Mutations in *CAV3* cause mechanical hyperirritability of skeletal muscle in rippling muscle disease

Hereditary rippling muscle disease (RMD) is an autosomal dominant human disorder characterized by mechanically triggered contractions of skeletal muscle[1–4]. Genome-wide linkage analysis has identified an RMD locus on chromosome 3p25. We found missense mutations in positional candidate *CAV3* (encoding caveolin 3; ref. 5) in all five families analyzed. Mutations in *CAV3* have also been described in limb-girdle muscular dystrophy type 1C (LGMD1C; refs. 6,7), demonstrating the allelism of dystrophic and non-dystrophic muscle diseases.

In RMD, mechanical stimulation leads to electrically silent muscle contractions that spread to neighboring fibers and cause visible 'ripples' to move over the muscle. To identify the causative gene defect, we pursued a systematic linkage analysis in three previously described German families[2,4]. We found evidence of linkage with *D3S1597*, with a lod score of 4.68 (θ=0.05), which we confirmed by fine mapping with 22 microsatellite markers. We obtained a maximal two-point lod score of 6.95 at θ=0.00 for *D3S3691*. In the largest family, we identified an 11-cM spanning haplotype between markers *D3S1560* and *D3S1597* that segregated in all affected members (Fig. A).

The gene *CAV3* is located within this interval and represented an attractive candidate gene given its involvement in two distinct muscle disorders: LGMD1C (refs. 6,7) and idiopathic elevation of serum creatine kinase[8] (also called 'hyperCKemia').

We derived intronic primers to amplify the two coding exons of *CAV3* from human PAC clone AF176315 (primer sequences and PCR conditions are available on request). By direct sequencing, we discovered *CAV3* mutations in a heterozygous state in all three families (Table 1, CAV3 mutants A45T, R26Q and P104L). We also identified *CAV3* missense mutations in two additional families with RMD, resulting in the CAV3 mutants A45V (family B, ref. 2) and A45T in the first RMD family described[1] (Table 1). All mutations cosegregated with the disease and were not detected in 400 control chromosomes, as shown by allele-specific restriction analyses. Three have been described in different muscle diseases (Table 1), emphasizing their pathogenic nature and the allelism between sporadic hyperCKemia, LGMD1C and RMD.

As in LGMD1C and hyperCKemia[6–8], a muscle biopsy from a patient with RMD carrying the A45T variant showed decreased surface expression of *CAV3* (Fig. 1*a*,*c*). In transiently transfected fibroblasts, the decreased surface expression of the caveodin 3 mutant P104L was due to its retention in the Golgi apparatus[9]. We studied the effect of *CAV3* mutations in the context of differentiating skeletal muscle cells by establishing stably transfected mouse skeletal muscle C2C12 cells. We introduced point mutations into a human *CAV3* cDNA cloned into the mammalian expression vector pCIneo (Promega). We transfected constructs into C2C12 cells using lipofectamine. We cultured proliferating C2C12 cells in 'high-mitogen' medium (DMEM and 10% FBS) and induced them to differentiate in 'low-mitogen' medium (DMEM and 3% horse serum). All RMD mutations resulted in a reduced plasma membrane expression of *CAV3* (Fig. 1*e*,*g*).

As caveolin 3 interacts with neuronal nitric oxide synthase[10,11] (nNOS), we investigated expression of nNOS using immunocytochemistry. We did not find a detectable change in the RMD muscle biopsy (Fig. 1*b*,*d*) or in mutant C2C12 myotubes (Fig. 1*f*,*h*). This is in agreement with data from *Cav3*-null mice and mice transgenic for the mutation producing the P104L mutant (refs. 12,13), but is in contrast with a reduction in nNOS in a biopsy from a patient with LGMD1C (ref. 7). As NOS activity is inhibited by caveolins[10], we measured cytokine-stimulated NOS activity in C2C12 myotubes as described[14]. All mutant cells demonstrated a significant increase of 30–40% in NO production compared with that of cells transfected with wildtype *CAV3* ($P<0.05$, Student's *t*-test; Fig. 1*i*). This is in agreement with an increased nicotinamide dinucleotide phosphate diaphorase activity in the P104L transgenic mouse[13].

The mislocalization of CAV3 and the associated increased inducibility of nNOS may contribute to the mechanically induced muscle contractions in RMD (refs. 11,15). The finding that not only the same gene but also identical mutations cause distinct muscle diseases (RMD, LGMD1C and hyperCKemia) poses a challenge: to identify genes or other factors that 'co-determine' the phenotypical outcome of these disorders.

Note: Supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

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Table 1 • *CAV3* missense mutations in autosomal dominant RMD, LGMD1C and idiopathic hyperCKemia

CAV3 mutant	Phenotype(s)
R26Q	AD-RMD (kindred B, ref. 4) Sporadic hyperCKemia without muscle weakness or overt signs of mechanical hyperirritability in two unrelated children (ref. 8)
A45T	AD-RMD in two unrelated families from Germany and Norway (kindred A, ref. 4; first-described RMD family, ref. 1) Sporadic patient with hyperCKemia, muscle cramps, myalgia and dystrophic muscle biopsy yet without muscle weakness or overt signs of mechanical hyperirritability (ref. 7)
A45V	AD-RMD (family B, ref. 2)
P104L	AD-RMD (family A, ref. 2) AD-LGMD1C (family A, ref. 6), patients presenting with muscle cramping, calf hypertrophy, mild-to-moderate proximal muscle weakness and nonspecific myopathic changes yet no overt signs of mechanical hyperirritability

Left, missense mutations in *CAV3* identified in this study. Right, mutation linked to the corresponding RMD family and an overview of the clinical phenotype for the responsible mutation in previous publications describing patients with LGMD1C and hyperCKemia AD-RMD, autosomal dominant rippling muscle disease.

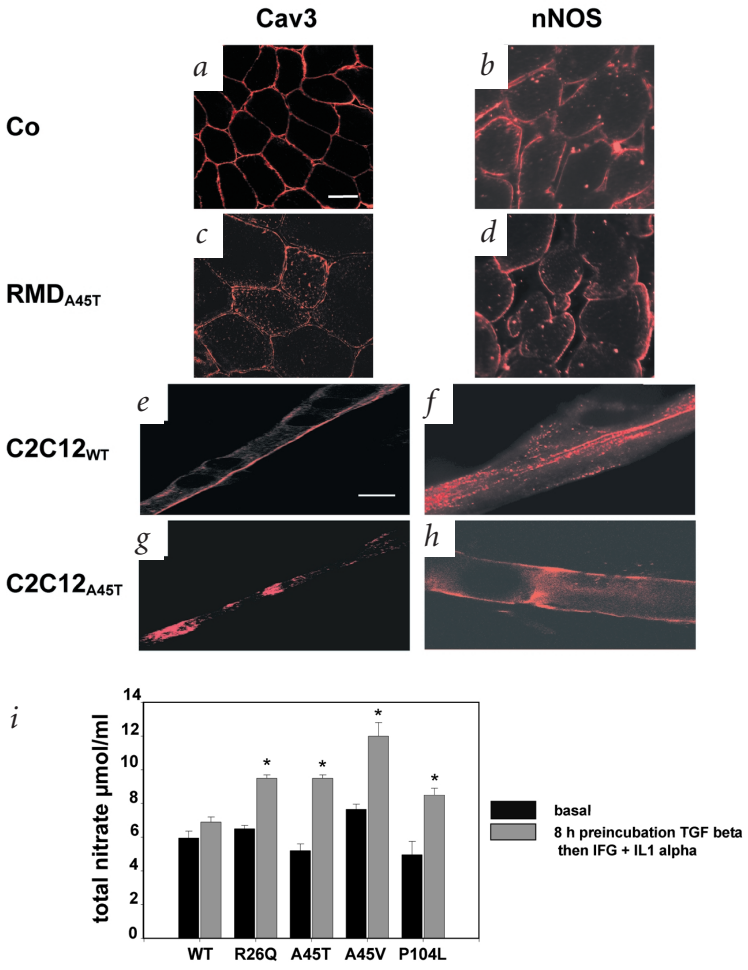

Fig. 1 Expression of caveodin 3 and nNOS, and NO stimulation assay. ***a*–*d***, Skeletal-muscle sections from a control individual (*a*,*b*) and a patient with RMD with caveodin 3 mutant A45T (*c*,*d*). ***e*–*h***, C2C12 myotubes transfected with wildtype or mutated *CAV3*. Left, *CAV3* expression; right, nNOS staining. For immunocytochemistry, we grew cells on glass coverslips and fixed them with paraformaldehyde. We detected binding of the primary antibodies (Transduction Lab) with Cy3-conjugated goat anti-mouse or Cy2-conjugated donkey anti-rabbit IgG (Dianova) using confocal fluorescence microscopy (Leica TCS-NT). caveolin 3 shows homogeneous sarcolemmal staining in the control biopsy (*a*), whereas there is decreased surface expression in RMD (*c*). For nNOS, there is sarcolemmal staining in the control (*b*) and RMD (*d*) biopsies. A multinuclear myotube transfected with wildtype *CAV3* shows strong sarcolemmal staining (*e*), whereas RMD mutations lead to a decreased surface expression of caveolin 3, as shown for A45T (*g*). Staining for nNOS is not altered in the A45T mutant (*h*) compared with that of a cell transfected with wildtype *CAV3* (*f*). Scale bars represent 30 µm (*a*) and 20 µm (e). ***i***, Cytokine-induced total nitrate production assay in stably transfected C2C12 cells. We measured total NO content using a nitrate/nitrite assay, following the manufacturer's instructions (R&D systems). Data were normalized to the protein content determined by the BCA assay (Pierce). After induction, there is a significant increase of 30–40% in nitrate production in mutant cell lines (n=8; P<0.05, Student's t-test). C2C12$_{A45T}$, C2C12 cells stably transfected with mutated *CAV3* producing the CAV3 mutant A45T; C2C12$_{WT}$, C2C12 cells transfected with wildtype *CAV3*; Co, control; IFG, interferon gamma; IL1, interleukin-1; RMD$_{A45T}$, muscle biopsy from a patient with RMD with the CAV3 mutant A45T; TGF, transforming growth factor.

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