



# Rare Diseases

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## Dystrophin: The dead calm of a dogma

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Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease leading to severe disability and death of young men. Current interventions are palliative as no treatment improves the long-term outcome. Therefore, new therapeutic modalities with translational potential are urgently needed and abnormalities downstream from the absence of dystrophin are realistic targets. It has been shown that DMD mutations alter extracellular ATP (eATP) signaling via P2RX7 purinoceptor upregulation, which leads to autophagic death of dystrophic muscle cells. Furthermore, the eATP-P2RX7 axis contributes to DMD pathology by stimulating harmful inflammatory responses. We demonstrated recently that genetic ablation or pharmacological inhibition of P2RX7 in the *mdx* mouse model of DMD produced functional attenuation of both muscle and non-muscle symptoms, establishing this receptor as an attractive therapeutic target. Central to the argument presented here, this purinergic phenotype affects dystrophic myoblasts. Muscle cells were believed not to be affected at this stage of differentiation, as they do not produce detectable dystrophin protein. Our findings contradict the central hypothesis stating that aberrant dystrophin expression is inconsequential in myoblasts and the DMD pathology results from effects such as sarcolemma fragility, due to the absence of dystrophin, in differentiated myofibres. However, we discuss here the evidence that, already in myogenic cells, DMD mutations produce a plethora of abnormalities, including in cell proliferation, differentiation, energy metabolism, Ca<sup>2+</sup> homeostasis and death, leading to impaired muscle regeneration. We hope that this discussion may bring to light further results that will help re-evaluating the established belief. Clearly, understanding

how DMD mutations alter such a range of functions in myogenic cells is vital for developing effective therapies.

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DMD mutations result in loss of dystrophin causing progressive muscle loss with sterile inflammation, accompanied by cognitive and behavioral impairments. This diversity of symptoms illustrates the importance of dystrophin in various cells. Dystrophin is readily found in myotubes/myofibres, where it protects against sarcolemma fragility.<sup>1</sup> It has become an established belief, which hardened into a dogma, that, because dystrophin is not present at detectable levels using standard methods in myoblasts, the focus of research into aberrant dystrophin expression should be on its role in differentiated muscle cells.

In this paper, we provide arguments that DMD mutations also affect myogenic cells and, thus, that this central dogma is not broad enough and may hold back the search for effective DMD treatments. Pivotal to our argument is the evidence that DMD gene mutations impact a whole spectrum of satellite cell and myoblast functions. A decade ago, we described one of the effects of DMD gene mutations in myoblasts, namely, a purinergic phenotype.<sup>2</sup> We further discuss our recent work that demonstrates relationships between DMD and P2RX7 purinergic receptor activities that shed new light on the pleiotropic effects of aberrant dystrophin gene expression and raises the possibility of using pharmacological purinergic interventions to alleviate DMD pathology.<sup>3</sup>

### **Purinergic signalling and DMD inflammation**

Chronic sterile inflammation of muscles is an important feature in both DMD and the *mdx* mouse model.<sup>4</sup> This potent inflammatory response contributes to both damage and fibrosis, but also muscle regeneration.<sup>5</sup> Intracellular ATP is abundant in muscle fibers<sup>6</sup> and released into the extracellular space following cell damage and death. Increased extracellular ATP (eATP) becomes one of the Danger-Associated Molecular Patterns (DAMPs) activating inflammatory responses.<sup>7</sup> The dystrophin-associated protein,  $\alpha$ -sarcoglycan, is a muscle-specific ATP-hydrolase responsible for degradation of ~25% of eATP.<sup>8</sup> In DMD,  $\alpha$ -sarcoglycan is lost from the sarcolemma,<sup>1</sup> which raises

already high eATP levels even further. Such very high eATP levels are uniquely capable of activating a specific purinergic receptor – the P2RX7.<sup>9</sup>

P2RX7 belongs to a family of ATP-gated ion channels but in response to prolonged, high eATP stimulation, it can exhibit a further open state with a considerably wider permeation that may be associated with cell death.<sup>10,11</sup> P2RX7 is considered a key activator of the “danger mode” of the immune response.<sup>7</sup> Importantly, it is involved in many diverse pathologies with an inflammatory component<sup>12</sup> and crucially also in Limb-Girdle Muscular Dystrophy (LGMD)-2B.<sup>13</sup>

### **P2RX7 purinoceptor as a therapeutic target for ameliorating the symptoms of DMD**

We used genetic ablation of P2RX7 in *mdx* mouse model to establish the impact of this receptor on the dystrophic pathology and to assess its suitability as a therapeutic target.<sup>3</sup> We found that ablation of P2RX7 produced a widespread functional attenuation of disease symptoms in the acute phase of the disease with improved muscle structure, increased muscle strength both *in vitro* and *in vivo*, lowered serum creatine kinase levels and decreased inflammation. Moreover, our RNA-Seq analyses revealed a pro-fibrotic gene expression signature in 4 week old *mdx* leg muscle. Unlike DMD, the *mdx* mouse model shows little fibrosis in limb muscles but this novel finding demonstrated that *mdx* shares this molecular defect with DMD. Notably, this early fibrotic signature was reduced by P2RX7 ablation.<sup>3</sup> These improvements in leg muscles during the acute phase of the disease were also evident at 20 months in leg, diaphragm, and heart muscles. In addition, reduced cognitive impairment and bone structure abnormalities were also apparent. To our knowledge, this is the first demonstration that a single treatment can improve both muscle functions and also correct cognitive impairment and bone loss caused by DMD mutations.

This interrelationship between P2RX7 function and disease progression led us to investigate the effects of pharmacological interference with P2RX7 in *mdx* mice and improvements in pathological markers were observed following administration of various P2RX7 antagonists.<sup>3</sup> Notably, a related study confirmed that pharmacological purinergic blockade can delay disease progression in DMD mice.<sup>14</sup> Therefore, the P2RX7 receptor is an attractive target for translational research; existing drugs with established safety records could be re-purposed for the treatment of this lethal disease relatively easily.

Equally noteworthy is the mechanism by which the ablation or blockade of P2RX7 can produce such wide-ranging improvements. Clearly, given its unique involvement in inflammation, lack of P2RX7 is bound to have a significant impact,<sup>14</sup> just as targeting the innate immunity was shown to slow the disease progression.<sup>15</sup> However, we have discovered an additional mechanism: Muscles with DMD gene mutations have significantly upregulated P2RX7 receptor expression and function.<sup>14,16</sup> This upregulation in dystrophic muscle cells is pathologically important. By analysing the consequences of P2RX7 activation in *mdx* muscle cells, we found a novel mechanism of autophagic cell death. P2RX7-evoked autophagic flux was triggered by the large pore formation but not by the canonical P2RX7-evoked signals and required HSP90.<sup>17</sup> Blockade of P2RX7 protected against eATP-induced death both *in vitro*<sup>17</sup> and *in vivo*.<sup>3</sup> Thus, P2RX7 seems to contribute to damage directly by causing death of dystrophic muscles and by stimulating harmful inflammatory responses.

#### **DMD mutations cause abnormalities in myogenic cells**

Astonishingly, we found that these purinergic abnormalities, including P2RX7 upregulation, eATP-evoked autophagic death, increased ERK phosphorylation, and Ca<sup>2+</sup> influx manifest already in undifferentiated *mdx* myoblasts.<sup>2,16</sup> Importantly, this purinergic phenotype has also been described

in human DMD lymphoblasts<sup>18</sup> and these cells are used for molecular diagnostics because they express full-length dystrophin transcripts but do not have detectable dystrophin protein; just like myoblasts.

Further studies, from our lab as well as from others, revealed that myoblasts with the mutant DMD gene have abnormalities of other important functions: Both myoblasts isolated from Duchenne patients<sup>19,20</sup> and *mdx* mice<sup>21</sup> show altered proliferation and differentiation. Furthermore, there are defects of energy metabolism with disorganized mitochondrial networks<sup>22</sup> and in store-operated calcium entry.<sup>23</sup>

Our quantitative phospho-proteomics analyses identified further cell-autonomous differences between wild-type and dystrophic myoblasts.<sup>17</sup> So far, one set of these alterations, namely in heat shock proteins (HSPs), proved pathologically significant as it was linked to the aforementioned novel mechanism of autophagic death in dystrophic muscles.<sup>17</sup>

Notably, the absence of normal DMD gene expression also affects stem cells. It has been previously described that DMD mutants show well-defined phenotypic defects leading to the intrinsic exhaustion of stem cells, compromising dystrophic muscle regeneration vital to the disease progression.<sup>24,25</sup> Recently, this abnormality has been firmly linked to the presence of the full-length dystrophin in activated satellite cells, where it regulates cell polarity: In *mdx*, loss of dystrophin results in abnormal asymmetric cell divisions and reduced number of myogenic progenitors.<sup>26</sup> Our data demonstrated that the loss of the regenerative potential is further exacerbated by the P2RX7-evoked death of dystrophic myoblasts in the high eATP environment of the damaged muscle.

Importantly, all these abnormalities are cell-autonomous as they were found in both primary and immortalized cells kept long-term in culture, thus excluding the influence of the dystrophic muscle environment.<sup>2,16-26</sup>

These findings provide compelling support for the proposition to re-consider the established belief that dystrophin expression is important only in myofibres because at this stage there is only sufficient protein to convey its anchoring and sarcolemma stabilization functions.

Further support is provided by the reports that RNAi-mediated knock-down of the full-length dystrophin in adult mouse did not trigger muscle degeneration despite the loss of protein from the sarcolemma<sup>27</sup> and the observations that dystrophin is expressed early in embryogenesis (mouse E9.5) and its loss in *mdx* mouse severely disrupts muscle development due to stem cell depletion and disrupted muscle patterning.<sup>28</sup> Moreover, although the absence of dystrophin during muscle development has not been given sufficient attention so far, its deficiency in brain cells, which express distinct dystrophin isoforms,<sup>29</sup> is linked with developmental abnormality and the DMD-associated cognitive defect.<sup>30</sup>

We hope that this discussion will bring to light further results contradicting this established belief and which did not find their way to publication due to researchers' self-doubt or impediments in the editorial process.

An unanswered question is how DMD gene mutations could produce the effects in proliferating myoblasts in the absence of detectable protein? One possibility is that the very small amounts of dystrophin protein concentrated in specific microdomains play a key role, analogous to dystrophin expression at postsynaptic densities in a subset of CNS synapses. Its absence in dystrophic neurons alters the molecular machinery defining the precise spatio-temporal pattern of synaptic transmission

within specific brain areas.<sup>31</sup> Another possibility is that dystrophin is expressed in bursts at specific checkpoints, just like in satellite cells when establishing polarity and asymmetric divisions.<sup>26</sup> Such burst of expression would not be easily detectable in non-synchronised myoblasts *in vivo* or in culture.

Full-length dystrophin loss in myogenic tumours was recently associated with progression to lethal high grades, making DMD a tumour suppressor gene. The reason behind the mutation rate in myogenic tumours being particularly high is unclear but it might involve mechanisms such as transcription-associated recombination.<sup>32</sup>

A radical but speculative explanation may be provided by the regulatory effects of the DMD transcriptome. DMD is the largest gene, encompassing 0.1% of the entire human genome with 7 promoters and 79 exons arranged into 3 full-length (for which transcription takes over 16 h<sup>33</sup>) and 4 progressively-truncated transcripts, heavily alternatively spliced and expressed in a temporally-controlled and tissue-specific manner.<sup>34</sup> Just one of these mRNAs is expressed in mature muscle fibres and its internally truncated in-frame variants can support functional muscles as evidenced in Becker MD and by some therapeutic effects.<sup>1</sup> DMD mutations in myogenic cells might also be pathogenic by altering transcriptional control of other protein-coding as well as non-coding (lncRNAs, miRNAs) genes. The DMD locus encodes specific lncRNAs<sup>35</sup> and DMD mutations could deregulate expression of such lncRNAs, which, in turn, can trigger additional abnormalities e.g. by altering miRNA sponging.<sup>35-37</sup> Notably, the levels of one such lncRNA have been found significantly reduced in myoblasts isolated from DMD patients.<sup>38</sup>

## Conclusion

Clearly, understanding how DMD mutations alter such a range of functions is vital for developing effective therapies. Molecular approaches aiming at restoration of dystrophin are currently the main focus of pre-clinical and clinical trials. Unfortunately, so far none of these proved effective, and some were even discontinued. Therefore, alternative strategies must be investigated. These should include treatments aiming at alterations downstream from the absence of dystrophin. After all, other genetic disorders such as hemochromatosis, Wilson's disease or phenylketonuria are treated in such a way. Although not curative, such approaches could succeed in treating important disease manifestations and several have already shown some therapeutic promise.<sup>39,40</sup> Most recent study demonstrated that functional muscle can exist in dystrophin-deficient animals thanks to a genetic modifier.<sup>41</sup> Notably, increased expression of the rescue gene, *Jagged 1* was found in muscle regeneration at a time point when myoblasts proliferate and fuse.<sup>41</sup>

Moreover, just as there is increasing evidence that disrupted muscle signaling is a good therapeutic target,<sup>3,14,42</sup> also targeting signaling pathways using pharmacological agents is currently more achievable than restoration of structural proteins *via* molecular manipulations. The reason might be, as suggested, that achieving the 15-20% level of dystrophin expression required to fully protect muscle fibers<sup>43</sup> is still elusive. However, if DMD pathology really begins in undifferentiated muscle cells, the expression of dystrophin cDNAs or forced translation of mutant transcripts in myotubes may not cure the disease. Indeed, while dystrophin absence causes a severe myogenic cell abnormality, these cells are not the prime target for molecular interventions. What if the positive outcomes of such treatments stem from dystrophin transcript re-expression or stabilisation in myoblasts before they fuse into or with myotubes? Could the prevailing dogma be steering us into a dead calm of conviction that we know what is wrong and just need more time and money to perfect the fix?

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