in Dysf−/− and chromosome 15 in Dmd−/− tumors indicating a probable MD-specific preference (Figure 2C).

Sarcoma and dystrophic muscle display similar patterns of genomic instability

More than 50% of the measured chromosome 8/15 ratios were consistent with gains throughout the tumor, implying the presence of trisomies in more than 90% of tumor cells. This suggested that together with losses at Cdkn2a and Nf1 loci the recurrent duplications of these chromosomes belong to early events in sarcoma development. Thus, we argued that such events might occur in skeletal muscles of MD-mice prior to formation of clinically identifiable tumors.

To test this hypothesis, we assessed chromosome 8 and chromosome 15 copy numbers in DNA samples extracted from a panel of typically tumor-prone limb muscles (n = 101), which were obtained from different animals (n = 31) that were sacrificed at advanced ages (comparable to the mean age of mice with sarcomas in the respective MD models) but had not developed visible tumors until then (Figure 3A). We found elevated levels of chromosome 8 and/or 15 in ~30% of muscles from MD-mice but
Figure 2. Common non-random genetic lesions in MD-mouse sarcomas. (A) Chromosomal aberration profiles from Agilent aCGH experiments performed on tumor DNA isolated from sarcomas of n = 4 Dmd2/2 (black) and n = 4 Dysf2/2 (grey) mice revealing gross genomic instability, specific losses at the Cdkn2a, Nf1, and Trp53 tumor suppressor loci, amplification of Met and Jun oncogenes, and recurrent gains of whole chromosome 8 and 15. (B) Real-time PCR quantification of Met and Jun oncogene amplification, losses at the Cdkn2a and Nf1 loci, and chromosome 8 and 15 copy numbers in n = 98 sarcomas from Dmd2/2 (X), Dysf2/2 (J), Dmd2/2 Dysf2/2 (XJ), and Dmd2/2 Capn32/2 (CX) mice. Relative copy numbers (shown as log2) were calculated by the ΔΔCt-method. Dashed lines indicate log2-thresholds that were set under the assumption of 80% tumor cell content: gene amplification (4-fold; Met and Jun), deletions (first line for heterozygous losses, 0.5-fold, second line for homozygous losses; Cdkn2a and Nf1), and chromosome copy numbers (3 and 4). (C) Frequency plots for Met and Jun amplification, Cdkn2a and Nf1 deletions, and chromosome 8/15 gains from the n = 98 sarcomas shown in (B).

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A

Dmd-/-

Dysf-/-

B

chr8

chr15

Cdkn2a

C

1

2

3

D

H&E

Cdk4

p27
never in wild-type mice (Figure 3B). Also occasional copy number aberrations of the Cdkn2a, Nf1, Met and Jun genes were detected in dystrophic muscles (~12%). Because the extents of some of these findings were clearly compatible with the presence of malignant cell clones within the tested muscles, we next analyzed these muscles microscopically. Indeed we found variably sized microscopic tumor masses residing between muscle groups and within single muscle fascicles (Figure 3C). Immunohistochemical examination of these tumors in situ revealed intense staining of cell proliferation markers (p27, PCNA) as well as Cdk4 (Figure 3D), compatible with high proliferative activity.

These findings clearly showed (i) that tumor pre-stages and neoplastic lesions are already present in dystrophic muscle and (ii) that the actual sarcoma incidence of MD-mice is much higher than that solely based on the occurrence of visible tumors. Because none of these DNA-abnormalities were present in non-muscle tissues (i.e. brain, liver, and lung) we concluded that these somatic aberrations are specific to dystrophic skeletal muscle.

Recurrent patterns of somatic aneuploidy in various types of human muscular dystrophies

To address whether aneuploidy affects also human MD, we analyzed primary myoblast lines from DMD and LGMD2B patients. In myoblast DNA samples from a DMD and a LGMD2B patient as well, arrayCGH revealed profiles indicating borderline gains of several chromosomes. In particular, aberration scores indicated gains of chromosome 19 (Figure 4A). To confirm this finding, interphase fluorescent in situ hybridization (I-FISH) experiments were performed on cytospin preparations from early-passage myoblast cell cultures of DMD (n = 4), and LGMD2B (n = 3) patients, as well as healthy donors (n = 2). In contrast to normal cells, myoblasts from DMD and LGMD2B patients frequently harbored tetrasomies of chromosome 19 (13–27%; Figure 4B). Additional analyses for other chromosomes revealed multiple aneusomies, such as tri- and tetrasomies of chromosomes 1 (5–8%), 2 (3–6%), and 8 (4–20%; Figure 4C). In a DMD myoblast cell line, metaphase spreads displayed formation of diplochromosomes (i.e. pairs of sister chromosomes, generated by endoreduplication) (Figure 4D), which are indicative for heterogeneous chromosomal instability and aneuploidies. DNA content analyses by FACScan profiling of propidium iodide-stained cells revealed that myoblasts from DMD and LGMD2B patients contained abnormally high proportions of nuclei with aberrant DNA-content, indicated by prominent G0+ peaks (Figure 4E). Targeted FISH analysis of nuclei isolated through sorting of such G0+ peaks verified the presence of genomes harboring chromosome 8 aneuploidies (Figure 4E, insets). Moreover, the occasional presence of micronuclei implied the continual induction of numerical or structural chromosomal damage in MD-myoblast lines.

In order to preclude that the observed chromosomal copy number aberrations had been acquired or at least amplified in vitro, as reported for embryonic stem cells [15] and committed progenitor cells [16], we asked whether aneuploidies also represent an in vivo genotype and do exist in skeletal muscle tissue of MD patients. To this end, interphase nuclei from frozen muscle biopsies from human MD-patients were isolated and probed by I-FISH. We detected tri- and/or tetrasomies of chromosomes 2 and/or 19 in ~5–12% of the nuclei isolated from DMD muscle (n = 4) (Figure 4F). In contrast, counts of chromosome 13, for which normal copy numbers were found in myoblasts, were readily comparable to control muscles (Table 2). Similarly, aberrant chromosome 2 and 19 counts were detectable in muscle biopsies from patients with LGMD2A (n = 3, CAPN3 mutations), LGMD2I (n = 3, FKRPM mutations), as well as LGMD2B (n = 1, DYSF mutations) (Figure 4G). Notably, LGMD2A muscles exhibited slightly aberrant counts also for chromosome 13 (4.6% versus 1% in controls). Generally, poly-/aneusomic nuclei further displayed features like enlargement, more irregular shape, and micronucleus formation, when compared to disomic nuclei. I-FISH signals in nuclei with aneuploidic configurations frequently appeared either as highly condensed doublet signals (in particular for chromosome 19) or as bizarre structures with highly elongated conformation, indicating increased variability of differential (probably abnormal) states of chromatin condensation (Figure 4F, 4G). In order to learn if the degree of aneuploidy correlates with the disease progression of muscular dystrophies, we also studied fetal muscle obtained during autopsy of aborted fetuses with prenatal diagnosis of DMD or MDC1C. Indeed, these fetal muscle tissues contained much less chromosomal copy number aberrations (chr2: ~1% versus 0.2% in controls; chr13: 0.6% versus 0%; chr19: ~3% versus 1%). Thus, compared to age-matched control muscles, we observed an age-dependent increase of the frequency of aneuploidic nuclei in MD patients (Figure 4H, 4I).

Widespread activation of the DNA damage response in muscular dystrophies

The finding of cancer-like mutations and somatic aneuploidy in dystrophic muscle prompted us to speculate that this might be caused by damage to DNA induced e.g. by oxidative or replication stress. The formation of interstitial deletions and intrachromosomal amplifications, which we found in pre-neoplastic lesions and sarcomas arising in murine MDs, belong to typical genetic aberrations that result from unrepaired DNA double-strand breaks (DSB) [17] and represent early events in the development of cancer [18]. To explore whether damage to genomic DNA precedes sarcoma development, we studied the canonical DNA damage response pathways in skeletal muscle from dystrophic mice. When analyzing muscle tissue from Dmd+/− mice, pronounced activation of the two major DNA damage response
pathways was observed, characterized by high expression of Ser1981-posphorylated ATM (p-ATM, ataxia-telangiectasia mutated kinase) and Ser428-posphorylated ATR (p-ATR, ATM and Rad3-related), and of their downstream signaling targets Chk1 and Chk2 (not shown). We next investigated histone H2A.x, which represents a target of the ATM pathway that signals the presence of DSBs and constitutes a key protein of the DNA damage response by accumulating at large stretches of chromatin surrounding DSBs and recruiting repair factors [19]. In contrast to normal controls, muscle from MD mice was characterized by intense immunoreactivity with an antibody specifically detecting Ser139-phosphorylated histone H2A.x (γ-H2A.x), similar to the reactivity observed in sarcomas (Figure 5A–5C).

We then examined the DNA damage response in muscle biopsies obtained from human DMD patients. In contrast to healthy control muscles, γ-H2A.x immunostainings revealed high levels of DSBs in muscle biopsies from all DMD patients (n = 4) tested, with multiple nucleoplasmic foci formation belonging to

Table 2. Somatic aneuploidy in human skeletal muscle from MD patients.

| Group       | Sample ID | Age (a) | chr2 | chr13 | chr19 | chr2 | chr13 | chr19 |
|-------------|-----------|---------|------|-------|-------|------|-------|-------|
| Controls    |           |         |      |       |       |      |       |       |
| Control-fetal | FK       | prenatal | 88   | 91    | 85    | 0.0  | 0.0   | 1.0   |
| Control-fetal | FP       | prenatal | 91   | 94    | 88    | 0.5  | 0.0   | 1.4   |
| Control     | M2232     | 2       | 87   | 81    | 79    | 0.7  | 1.0   | 1.3   |
| Control     | M1856     | 3       | 87   | 67    | 81    | 0.8  | 2.1   | 1.1   |
| Control     | M2066     | 4       | 91   | 93    | 84    | 1.1  | 0.0   | 0.7   |
| Control -adult | M826   | 44      | 93   | 87    | 79    | 0.5  | 0.5   | 0.5   |
| Control -adult | M1006  | 49      | 87   | 79    | 70    | 1.1  | 1.4   | 0.0   |
| Control -adult | M983   | 52      | 87   | 91    | 70    | 2.3  | 0.0   | 1.1   |
| Control -adult | M689   | 52      | 84   | 74    | 77    | 1.1  | 1.6   | 1.7   |
| DMD         |           |         |      |       |       |      |       |       |
| DMD-fetal   | FL        | prenatal | 85   | 93    | 86    | 2.2  | 0.6   | 4.9   |
| DMD-fetal   | F8        | prenatal | 83   | 92    | 81    | 0.0  | 0.5   | 1.4   |
| DMD         | M2006     | 1       | 82   | 88    | 84    | 2.7  | 0.0   | 3.3   |
| DMD         | M1994     | 7       | 71   | 82    | 70    | 8.3  | 2.4   | 6.8   |
| DMD         | M1895     | 8       | 76   | 86    | 66    | 5.9  | 3.6   | 8.8   |
| DMD         | M1959     | 15      | 58   | 75    | 54    | 11.2 | 2.0   | 6.1   |
| DYSF        |           |         |      |       |       |      |       |       |
| LGMD2B-adult | M2057    | 62      | 58   | n.a.  | 46    | 17.7 | n.a.  | 7.5   |
| FKRP        |           |         |      |       |       |      |       |       |
| MDC1C-fetal | M2166     | prenatal | 88   | 89    | 83    | 1.7  | 0.5   | 2.6   |
| LGMD2I      | M1787     | 10      | 69   | 81    | 55    | 7.3  | 1.9   | 6.1   |
| LGMD2I -adult | M2190 | 28      | 62   | 80    | 62    | 7.1  | 0.7   | 3.3   |
| CAPN3       |           |         |      |       |       |      |       |       |
| LGMD2A      | M1883     | 9       | 77   | 84    | 66    | 3.9  | 4.1   | 4.6   |
| LGMD2A      | M2207     | 13      | 79   | 87    | 77    | 6.1  | 6.0   | 4.6   |
| LGMD2A-adult | M2219  | 25      | 73   | 84    | 70    | 3.8  | 3.5   | 2.8   |

n.a.: not analyzed

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muscle fibres and moreover to non-muscle cells within the endomysial connective tissue, such as interstitial fibroblasts and endothelial cells (Figure 5D, 5E). We further found that DNA-damage response was already present in pre-pathologic muscle from very young patients (9–11 months) and a DMD fetus, which suggested that DNA-double strand breaks very likely occur prior to clinical onset of muscle weakness, wasting, and the concomitant inflammatory response. Also muscle tissue in samples from LGMD2A (CAPN3, n = 3), LGMD2I (FKRP, n = 2), MDC1C (FKRP), and LGMD2B patients (DYSF) exhibited intense γ-H2A.x immunoreactivity and multiple nucleoplasmic foci formation (not shown). That both muscle-fiber nuclei and non-muscle cell nuclei displayed massive γ-H2A.x accumulation prompted us to specifically assess the DSB response in myogenic precursor cells. We investigated primary muscle cell cultures generated from DMD and LGMD2B patients. In contrast to myoblasts from healthy donors, nuclei from DMD and LGMD2B myoblasts showed pronounced accumulation of γ-H2A.x foci (Figure 5F–5I). The formation of distinct nuclear immunofluorescent foci was observed in 49% of cells from DMD and 59% from LGMD2B (compared to 24% in controls) and the number of cells with multiple ($\geq$3) foci was also

**Figure 5. Pronounced DNA damage response in murine and human dystrophic muscle.** (A–C) Intense immunoreactivity with an antibody detecting Ser139-phosphorylated histone H2A.x (γ-H2A.x) in skeletal muscle from a dystrophin-deficient (mdx) mouse (B) and in a sarcoma from a mdx mouse as well (C), in contrast to wild-type (WT) muscle (A). (D–E) In contrast to a healthy control (D), γ-H2A.x immunostainings revealed high levels of DSBs in muscle biopsies from DMD patients (E), with multiple nucleoplasmic γ-H2A.x foci formation in nuclei belonging both to muscle fibres (arrows) and non-muscle cells (arrow-heads). Close-up panel (E’) shows two nuclei with intense foci formation. (F–H) Pronounced accumulation of γ-H2A.x foci in cultured myoblasts from LGMD2B patients (G, “362/03” and H, “90/01”), in contrast to myoblasts from a healthy donor (F, “363/07”), (I) Quantification of γ-H2A.x immunoreactivity revealed increased total reactivity and a prominent increase of cells with multiple ($\geq$3) foci (log10 = 0.5) in myoblasts from LGMD2B (“362/03”) and DMD (“88/07”) patients compared to a control myoblast line (“363/07”). Fluorescence intensity is shown in a log2 scale, foci number is log10. doi:10.1371/journal.pgen.1002042.g005
markedly increased (DMD: 32%; LGMD2B: 45%; controls: 10%).

Discussion

MD mice are prone to develop age-related mixed rhabdo-fibro-liposarcomas

Here we show that different types of MD mouse models develop with increasing age mixed soft-tissue sarcomas (STS), presenting as rhabdo-fibro-liposarcomas. While the spontaneous occurrence of RMS has been previously reported in mdx mice [20] and in addition in mice deficient of z-sarcoglycan [21] (Sgca−/−, a model for the human LGMD2D), this is the first report of sarcomas in mice lacking dysferlin, calpain 3, or Large. Our work further shows for the first time that also mice lacking dystrophin due to other mutations than mdx and on different genetic backgrounds are prone to develop age-related STS. In contrast to the previous reports, we found that virtually all sarcomas from MD mice histologically present as mixed sarcomas consisting of RMS and of two additional components with fibro- and liposarcomatous differentiation. Macroscopically, sarcomas feature considerable heterogeneity regarding visual appearance and consistency of tumor mass. Similar to the high complexity and histological diversity inherent to human sarcomas, we found it extremely difficult to exactly stage individual tumors due the highly complex and heterogeneous structure and significant sectional plane divergence. Therefore, our finding of mixed sarcomas in mdx and other MD mice rather extends than rebuts the previous reports by Chamberlain et al. [20], who reported alveolar RMS in mdx, and Fernandez et al. [21], who described embryonal RMS in mdx and also Sgca−/− mice. As a further difference, sarcoma incidence in our C57BL/10 mdx mice (39%) was clearly higher compared to the previously reported RMS incidences (~6–9%). It remains elusive if these differences are due to different housing conditions or other unknown environmental or strain-specific factors.

It is, however, remarkable that the three main components of malignant cell-types, i.e. myo-, fibro-, and lipocytes, which we observed in our MD-mouse tumors, correspond exactly to the same cell- and tissue types that are crucially characterized by progressive proliferation in MDs. Thus, the MD-associated proliferation of fat and connective tissue might create the molecular context permitting sarcoma development arising from a multipotent mesenchymal or muscle-derived stem cell.

MD-genes display some features similar to tumor suppressors

Several observations in our study lend support to the speculative view that MD-genes might have a role as tumor suppressors. We found that strain backgrounds with C57BL/6 proportions obviously exerted protective effects with regard to tumor latency and that tumor penetrance was lower in Dmd−/− mice on C3H or BALB/c backgrounds compared to C57BL/10. In line with our observation, C57BL/6 is known for its resistance to Pchh1+/−-induced rhabdomyosarcomas [22]. Genetic background also clearly influenced tumor gender specificity in Dmd −/− mice (male preference in BALB/c, female in C3H) and tumor site predilection in Dysf−/− mice (~60% abdominal wall tumors in C57BL/10 x B6C3Fe compared to ~20% in C57BL/10). Such strain-specific modulation of incidence, latency, location spectrum, and gender preference has been well documented for other cancer models, such as the p53-deficient mouse [23]. The significantly reduced sarcoma latency in double-mutant Dmd−/− Dysf−/− and Dmd−/− Capn3−/− mice also resembles a common feature of tumor suppressor mouse models, as exemplified by the synergistic effect of a combined loss of p53 and NF1, which accelerates soft-tissue sarcoma development [24]. Thus, the effects we observed for MD-gene losses represent classical credentials of tumor suppressor genes. In support of this view, dystrophin has been linked to human cancer, as its frequent inactivation was shown to be involved in the pathogenesis of malignant melanoma [25]. Notably, in melanoma cell lines dystrophin knock-down enhanced migration and invasion, whereas re-expression attenuated migration and induced a senescent phenotype, fully in line with a tumor suppressor role of dystrophin [25]. Moreover, utrophin, the highly related autosomal paralog of dystrophin, represents a tumor suppressor candidate, owing to its frequent disruption in human malignant tumors and its capability to inhibit breast cancer cell growth [26]. Notably, aberrations of the DG have been associated with several types of human cancer [27–30], suggesting a potential role also in tumorigenesis. In particular, a tumor suppressor function has been suggested for laminin-binding glycans on z-dystroglycan [31], whose loss can be caused by silencing of the LARGE gene in several metastatic epithelial cell lines [30]. For both, dystrophin [32] and dysferlin [33] interactions with the microtubule network have been recently described, which suggests their hypothetical implication in microtubule-mediated cell functions, such as mitosis and cell migration. Future studies will be needed to clarify whether MD-genes act as tumor suppressors, which is suggested but not proven by our data.

Recurrent non-random genetic lesions in MD sarcomas

We found that marine sarcomas from MD-mice frequently harbor non-random, recurrent genetic lesions that provide links to human mesenchymal cancers. The pivotal p53 and retinoblastoma (RB) cell cycle control pathways were frequently incapacitated by the disruption of the Cdk4 kinase inhibitor p16INK4a and the Mdm2-p53 regulator p19ARF, both of which play an important role in the development and progression of many human cancer types. Deletions at Tp53 and Nf1 loci established a genetic link to human soft-tissue sarcomas, which are characterized by frequent p53 mutations [34–36], as well as to syndromes associated with increased RMS incidence due to germ-line disruption of these tumor suppressor genes (Li-Fraumeni, TP53; Neurofibromatosis type I, NF1) [37]. More recently, human myxofibrosarcoma and pleomorphic liposarcomas were shown to frequently harbor NF1 mutations [14]. Thus, the disruption of Nf1 in sarcomas from MD-mice parallels specific - non myogenic - subtypes of human soft-tissue sarcomas and suggests a more general role for Nf1-lesions in the genesis of mesenchymal cancers. A high fraction of sarcomas from MD-mice harbored amplifications of the Met or Jun oncogenes. The Met oncogene amplification constitutes a critical path to aberrant activation of the Hgf/c-Met axis, which is known to promote tumorigenesis and to be involved in the progression and spread of multiple human cancers. Amplification of the JUN oncogene has been reported in human liposarcomas [38–39], in sound accordance with herein discovered frequent Jun amplification in MD mixed sarcomas.

Our finding of recurrent chromosome 8 and/or 15 gains in MD sarcomas provides a link to other marine cancers. Chromosomes 8 and/or 15 are frequently duplicated in T cell tumors [40–41] or transgenic mouse models of acute promyelocytic leukemia [42], and probably contribute to elevated expression of the Jund and/or Myc oncogenes, as suggested for Myc in T cell lymphomas [41]. Notably, several human malignancies, amongst them myxoid/ round cell liposarcoma [14], are known to harbor recurrent gains of chromosome 8. Most importantly, the human chromosome 8
Dystrophic skeletal muscle from mice and humans harbors multiple regions that are syntenic to both murine chromosomes 8 and 13, which we found to be regularly gained and harbor genomic instability similar to pre-cancerous lesions. Dystrophic skeletal muscle of MD-mice harbors microscopic tumors infiltrates prior to development of macroscopic visible tumors. In particular, our findings suggested that somatic aneuploidy, indicated by recurrent gains of chromosomes 8 and 15, contributes to sarcoma formation in muscular dystrophy (MD) mice. Thus, the frequent occurrence of chromosome 8/15 gains together with specific losses at the Calr2 locus might represent early stages occurring in cancer pre-stage and promoting malignant transformation [43]. Importantly, these findings also suggested that the actual sarcoma incidence of MD-mice is much higher than that solely based on the occurrence of visible tumors. In the light of our results, sarcoma formation might be regarded as the disease end-stage of a MD in mice.

The finding of cancer-like genomic aberrations and DNA damage in the skeletal muscle from MD-mice inspired us to search for such aberrations in skeletal muscle of human MD patients. We focused on DMD and LGMDs caused by DYSF, CAPN3, or FKRPs mutations, representing the most frequent MDs, and found that all of them are associated with somatic aneuploidy and widespread DNA damage in skeletal muscle tissue in vivo. Also, in vitro, cultured myogenic stem cells from DMD and LGMD2B patients exhibited DNA damage and aneuploidy. In our study, somatic aneuploidy appeared to be a feature occurring with the outbreak of pathology in dystrophic muscle and to increase with age in human MD patients. In contrast, high levels of DSBs were already evident in fetal muscle from DMD and MDC1C individuals and in muscle biopsies from DMD infants (<1 year), which suggested that DNA damage precedes the clinical manifestation and therefore cannot be solely related to replication stress. While somatic aneuploidy has been reported in multiple human pathologies, such as Alzheimer's disease, this is the first report on gross somatic aneuploidy in MDs. Genetic instability has been reported in laminopathy-based premature ageing [44], a condition caused by mutations in lamin A/C, notably another MD-related molecule. DNA damage was shown recently in Friedreich's ataxia, caused by mutations in lamin A/C, notably another MD-related molecule. DNA damage was shown recently in Friedreich's ataxia, caused by mutations in lamin A/C, notably another MD-related molecule. DNA damage was shown recently in Friedreich's ataxia, caused by mutations in lamin A/C, notably another MD-related molecule.
of Neurology, Ludwig-Maximilians-University Munich (Germany). DM1: "Essen 88/07" (14 a, del45-50); "72/05" (7 a, dup-exd4-29); "Essen 8/02" (4 a, del-ex51-53); "166/00" (6 a, 2bp-deletion in exon 6); LGMD2B: "90/01" (36 a, female, c. [6360C>T]+ [5249delG]); "176/01" (32 a, male, c. [2367C>G]+[5579dupA]); "362/03" (male, 33 a, c. [exon 5 p.Pro134Leu]+[5022delT]); controls: "363/07" (21a, male); "179/07" (21a, female). Cells were maintained in Ham's F-12 medium supplemented with 15% fetal bovine serum, GlutaMax (L-Glutamine 200 mM), glucose (6.6 mM), fetuin (0.47 mg/mL), bovine serum albumin (0.47 mg/mL), dexamethasone (0.38 μg/mL), insulin (0.2 μg/mL), epidermal growth factor (10 ng/mL), Pen-Strep (penicillin G 5000 units/mL, streptomycin 5 mg/mL), and fungizone (amphotericine B 0.5 μg/mL) at 37°C in a humidified atmosphere of 5% CO2: 95% air. For experimental purposes, cells were harvested after 3 or 4 passages. DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO). Cells were stained with BD CycleTest Plus, DNA Reagent Kit for DNA content analysis by flow cytometry (BD Biosciences, San Jose, CA).

Mice

Mice stocks were maintained at the Division for Laboratory Animal Science and Genetics (Medical University Vienna, Himberg, Lower Austria) under institutionally approved protocols for the humane treatment of animals. Mice were cared for in our facilities under conventional housing conditions and received food and tap water ad libitum. Mice presenting with weakness received intensive care, were fed with food pellets soaked in tap water, and were examined daily. Aged mice were checked daily for the humane treatment of animals. Mice were crossed to C57BL/10, C57BL/6, and C3H/HeN inbred strains for the humane treatment of animals. Mice were crossed to B6C3Fe/C57BL/10 mice were crossed to B6C3Fe/C57BL/10, C57BL/10, SJL/LtJ, and C57BL/6 mice were maintained on a 129/Sv x C57BL/6 background. In all cases, litter-matched males were used. Inbred and outbred strains were used in this study. For histomorphological examination. Sections were stored at −80°C and then subjected to tissue lysis and nucleic acid purification according to the QIAamp DNA Mini Kit protocol (Qiagen). Mouse tail DNA was isolated using the same protocol starting from lysates prepared by directly lysing 2–3 mm tail tips. DNA concentrations were measured using the NanoDrop spectrophotometer (Peqlab, Erlangen, Germany), DNA samples were diluted (10 μg/μL) and stored at −20°C until use. RNA was extracted from serial 1 μm cryosections by lysis in 1 TRI Reagent (Sigma-Aldrich), chloroform extraction, and precipitation with isopropanol. RNA samples were measured by spectrophotometry (NanoDrop) and quality controlled using BioAnalyzer LabChips (Agilent Technologies, Santa Clara, CA).

Histology and immunohistochemistry

Cryosections were stained with haematoxylin and eosin (HE). Sudan Black B was used for lipid staining. For immunohistochemistry, 10 μm cryosections were fixed using 3.7% paraformaldehyde (5 min), treated with 0.1% Triton-X100 (5 min), rinsed in PBS, and subsequently incubated with primary antibodies. For immunocytochemistry, cytochrome c was stained using primary antibodies. Mouse primary antibodies were raised against the following antigens. Myogenin (Santa Cruz Biotechnology, CA; sc-576), Myf-5 (sc-302), desmin (Millipore, Billerica, MA, MAB3430), CD44 (sc-260), PCNA (sc-7907), p27 (sc-776), p-Ser1981-ATM (Cell Signaling Technology, Danvers, MA; #4526), phospho-Ser428-ATR (#2835), p-Ser296-Chk1 (#2349), p-Thr68-Chk2 (#2661), p-9139-Histone H2AX (#9718). Secondary antibodies were conjugated to Alexa-Fluor 488, Alexa-Fluor 594 (Molecular Probes Invitrogen, Carlsbad, CA), Cy3 (Dianova, Hamburg, Germany), or to horseradish peroxidase. Where indicated, immunostained sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and then analyzed by confocal microscopy using either Olympus Fluoview or Zeiss Axioplan2 microscopes. Fully automated software-assisted quantification of DNA damage (γ-H2AX foci) in myoblasts was performed using the software Metafer (MetaSystems, Altlussheim, Germany). Graphical representations (plots of fluorescence intensity versus foci numbers) were generated in R.

Array comparative genomic hybridization (aCGH)

Matched pairs of sarcoma and tail-tip DNA (as reference) samples from the same mice were analyzed using the AGilent mouse genome CGH 44K assay (Giardina S.L. 2011) and 244K (14495) oligonucleotide microarrays (Agilent Technologies). Human myoblast DNA samples were analyzed on Agilent human genome CGH 44K arrays (14495), using as reference human genomic DNA from multiple anonymous male donors that was purchased...
Quantitative PCR (qPCR)

To screen for Met (chromosome [chr] 6) and/or Jun (chr 4) oncogene amplification, tumor DNA samples (25 ng) were subjected to a quantitative endpoint PCR, consisting of 0.4 μM each primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, (NH₄)₂SO₄-containing amplification buffer, and 0.5 units Taq DNA polymerase (from Fermentas, St. Leon-Rot, Germany). Competitive co-amplification of internal control targets (with similar amplicon size) allowed the unambiguous determination of ≥4-fold amplification levels. Primer sequences (5’→3’)

For I-FISH experiments on myoblasts, cells were fixed using 4% formaldehyde. FISH analysis on interphase nuclei extracted from wild-type and mdx mice. RNA abundance in tumor samples was compared to murine C2C12 myoblast cells.

Reverse transcriptase PCR (RT-PCR)

To study whether mixed sarcomas from MD-mice express select human sarcoma-related genes, we subjected RNA isolated from primary tumor samples as well as from tumor cell cultures to quantitative RT-PCR. Total RNA (1 μg) was reverse-transcribed by standard oligo-dT primed cDNA synthesis using M-MuLV Reverse Transcriptase in a reaction buffer containing 50 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 4 mM MgCl₂, 10 mM DTT, and 1 mM dNTPs (Fermentas). An aliquot corresponding to 10 ng of the initial RNA sample was subjected to a quantitative endpoint PCR, consisting of 0.4 μM each primer, 0.2 mM dNTPs, 2 mM MgCl₂, (NH₄)₂SO₄-containing amplification buffer, and 0.25 units DreamTaq Green DNA Polymerase (reagents from Fermentas) in a 25 μl reaction volume. Primer sequences for the human rhabdomyosarcoma-marker genes (Myf, Myh, IGF2, Proxl), a fibrosarcoma gene (Vcan), and liposarcoma-related genes (Pparg, Myo5a, Hoxa5, Plau) are available from the authors on request. After cycling (3 min 95°C, 30 x [40 sec 95°C, 40 sec 60°C, 1 min 72°C], 3 min 72°C), 5 μl aliquots of reaction products were analyzed on ethidium bromide-stained 1.5% agarose gels and quantified from captured images using ImageJ. Relative Met, Jun, Cdk4, and Mdm2 copy number levels were calculated by normalization to the internal standard (S100a8) on chr3 and Lg3 on chr11, respectively. Tumor samples with copy numbers indicating oncogene amplification were also subjected to verification by real-time PCR (see below). Deletions in Cdk2a (chr 4) and Nfl (chr 11) loci were measured using a quantitative real-time PCR (qRT-PCR) SybrGreen assay (ΔCt method), involving separate amplification of target genes and an internal reference (Lig3). Primers were designed for Cdk2a exon 2, which encodes parts of both p16INK4a and p19ARF. CGH 244K data from one Dsorf/− tumor revealed a compound loss at the Nfl locus consisting of a large ~0.4 Mb deletion encompassing the whole gene and a smaller ~42 kb deletion spanning exons 9-28. To screen for Nfl deletions in other tumors, two different exons (23 and 36) were chosen as qRT-PCR targets. Primer sequences were as follows: Cdk2a_f: GTA GCA GCT CTT CTT CTT AAC TAC; Cdk2a_r AAT ATC GCA GTA TGT CTT GAT GT; Nfl_f122_f TGA TGA AGT AGT TGG CCA TTA TTC; Nfl_f123_r TTG CCA TGA TTA CAA CTA ACT; Nfl_f133_f CTC TCG CTT ATT CTA CTA TCT

Interphase fluorescence in situ hybridization (I-FISH)

For I-FISH experiments on myoblasts, cells were fixed using 4% formaldehyde. FISH analysis on interphase nuclei extracted from cryofixed tissues was performed according to a previously published protocol with modifications [56]. In brief, thirty 20 μm-cryosections were fixed in PBS-buffered 4% paraformaldehyde (2–3 h at ambient temperature), rinsed twice with 0.9% NaCl and stored at 4°C overnight until further use. Fixed tissue sections were then transferred into a 90 μm nylon mesh and subjected to proteinase K digestion (0.05%; 10–15 min 37°C). After harvesting by cytopinning through the mesh, nuclei were
air-dried, fixed with paraformaldehyde solution (4% in PBS), washed with 1× PBS (2×3 min), pre-treated with sodium thiocyanate (1 M, 10′ 1 min), and subjected to digestion with proteinase K (1 min at 37°C). After fixation, slides were air-dried, followed by heating to 78°C (5 min) for denaturing. Slides were then hybridized with digoxigenin- or biotin labeled chromosome probes (2p, 18cen, 19q from Dr. M. Rocchi, Molecular Cytogenetic Resource Centre, Bari, Italy; chr1 from Dr. Howard J. Cooke [57]; 8cen purchased from Kreatech Diagnostics, Amsterdam, The Netherlands; chr13 FKHR and 19p/19q from Vyysis, Abbott Laboratories, IL) for hybridization overnight at 37°C. Slides were washed in 2× SSC 50% formamide, and 2× SSC at 42°C, and incubated with Cy3-labelled anti-biotin (Dianova, Hamburg, Germany) or FITC-labeled anti-digoxigenin antibodies in 2% BSA for 30 min at 37°C in a humid chamber. After washing in 4× SSC 0.1% Tween-20 (2×7 min at 42°C), slides were incubated with secondary antibodies labeled with Cy3 or FITC (Dianova) in 2% BSA for 30 min at 37°C, washed again as above, ethanol-dried, and mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Slides were analyzed using an Axioplan2 (Zeiss) microscope and image analysis was performed using the ISIS software (both from, MetaSystems, Altlussheim, Germany). For each sample 500 nuclei were automatically detected by the software and subsequently visually inspected by two independent investigators. Data presented were calculated from an average of 200 nuclei eligible for analysis.

Supporting Information

Figure S1 Expression of myogenic and human sarcoma biomarker genes in murine MD mixed sarcomas. To study whether mixed sarcomas from MD-mice express select human sarcoma-related genes (rhabdomyosarcoma-marker genes: Myog, Myf5, Igf2, Prov1, a fibrosarcoma gene: Vian, and liposarcoma-related genes: Phog, MyoD, Hexad, Fluc), we subjected RNA isolated from primary tumor samples as well as from tumor cell cultures to quantitative RT-PCR. The figure shows a heatmap representation of expression levels corresponding to human sarcoma-related genes, revealing high abundance of not only rhabdomyosarcoma (Rhabdo) marker genes but also of genes related to human fibrosarcoma (Fibro) and liposarcoma (Lipo) in both, primary sarcomas (A) and in vitro tumor cell cultures (B). (PDF)

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Author Contributions

Supplied mice used in the study: HH. Conceived and designed the experiments: WMS REB IMA PFA HH CP WB. Contributed reagents/materials/analysis tools: PFA WB. Wrote the paper: WMS REB.

References

1. Davies KE, Nowak KJ (2006) Molecular mechanisms of muscular dystrophies: old and new players. Nat Rev Mol Cell Biol 7: 762–773.
2. Nowak KJ, Davies KE (2004) Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. EMBO Rep 5: 872–876.
3. Hoffman EP, Brown RH, Jr., Kunkel LM (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51: 919–928.
4. Bulfield G, Siller WG, Wight PA, Moore KJ (1984) X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci U S A 81: 1189–1192.
5. Cox GA, Phelps SF, Chapman VM, Chamberlain JS (1993) New mdx mutation disrupts expression of muscle and nonmuscle isoforms of dystrophin. Nat Genet 4: 87–93.
6. Bashir R, Britton S, Strachan T, Kears S, Vafaiaki E, et al. (1998) A gene related to Caenorhabditis elegans spermagoneiosis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B. Nat Genet 20: 37–42.
7. Liu J, Aski M, Ilii I, Wu C, Fardeau M, et al. (1998) Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy. Nat Genet 4: 87–93.
8. Nowak KJ, Davies KE, Anderson LV, Burkhardt E, Bashir R, Vafaiaki E, et al. (1999) Dysferlin deletion in SJL mice (SJL-Dysf) defines a natural model for limb girdle muscular dystrophy. Proc Natl Acad Sci U S A 96: 11909–11912.
9. de Moorre A, Latie Hutak D, Impagliazzo A, van Haagen HH, de Galan P, et al. (2004) GammaH2AX and cancer. Nat Rev Cancer 8: 957–967.
10. Sareen D, McMillan E, Ebert AD, Shelley BC, Johnson JA, et al. (2009) Chromosome 7 and 19 trisomy in cultured human neural progenitor cells. PLoS ONE 4: e7630. doi:10.1371/journal.pone.0007630.
11. Richard I, Roudaut C, Marchand S, Baghdiguian S, Herasse M, et al. (2000) Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. Faseb J 14: 2195–2204.
12. Barretina J, Taylor BS, Banerji S, Ramos AH, Lagos-Quintana M, et al. (2010) Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. Nat Genet 42: 715–721.
13. Baker DE, Harrison JS, Malby E, Smith K, Moore HD, et al. (2007) Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotechnol 25: 207–215.
14. Sareen D, McMillan E, Ebert AD, Shelley BC, Johnson JA, et al. (2009) Chromosome 7 and 19 trisomy in cultured human neural progenitor cells. PLoS ONE 4: e7630. doi:10.1371/journal.pone.0007630.
15. Pipiras E, Coquelle A, Bieth A, Debatine M (1998) Intestinal deletions and intrachromosomal amplification initiated from a double-strand break targeted to a mammalian chromosome. Embry J 17: 325–333.
16. Duerberg P, Li R, Fabarius A, Helmann R (2005) The chromosomal basis of cancer. Cell Oncol 27: 295–318.
17. Bonnet WM, Redon CE, Dickey JS, Nakamura AJ, Sedelaikova OA, et al. (2008) GammaH2AX and cancer. Nat Rev Cancer 9: 957–967.
18. Chamberlain JS, Metzger J, Reyes M, Townsend D, Faulkner JA (2007) Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. Faseb J 21: 2195–2204.
19. Fernandez K, Sermagao yol, Hammond S, Martin LT, Martin PT (2010) Mice lacking dystrophin or alpha sarcoglycan spontaneously develop embryonal rhabdomyosarcoma with cancer-associated p53 mutations and alternatively spliced or mutant Mdm2 transcripts. Am J Pathol 176: 416–434.
20. Hahn H, Nikita F, Schorban T, Hemmerlen B, Threadgill D, et al. (2004) Genetic mapping of a Pch1-associated rhabdomyosarcoma susceptibility locus on mouse chromosome 12. Genomics 84: 853–858.
21. Donehower LA, Harvey M, Vogel H, McArthur MJ, Montgomery CA, Jr., et al. (1995) Effects of genetic background on tumorigenesis in p53-deficient mice. Mol Carcinog 14: 16–22.
22. Vogel KS, Klece J, Velasco-Miguel S, Meyers K, Rushing EJ, et al. (1999) Mouse tumor model for neurofibromatosis type 1. Science 286: 2176–2179.
23. Korrer H, Eppenhans R, Berking C, Schüler-Thurner B, Speicher MR, et al. (2007) Digital karyotyping reveals frequent inactivation of the dystrophin/DMD gene in malignant melanoma. Cell Cycle 6: 189–198.
24. Li Y, Huang J, Zhao YL, He J, Wang W, et al. (2007) UTRN on chromosome 6q24 is mutated in multiple tumors. Oncogene 26: 6220–6228.
27. Muschler J, Levy D, Boudeau R, Henry M, Campbell K, et al. (2002) A role for dystroglycan in epithelial polarization: loss of function in breast tumor cells. Cancer Res 62: 7102–7109.

28. Sgambato A, Brancaccio A (2005) The dystroglycan complex: from biology to cancer. J Cell Physiol 205: 163–169.

29. Martin LT, Glass M, Dosanua E, Martin PT (2007) Altered expression of native glycosylated alpha dystroglycan in pediatric solid tumors. Hum Pathol 38: 1657–1668.

30. dr Bernabe BR, Inamori K, Yoshida-Moriguchi T, Weydert C, Harper HA, et al. (2009) Loss of alpha-dystroglycan laminin binding in epithelium-derived cancers is caused by silencing of LARGE. J Biol Chem 284: 11279–11284.

31. Bao X, Kobayashi M, Hatakeyama S, Angata K, Gollberg D, et al. (2009) Tumor suppressor function of laminin-binding alpha-dystroglycan requires a distinct beta3-N-acetylglucosaminyltransferase. Proc Natl Acad Sci U S A 106: 12109–12114.

32. Prins KW, Humston JL, Mehta A, Tate V, Rahston E, et al. (2009) Dystrophin is a microtubule-associated protein. J Cell Biol 186: 363–369.

33. Azakir BA, Di Fulvio S, Therrien C, Sinnreich M (2010) Dysferlin interacts with tubulin and microtubules in mouse skeletal muscle. PLoS ONE 5: e10122. doi:10.1371/journal.pone.0010122.

34. Felix CA, Kappel CC, Minaudomi T, Nau MM, Tsokos M, et al. (1992) Frequency and diversity of p53 mutations in childhood rhabdomyosarcoma. Cancer Res 52: 2243–2247.

35. Yoo J, Lee HK, Kang CS, Park WS, Lee JY, et al. (1997) p53 gene mutations and p53 protein expression in human soft tissue sarcomas. Arch Pathol Lab Med 121: 395–399.

36. Castrisana JS, Rubio MP, Gomez L, Kreiebergs A, Zetterberg A, et al. (1995) Detection of TP53 gene mutations in human sarcomas. Eur J Cancer 31A: 735–738.

37. Xia SJ, Persey JG, Barr FG (2002) Molecular pathogenesis of rhabdomyosarcoma. Cancer Biol Ther 1: 97–104.

38. Mariani O, Borennet G, Coindre JM, Gueul N, Ganem C, et al. (2007) JUN oncogene amplification and overexpression block adipogenic differentiation in highly aggressive sarcomas. Cancer Cell 11: 361–374.

39. Snyder EL, Sandstrom DJ, Law K, Fiore C, Sicinska E, et al. (2009) c-Jun amplification and overexpression are oncogenic in liposarcoma but not always sufficient to inhibit the adipogenic differentiation programme. J Pathol 218: 292–300.

40. Wirsching S, Tschiri P, Klein G, Summer J (1986) Rearrangement of c-myc, pim-1 and Mblv-1 and trisomy of chromosome 15 in MCF- and Moloney-MuLV-induced murine T-cell leukemias. Int J Cancer 38: 739–745.

41. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Daumen J, et al. (2003) Induction of tumors in mice by genomic hypomethylation. Science 300: 489–492.

42. Le Beau MM, Davis EM, Patel B, Phan VT, Suhal J, et al. (2003) Recurring chromosomal abnormalities in leukemia in PML-RARA transgenic mice identify cooperating events and genetic pathways to acute promyelocytic leukemia. Blood 102: 1072–1074.

43. Hahn WC, Weinberg RA (2002) Modelling the molecular circuitry of cancer. Nat Rev Cancer 2: 331–341.

44. Liu B, Wang J, Chan KM, Tjia WM, Deng W, et al. (2005) Genomic instability in laminopathy-based premature aging. Nat Med 11: 780–785.

45. Haugen AC, Dr Prospero NA, Parker JS, Finnin RD, Chou J, et al. (2010) Altered gene expression and DNA damage in peripheral blood cells from Friedreich's ataxia patients: cellular model of pathology. PLoS Genet 6: e1000812. doi:10.1371/journal.pgen.1000812.

46. Holland AJ, Cleveland DW (2009) Boxer revisited: chromosomal instability, aneuploidy and tumorigenesis. Nat Rev Mol Cell Biol 10: 478–487.

47. Weaver BA, Cleveland DW (2006) Does aneuploidy cause cancer? Curr Opin Cell Biol 18: 658–667.

48. Williams BR, Prabhu VR, Hunter KE, Glazier CM, Whittaker CA, et al. (2008) Aneuploidy affects proliferation and spontaneous immortalization in mammary cells. Science 322: 703–709.

49. Weaver BA, Silk AD, Montague C, Verderi-Pinard P, Cleveland DW (2007) Aneuploidy acts both oncogenically and as a tumor suppressor. Cancer Cell 11: 25–36.

50. Rodier F, Coppe JP, Patil CK, Hoangmakers WA, Munoz DP, et al. (2009) Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat Cell Biol 11: 973–979.

51. Webster C, Blau HM (1990) Accelerated age-related decline in replicative lifespan of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. Somat Cell Mol Genet 16: 557–565.

52. Sacco A, Mourkoti F, Tran R, Choi J, Llewellyn M, et al. (2010) Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in mdx/mTR mice. Cell 143: 1059–1071.

53. Campisi J (2003) Cancer and ageing: rival demons? Nat Rev Cancer 3: 339–349.

54. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, et al. (2000) Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 464: 641–645.

55. Rangarajan A, Hong SJ, Gifford A, Weinberg RA, Munoz DP, et al. (2009) Aneuploidy affects proliferation and spontaneous immortalization in mammalian cells. Science 322: 703–709.

56. Webster C, Blau HM (1990) Accelerated age-related decline in replicative lifespan of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. Somat Cell Mol Genet 16: 557–565.

57. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, et al. (2000) Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 464: 641–645.

58. Sokol P, Anbous M, Lion T, Haas OA, Zoubek A, et al. (1994) Detection of p53 gene mutations in human sarcomas. Eur J Cancer 31A: 735–738.

59. Sacco A, Mourkoti F, Tran R, Choi J, Llewellyn M, et al. (2010) Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in mdx/mTR mice. Cell 143: 1059–1071.

60. Campisi J (2003) Cancer and ageing: rival demons? Nat Rev Cancer 3: 339–349.

61. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, et al. (2000) Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 464: 641–645.

62. Sokol P, Anbous M, Lion T, Haas OA, Zoubek A, et al. (1994) Detection of p53 gene mutations in human sarcomas. Eur J Cancer 31A: 735–738.