where, as here, faithful recapitulation of molecular and pathological phenotypes is possible, our findings argue that primary human BSE prion infection, as well as secondary infection with vCJD prions by iatrogenic routes, may not be restricted to a single disease phenotype. These data, together with the recent recognition of probable iatrogenic transmission of vCJD prions to recipients of blood (*21*, *22*), including a *PRNP* codon 129 Met/Val heterozygous individual (*22*), reiterate the need to stratify all human prion disease patients by PrP^{Sc} type. This surveillance will facilitate rapid recognition of novel PrP^{Sc} types and of any change in relative frequencies of particular PrP^{Sc} subtypes in relation to either BSE exposure patterns or iatrogenic sources of vCJD prions.

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Rescue of Dystrophic Muscle Through U7 snRNA–Mediated Exon Skipping

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Most mutations in the dystrophin gene create a frameshift or a stop in the mRNA and are associated with severe Duchenne muscular dystrophy. Exon skipping that naturally occurs at low frequency sometimes eliminates the mutation and leads to the production of a rescued protein. We have achieved persistent exon skipping that removes the mutated exon on the dystrophin messenger mRNA of the *mdx* mouse, by a single administration of an AAV vector expressing antisense sequences linked to a modified U7 small nuclear RNA. We report the sustained production of functional dystrophin at physiological levels in entire groups of muscles and the correction of the muscular dystrophy.

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder caused by mutations in a gene that encodes dystrophin, a large cytoskeletal protein that complexes with other partners at the sarcolemma and is essential for membrane integrity of the muscle fiber. The dystrophin gene spans about 2.5 Mb and encodes a major 14-kb mRNA transcript processed from 79 exons. Full-length dystrophin (427 kD) is composed of several domains consisting of an actin-binding site at the N terminus; a central rod domain of 24 spectrin-like repeats; and a cysteine-rich domain, which binds other

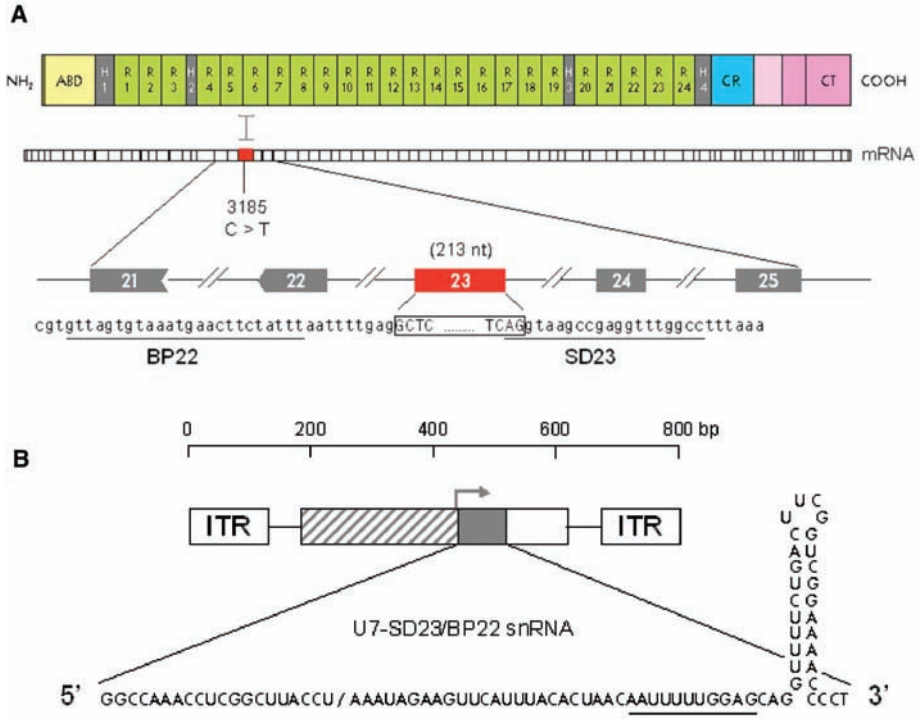

Fig. 1. (**A**) (Top) Dystrophin includes an actin-binding domain (ABD) at the N terminus, a central rod domain that contains 24 spectrin-like repeats (R) and four hinge segments (H), a β-dystroglycan binding, a cysteine-rich domain (CR), and a C-terminal domain (CT). (Middle) Position of exon 23 partly encoding repeats R5 and R6 in which a C to T mutation creates a stop codon in the *mdx* mouse. (Bottom) Target sequences for exon skipping at the branch point (BP22) upstream of exon 23 and at the downstream donor splice site (SD23). (**B**) Structure of the AAV(U7-SD23/BP22) vector. The U7-SD23/BP22 cassette includes the U7-promoter (position –267 to +1, hatched box), the U7SmOPT snRNA (gray box and sequence below) and downstream sequences down to position 116 (open box). It is shown between two AAV2 inverted terminal repeats (ITRs).

members of the sarcolemmal complex, near the C terminus. As a consequence of the modular structure of dystrophin, internally truncated proteins missing some of the repeats can be fully functional or at least partly active as seen in patients with mild (Becker) forms of DMD (*1*). About 70% of mutations in the dystrophin gene result in the absence of protein and are associated with a severe Duchenne phenotype, because they create a disruption of the translational frame of the mRNA. It is noteworthy that exon skipping that naturally occurs during dystrophin mRNA processing can restore the reading frame and give rise to rare "revertant" fibers that contain shortened proteins (*2*, *3*). Strategies for dystrophin rescue in the DMD muscle have been evaluated with the use of antisense oligonucleotides that cause the skipping of selected exons [reviewed in (*4*)]. In the *mdx* mouse, which carries a nonsense mutation in exon 23 of the dystrophin gene (*5*), although the local injection of 2′-*O*-methyl antisense oligoribonucleotides resulted in rescue of the protein, the effect remained localized and started to vanish after 4 weeks (*6*). Our goal here was to achieve a stable long-term expression of antisense sequences that would generate sustained therapeutic levels of rescued dystrophin in entire groups of muscles.

The activity of antisense sequences that can interfere with the mRNA maturation process is considerably enhanced when these are linked to small nuclear RNAs (snRNAs), because it allows for their proper subcellular localization and facilitates their inclusion into mRNA processing machines such as the spliceosome (*7*, *8*). U7, a nonspliceosomal snRNA normally involved in the processing of the histone mRNA 3′ end, can be engineered to bind the appropriate Sm proteins, redirected to the spliceosome, and used to deliver antisense sequences (*9*). The stable expression of modified U7 snRNAs (U7SmOPT) transfected into cells can result in a sustained and sequence-specific modification of the targeted mRNA structure (*10*, *11*). A number of antisense sequences have been used to skip the nonsense mutation containing exon 23 on the *mdx* dystrophin mRNA (Fig. 1A) (*12*). From these, we selected a 24-nucleotide sequence located across the splicing branching point in intron 22 (BP22) (*13*), and a 20-nucleotide sequence in intron 23 that corresponds to the U1 binding region at the donor site (SD23) (*14*) for the construction of a "double-target" U7SmOPT gene according to Suter *et al*. (*10*) (Fig. 1).

The modified U7 gene, along with its natural promoter and 3′ elements, was introduced into an AAV-2–based vector that was packaged into an AAV-1 capsid for high-efficiency gene transfer into the skeletal muscle (*15*). Adult *mdx* mice (n = 37) were injected in the tibialis anterior (TA) muscle with single vector doses of 0.2 to 1 × 10^{12} viral genomes (vg), and the results were analyzed at different time points between 2 and 13 weeks. One of the experimental group was also injected into the extensor digitorum longus (EDL) muscle with 0.4 to 2 × 10^{11} vg (table S1). In comparison with the endogenous U7 snRNA, the U7-SD23/BP22 snRNA was robustly expressed in the injected TA muscles after 2 weeks (Fig. 2A). The presence of the modified U7 snRNA was associated with the appearance in these samples of dystrophin transcripts lacking exon 23, as detected by reverse transcription polymerase chain reaction (RT-PCR) and analyzed by DNA sequencing (Fig. 2, B and C). The 688-bp product amplified from the skipped mRNA represented ~15% of the PCR products 2 weeks after injection and became the major species at 4, 6, and 13 weeks (Fig. 2B). This slow accumulation of skipped transcripts was not the result of a progressive transgene expression during the first weeks after AAV-mediated gene transfer (*16*), because the levels of modified U7 were already maximal at 2 weeks. Rather, it suggests a limited availability of the pre-mRNA and/or a slow turnover of the processed dystrophin mRNA in the muscle fiber.

Consistent with the generation of skipped transcripts, the dystrophin protein was readily detected both by Western blot on muscle extracts (Fig. 2D) and by immunofluorescence on tissue sections (Fig. 3). The levels of dystrophin mirrored those of the rescued mRNA (3% of normal at 2 weeks and 50 to 80% thereafter). The skipping procedure generated immunoreactive protein species with the expected mobility around 426 kD, without evidence for multiply deleted by-products. (Note: The expected 8-kD difference between wild-type and rescued proteins could not be resolved on this gel.) Virtually all fibers in the injected muscle stained positive from 4 weeks post injection onwards, and the protein was typically localized at the periphery of fibers (Fig. 3, D and E). The histology of the corrected muscles was essentially normal, with fibers displaying a "healthy" polygonal shape. Small-caliber

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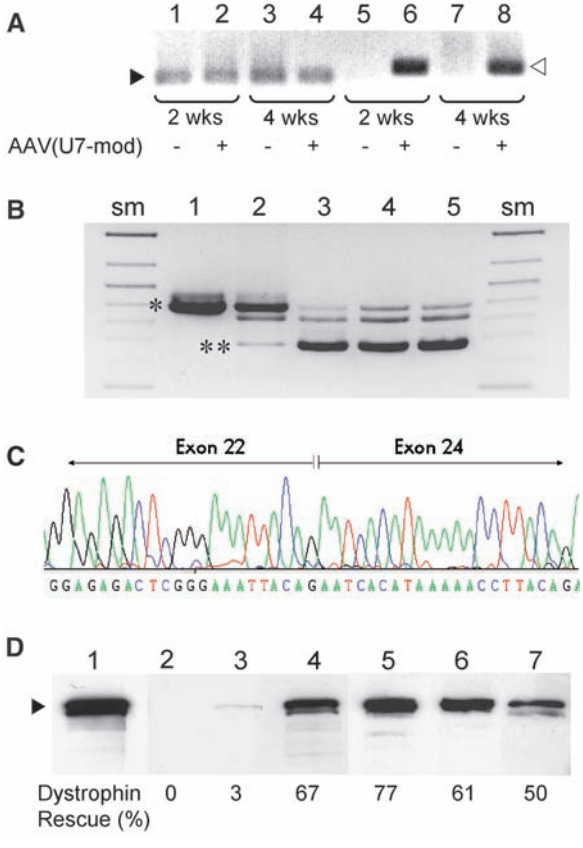

Fig. 2. (**A**) Detection of native and modified U7 snRNAs in *mdx* muscles. Total RNA from treated muscles (lanes 2, 4, 6, and 8) or contralateral untreated muscles (lanes 1, 3, 5, and 7) was analyzed by RT-PCR at 2 weeks (lanes 1, 2, 5, and 6) and 4 weeks (lanes 3, 4, 7, and 8). The 60- and 80-bp products corresponding to endogenous and newly expressed U7 snRNA, respectively, are shown by arrowheads. (**B**) Detection of exon 23–skipped dystrophin mRNA. RNA samples were analyzed at 0, 2, 4, 6, and 13 weeks by nested RT-PCR with primers in exons 20 and 26. The 901-bp band corresponding to the normal mRNA (*) is the only species detected at day 0 (lane 1), and it is progressively replaced by a 688-bp fragment (**) that corresponds to the exon 23–skipped mRNA (lanes 2 to 5). (**C**) DNA sequence of the 688-bp band. (**D**) Western blot of total protein extracted from injected *mdx* muscles stained with the NCL-DYS1 monoclonal antibody. Arrows indicate the full-length 427-kD dystrophin, as detected in normal C57BL6 sample (lane 1). Lanes 2 to 7 correspond to uninjected control and samples at 2, 4, 6, 8, and 13 weeks, respectively. Each lane was loaded with 40 μg of total protein. The same profile was obtained by using the NCL-DYS2 monoclonal antibody (*18*).

fibers indicative of previous regeneration activity were more abundant in the treated muscles. CD11b-positive monocytes/macrophages that massively infiltrate the dystrophic *mdx* muscle lesions (*17*) were completely absent after dystrophin rescue, which indicated that the process of necrosis and regeneration had been arrested. Moreover, no $CD4^+$ or $CD8^+$ cells were detected, consistent with an absence of immune response against the rescued dystrophin (see SOM).

A group of five *mdx* animals received the AAV-U7-SD23/BP22 vector by intra-arterial perfusion of the lower limb. This resulted, after 1 month, in the efficient rescue of dystrophin in >80% of the fibers in most muscles of the perfused leg, including tibialis anterior and extensor digitorum longus muscles (Fig. 3F), gastrocnemius, soleus, plantaris, and biceps femoris muscles (*18*). Along with the rescued dystrophin, the components of its associated glycoprotein complex, including α- and β-sarcoglycans and β-dystroglycan, were expressed at the periphery of the fibers in treated animals (Fig. 4). This indicates that the dystrophin produced from the skipped mRNA contains the C-terminal β-dystroglycan binding domain essential for membrane anchoring of the complex (*19*).

The contractile and mechanical properties of treated muscles were studied by measuring resistance to tetanic contractions accompanied by forced lengthening (Fig. 5A). For this assay, animals that had been injected in the EDL were analyzed after 6 weeks. Muscles from *mdx* animals were unable to sustain repeated elongations and lost 65% of their maximum force over five eccentric contractions. In contrast, treated muscles, displaying >70% of fibers with rescued dystrophin, were essentially normal by this criterion. One representative example where the treated muscle displayed 17% force drop compared with 15% for the wild type is shown in Fig. 5A. Exercise-induced damage was also evaluated by submitting TA-injected animals to extensive downhill running on a treadmill, followed by an intravenous injection of Evans blue, a cell-impermeable dye. Muscle lesions revealed by dye entry into the fibers in all untreated contralateral legs (Fig. 5B) were absent from muscles of the legs injected with AAV-U7-SD23/BP22 (Fig. 5C).

The accumulating data on AAV-mediated gene transfer into the skeletal muscle in rodent, canine, and primates, including human subjects, indicate that U7-mediated rescue may be permanent (*20*–*23*). The levels and stability of exon skipping that we report are significantly higher than those obtained using other U7-based constructs in myoblast cultures (*8*, *13*) or oligonucleotides injected in vivo (*6*, *14*, *24*, *25*). This may be, in part, related to the particular combination of target sequences that we have chosen and to the high efficiency of AAV-1–mediated gene transfer into mature skeletal muscle. It is possible, too, that the muscle fiber provides an especially favorable environment for U7-mediated targeting of antisense sequences. In this respect, it will be important to explore the potential of the AAV-U7 system

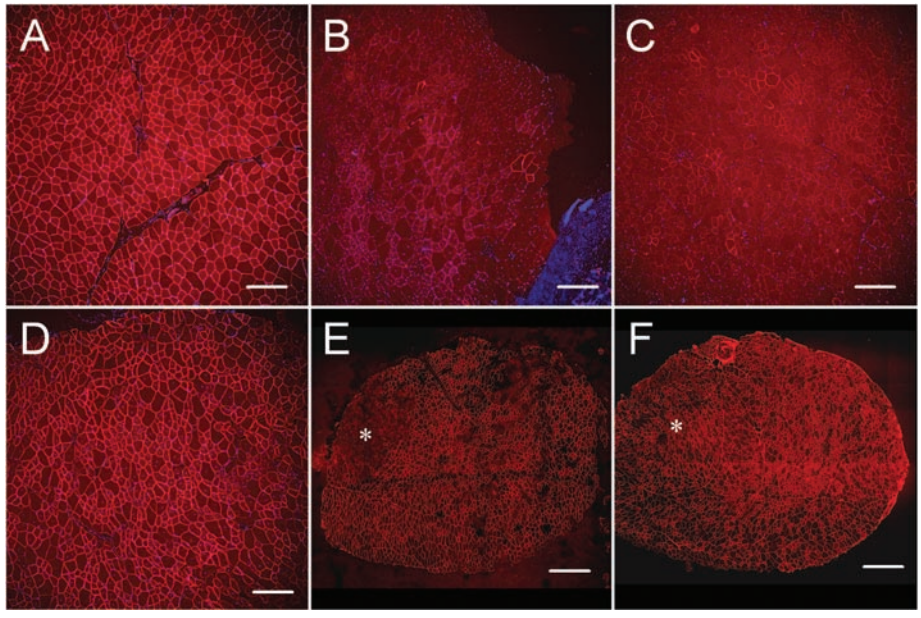

Fig. 3. Dystrophin rescue in *mdx* mice after administration of AAV(U7-SD23/BP22). NCL-DYS2 immunostaining of whole transverse sections from the hind limb anterior compartment (tibialis anterior and extensor digitorum longus* muscles) from normal C57BL6 (**A**), untreated *mdx* (**B**), *mdx* 2, 4, and 13 weeks after intramuscular injection (**C** to **E**), and *mdx* 4 weeks after intra-arterial vector delivery (**F**). Scale bars (A to D), 0.5 mm; (E and F), 1 mm.

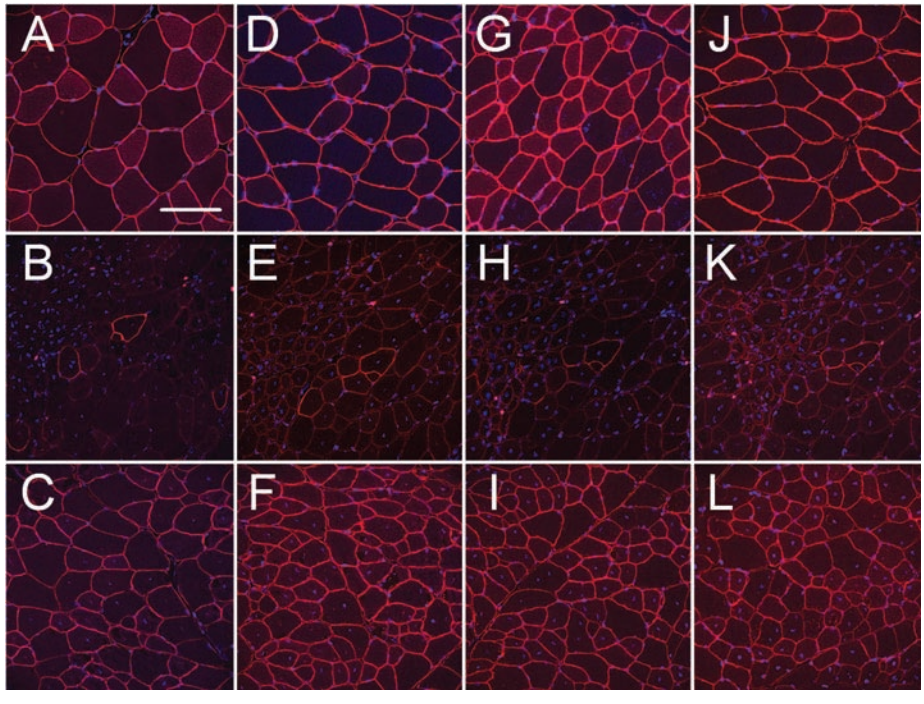

Fig. 4. Restoration of the dystrophin-associated protein complex in treated *mdx* muscles. Left, middle, and right columns show sections from TA muscles of C57BL6, untreated *mdx*, and *mdx*, respectively, 4 weeks after treatment. Sections were immunostained for (**A** to **C**) dystrophin, (**D** to **F**) α-sarcoglycan, (**G** to **I**) β-sarcoglycan, and (**J** to **L**) β-dystroglycan. The same cluster of revertant fibers displaying dystrophin, as well as the associated protein complex, is shown on the serial sections from untreated *mdx*.

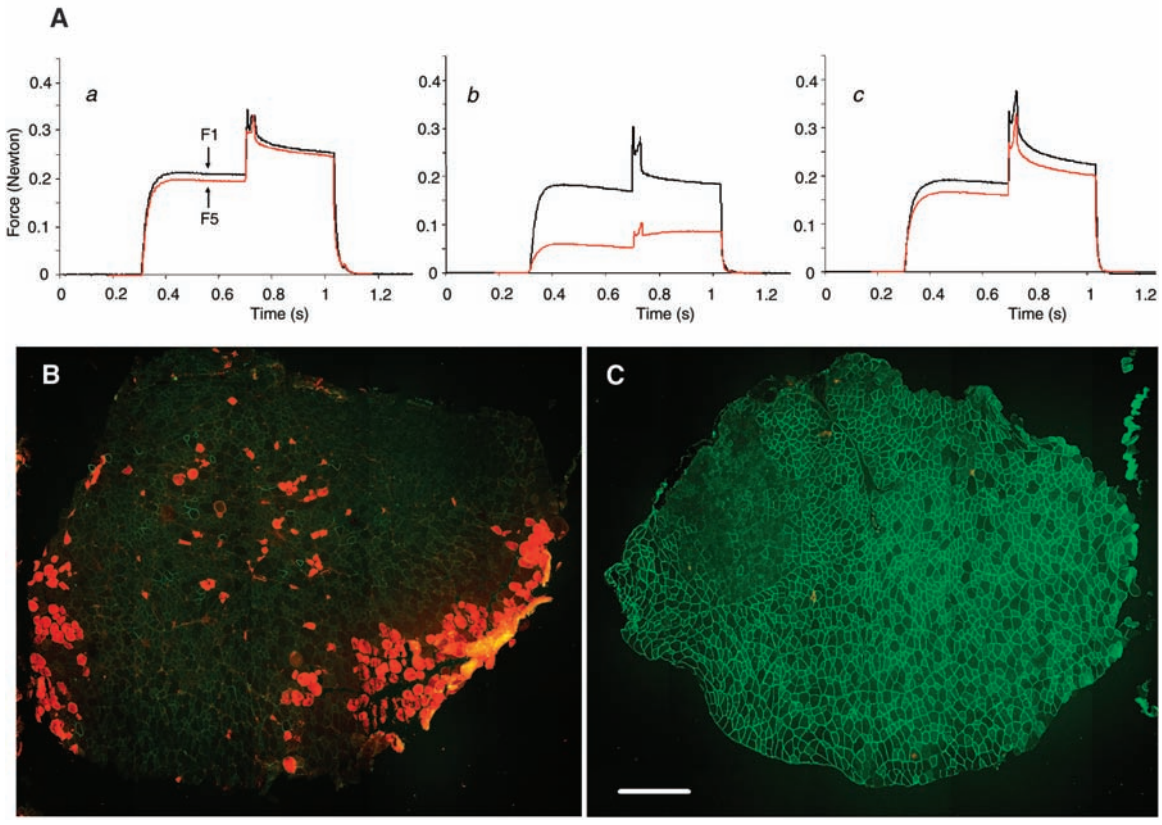

Fig. 5. Dystrophin rescue in AAV(U7-SD23/BP22)–treated *mdx* muscle restores normal susceptibility to exercise-induced damage. (**A**) Superimposed traces of tension produced by EDL muscles during five tetanic contractions with forced lengthening. F_1 is the isometric force developed just before lengthening in the first tetanus, and F_5 that of the fifth one. In the experiment shown, the force drop was 15% for C57BL6 muscle (a), 65% in *mdx* (b), and 17% in 6 week treated *mdx* (c). (**B** and **C**) Double staining of dystrophin and Evans blue detection of exercise-damaged muscle fibers in TA muscles of untreated (B) and treated (C) legs from the same *mdx* animal, 60 days after vector administration. Damaged fibers incorporate Evans blue, whose fluorescence is collected in the red channel, and dystrophin is revealed with NCL-DYS2 (green).

for modifying or inactivating various mRNA targets in the muscle, in comparison with current small interfering RNA tools (*26*).

AAV vectors can be safely and efficiently administered through the vascular route, resulting in the permanent modification of multiple muscle groups (*27*). Our study now defines a pathway for the development of effective therapies based on exon skipping for DMD and other neuromuscular diseases (*28*, *29*). DMD is uniquely suited to therapeutic exon skipping, given the modular and repetitive nature of some dystrophin domains. Among DMD patients registered in our database (Hôpital Cochin), 43% could benefit from skipping of a single exon, and this proportion might be increased if skipping of multiple exons can be accomplished (*4*). In most cases, an attenuated Becker-like phenotype would be obtained, but a fully functional rescued protein can be predicted for selected genotypes.

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