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Suppression of revertant fibers in *mdx* mice by expression of a functional dystrophin

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Duchenne muscular dystrophy (DMD) is characterized by progressive muscle degeneration that results from the absence of dystrophin. Despite null mutations in the dystrophin gene, many DMD patients display a low percentage of dystrophin-positive fibers. These 'revertant fibers' are also present in the dystrophin-deficient *mdx* mouse and are believed to result from alternative splicing or second mutation events that bypass the mutation and restore an open reading frame. However, it is unclear what role dystrophin and the dystrophic pathology might play in revertant fiber formation and accumulation. We have analyzed the role of dystrophin expression and the dystrophic pathology in this process by monitoring revertant fibers in transgenic *mdx* mice that express truncated dystrophins. We found that newborn transgenic mice displayed approximately the same number of revertant fibers as newborn *mdx* mice, indicating that expression of a functional dystrophin does not suppress the initiation of revertant fiber formation. Surprisingly, when the transgene encoded a functional dystrophin, revertant fibers were not detected in adult or old *mdx* mice. In contrast, adult transgenic mice expressing a non-functional dystrophin accumulated increasing numbers of revertant fibers, similar to *mdx* mice, suggesting that positive selection is required for the persistence of revertant fibers. Finally, we provide evidence that the loss of revertant dystrophin in transgenic *mdx* muscle fibers overexpressing a functional dystrophin results from displacement of the revertant protein by the transgene-encoded dystrophin.

INTRODUCTION

Duchenne muscular dystrophy (DMD) and the milder Becker muscular dystrophy (BMD) result from mutations within the dystrophin gene (1–3). Dystrophin is a large, 427 kDa multi-domain protein that binds multiple integral and peripheral membrane proteins, known as the dystrophin glycoprotein complex (DGC) (4). The DGC links the actin cytoskeleton to the extracellular matrix in muscle, helping to prevent damage to the sarcolemma in contracting and quiescent muscle fibers (5–8). Patients with dystrophin mutations display progressive muscle degeneration, leading to muscle weakness and eventual respiratory and/or cardiac failure (1).

An interesting phenomenon that occurs in ~50% of DMD patients is the presence of revertant fibers that express dystrophin (9–12). Little is known about the molecular mechanisms leading to revertant fibers, but they comprise only 1–7% of all muscle fibers (9,13,14). This phenomenon is not limited to humans, but has also been observed in the dystrophin deficient *mdx* mouse and the *cxmd* dog animal models (15–17). Revertant fibers are detectable during all stages of development. Newborn and fetal muscles display isolated, single revertant fibers which grow into clusters that increase in size and number with age (18). There appears to be no correlation between the number of revertant fibers and the severity of the disease, as they are not found in sufficient numbers to prevent dystrophy (9).

Closer analysis of individual revertant fibers has revealed the structure of revertant dystrophins present in *mdx* muscles. Immunofluorescent analysis using a panel of antibodies specific to the N- and C-terminal regions of dystrophin show that these domains are expressed in revertant fibers (19). However, antibodies recognizing portions of dystrophin encoded by exons near the *mdx* mutation (a nonsense mutation in exon 23) typically fail to detect the revertant dystrophin. These studies suggest that revertant fibers express dystrophins that arise from alternatively spliced transcripts lacking both the mutant exon and a variable number of adjacent exons (18). Alternatively spliced forms of dystrophin mRNA are detectable by RT–PCR in both dystrophic and wild-type muscle, indicating that such splicing may occur regardless of the muscle phenotype (18–21). It remains unclear what mechanism leads to the stable appearance of revertant dystrophin proteins in only a subset of dystrophic muscle fibers. It is also unclear whether the dystrophic phenotype induces the formation of revertant fibers or simply provides a selective environment to maintain such fibers once they have formed.

We sought to understand the process of dystrophy in the initiation and progression of revertant fibers by asking whether revertant fibers arise in non-dystrophic muscle. This question

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has been difficult to address since antibodies against dystrophin would not distinguish revertant from wild-type dystrophin in muscles from heterozygous (*mdx*/+) female mice. However, we have recently described several lines of transgenic mice on the *mdx* background that express highly functional dystrophins lacking small domains (22–24). These truncated dystrophins lack some epitopes present in full-length and revertant dystrophins, making it possible to distinguish the revertant from the transgene-encoded dystrophins by immunostaining. We found that mice expressing functional dystrophins exhibited normal numbers of revertant fibers in newborn muscle. Surprisingly, almost all revertant fibers disappeared by 2 months of age. We also show that the overexpression of transgene-encoded dystrophin significantly downregulates the endogenous dystrophin levels in wild-type mice, providing a possible explanation for the absence of revertant fibers in adult, transgenic *mdx* muscles.

RESULTS

Revertant fibers in adult transgenic mice

We initially sought to determine whether expression of dystrophin from transgenes might affect the number of revertant fibers found in *mdx* muscle. For this purpose, single muscle sections from a variety of muscle groups were immunostained with dystrophin antisera. The mice analyzed were adult C57Bl/10, *mdx*, and transgenic mice expressing either the Δ71–78, ΔH2–R19 or Dp71 forms of dystrophin (Fig. 1A). One-year-old Δ71–78 mice displayed no revertant fibers in quadriceps, extensor digitorum longus (EDL), tibialis anterior (TA), diaphragm or soleus muscle (Fig. 1B). Similarly, no revertant fibers were detected in the TA or EDL from 2-year-old ΔH2–R19 mice. In contrast, mice expressing the Dp71 isoform of dystrophin displayed large numbers of revertant fibers, similar to the number in *mdx* mice (Fig. 1). The number of revertant fibers observed in each muscle group is tabulated in Table 1. Since we observed so few revertant fibers in the Δ71–78 mice, we chose to examine a larger number of sections from several different muscles of young (2 months) and old (2 years) mice. Analysis of 100 sections from each muscle of the Δ71–78 and ΔH2–R19 mice identified very few revertant fibers, all of which were found in a single muscle from each strain (Table 2). Two isolated revertant fibers were detected in the TA of one 2-year-old Δ71–78 mouse. The ΔH2–R19 mice displayed a single cluster of five revertant fibers in the soleus muscle from a 2-year-old mouse.

Revertant fibers in newborn transgenic mice

Since the 2-month-, 1-year- and 2-year-old Δ71–78 and ΔH2–R19 mice displayed a striking paucity of revertant fibers, we decided to also examine neonatal muscles. Quadriceps muscles from 5-day-old Δ71–78 mice displayed a similar number of revertant fibers as did those from *mdx* mice (Fig. 2; Table 2). These revertant fibers were approximately the same length (~30 µm) and were present as single isolated fibers (not clustered) in both strains. Immunostaining with antisera against the N-terminus of dystrophin confirmed that the Δ71–78 protein was expressed at this age (Fig. 2).

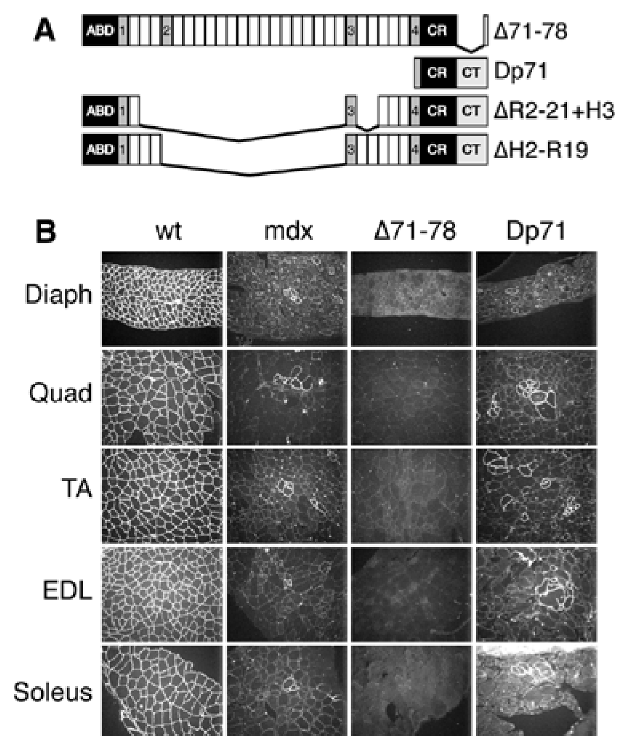

Figure 1. Revertant fibers in various mouse strains. (**A**) Dystrophin cDNA constructs used to generate transgenic lines. Shown are the constructs deleted for either the C-terminal domain of dystrophin (Δ71–78), or different combinations of spectrin-like repeat (R) and hinge (H) regions (ΔR2–21+H3 and ΔH2–R19). Also shown is the construct encoding the Dp71 isoform, which contains only the second half of hinge 4 plus the cysteine-rich (CR) and C-terminal (CT) domains, but lacks the actin-binding domain (ABD) and the central rod domain. The *mdx* mutation is a nonsense codon located in the central rod domain of dystrophin (15). (**B**) Muscle cryosections were prepared from diaphragm, quadriceps, TA, soleus and EDL, and stained with dystrophin antisera. Immunofluorescence of WT, mdx and Δ71–78 muscle was performed with antisera against the dystrophin CT domain. Muscle sections expressing the dystrophin Dp71 isoform were stained with antisera against the dystrophin actin-binding domain. Scale bar is 50 µm.

Absence of revertant fibers is not likely due to mRNA downregulation

To determine whether the reduced number of revertant fibers in adult transgenic mice was due to a downregulation of the endogenous dystrophin transcript, we performed RT–PCR. Amplification of the dystrophin mRNA segment encoded by exons 74–76 generates a product from the endogenous, but not from the Δ71–78 transcript. Semi-quantitative RT–PCR was performed in C57Bl/10, *mdx* and Δ71–78 mice by amplifying this exon 74–76 fragment in parallel with a fragment from the hypoxanthine phosphoribosyltransferase (HPRT) gene transcript. When compared with HPRT controls, the dystrophin mRNA levels were approximately equal in muscle RNA isolated from Δ71–78 and *mdx* mice (Fig. 3). Dystrophin mRNA levels were higher in C57Bl/10 muscle than in *mdx* muscles as reported previously (25).

Table 1. The number of revertant fibers observed in a single muscle cross section from the indicated muscles

Line[a]	Age (months)	Quadriceps	Diaphragm	TA[b]	EDL[c]	Soleus
mdx	12	30	16	86	3	11
Δ71–78	12	0	0	0	0	0
Dp71	15	88	5	101	11	7

ND, not determined.
[a]Data were obtained from at least two animals of each strain.
[b]TA, tibialis anterior.
[c]EDL, extensor digitorum longus.

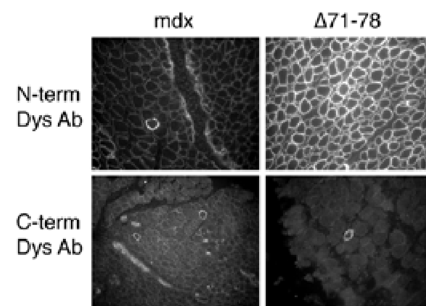

Figure 2. Immunofluorescence analysis of newborn mouse muscle. Five-day-old mdx and Δ71–78 muscle sections were stained with the N- and C-terminal dystrophin antisera. In Δ71–78 muscle, the N-terminal dystrophin antisera detects the transgenic and revertant protein, whereas the C-terminal antisera detects only revertant fibers.

Endogenous dystrophin levels are downregulated by overexpression of exogenous dystrophin

We next asked whether the transgene-encoded dystrophin could affect the accumulation of endogenous wild-type C57Bl/10 or revertant (*mdx*) dystrophin. For these studies, we initially examined two different transgenic mouse lines that express the Δ71–78 transcript at different levels on a wild-type (C57Bl/10) background (Fig. 4). Line 1 expresses less dystrophin Δ71–78 protein than Line 2, as detected with the N-terminal dystrophin antibody. Muscles with the highest expression of dystrophin Δ71–78 protein accumulated the least amount of endogenous dystrophin, as detected with the C-terminal dystrophin antibody. A similar downregulation of the endogenous protein was also seen in a transgenic line that expresses high levels of a functional, truncated dystrophin (ΔR2–21+H3). Furthermore, we have observed a similar downregulation of endogenous dystrophin in at least three other transgenic mouse lines (unpublished data). This striking downregulation of endogenous dystrophin on a wild-type background suggests that low levels of revertant dystrophin in *mdx* mice might be similarly suppressed by expression of stable and functional transgenic dystrophins.

DISCUSSION

The precise mechanism by which revertant fibers express dystrophin is unknown, but appears to involve exon skipping around mutations such that an open reading frame is restored (18–21). It is unclear what role dystrophin and the dystrophic pathology play in revertant fiber formation. We had the opportunity to study revertant fibers on a non-dystrophic background, and found evidence that a lack of functional dystrophin is required for the maintenance, but not the formation of revertant fibers.

Revertant fibers have previously been identified in newborn *mdx* mice before any signs of dystrophy were apparent (9,18). This observation suggested that dystrophic pathology is not required for the initial events leading to the formation of revertant fibers. We showed that overexpression of a highly functional dystrophin transgene does not prevent revertant fibers from forming in newborn mice. Therefore, the events leading to the initial formation of revertant fibers in *mdx* mice appear

Table 2. The number of revertant fibers observed in 100 serial muscle sections from the indicated muscles

Line[a]	Age	Quadriceps	Diaphragm	TA	EDL	Soleus
mdx	5 days	5	ND	ND	ND	ND
Δ71–78	5 days	3	ND	ND	ND	ND
Δ71–78	2 months	0	0	0	0	0
Δ71–78	2 years	0	0	2	0	0
ΔH2–R19	2 months	ND	ND	0	0	0
ΔH2–R19	2 years	ND	ND	0	0	5

ND, not determined.
[a]Data were obtained from at least two animals at each time point, except ΔH2–R19, which was from one animal at each time point.
[b]TA, tibialis anterior.
[c]EDL, extensor digitorum longus.

to be independent of a dystrophic phenotype and the presence of dystrophin.

Revertant fibers increase in size, number and length with age in both humans and the *mdx* mouse (18). Surprisingly, we found that in adult Δ71–78 mice, revertant fibers were almost entirely absent. This lack of revertant fibers indicates the importance of the dystrophic phenotype in growth and expansion of revertant fibers. Mice expressing the Dp71 isoform of dystrophin have the same number of, if not more, revertant fibers than do *mdx* mice. Since Dp71 is not able to prevent dystrophy in *mdx* mice (26), this result suggests that simple expression of an exogenous dystrophin protein is not sufficient to prevent the accumulation of revertant fibers. Instead, the exogenous dystrophin must be functional to suppress an increase in the number of revertant fibers. We have previously noted that Dp71 mice appear more dystrophic than *mdx* mice (26), suggesting that the severity of dystrophy may correlate with the number of revertant fibers present. We found it intriguing that the TA muscle was the only muscle group from the Δ71–78 transgenic mouse in which any revertant fibers were detected (Table 2). This muscle displays a partially

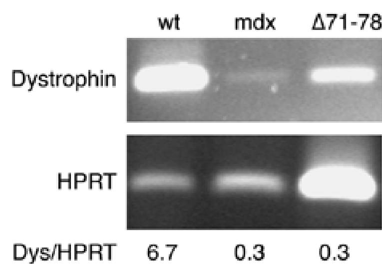

Figure 3. Dystrophin mRNA levels in various strains of mice. RT–PCR was performed on total RNA isolated from wild-type, *mdx* or Δ71–78/*mdx* muscles using primers specific for endogenous dystrophin and HPRT. Levels of the dystrophin product were estimated relative to the HPRT product by densitometry. The ratio of dystrophin to HPRT is listed below each lane. The ratios in *mdx* and Δ71–78/*mdx* mice were the same.

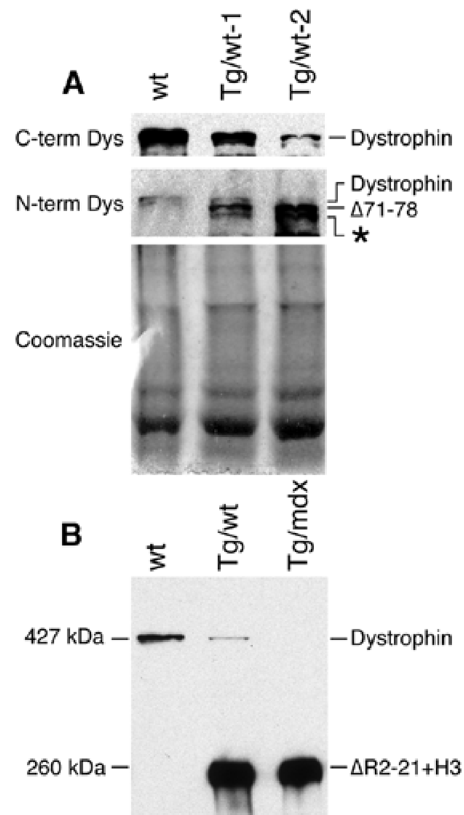

Figure 4. Western analysis of protein from different transgenic lines on the wild-type (Tg/wt) or *mdx* background (Tg/*mdx*). (**A**) Forty micrograms of protein from wild-type and two lines of Δ71–78 mice (Tg/wt-1 and Tg/wt-2) expressing different levels of dystrophin were analyzed by western blot. The N-terminal dystrophin antisera detects higher levels of dystrophin Δ71–78 protein in Tg/wt-2 than in Tg/wt-1. Dystrophin is detected as a doublet when probed with N-terminal antibodies. Note that the smaller Δ71–78 protein also migrates as a doublet (asterisk). The C-terminal dystrophin antibody detects less endogenous full-length dystrophin in Tg/wt-2 than Tg/wt-1. (**B**) Western analysis of protein isolated from transgenic mice expressing the ΔR2–21+H3 construct on the wild-type (Tg/wt) and *mdx* backgrounds (Tg/*mdx*) using monoclonal antibody Dys2. Expression of the ΔR2–21+H3 transgene resulted in downregulation of the endogenous full-length dystrophin.

mosaic expression of the transgene and significantly higher levels of centrally nucleated myofibers, indicating an increased rate of regeneration (22). These data suggest that ongoing regeneration may be required to maintain revertant fibers in adult mice, and support the idea that positive selection is necessary for persistence and expansion of revertant fibers. When normal dystrophin is absent, as in *mdx* mice, the revertant dystrophin may persist, mechanically protecting the revertant muscle fiber from injury.

Expression of a functional dystrophin not only prevented an increase in the number and size of revertant clusters, but also led to a dramatic decrease in detectable revertant dystrophin in adult mice. Semi-quantitative analysis of endogenous dystrophin mRNA and protein levels in transgenic/*mdx* and transgenic/wild-type mice suggested that functional transgenic dystrophin can actively downregulate endogenous dystrophin protein, but not mRNA. Although the mechanism of down-regulation is unclear, there are several possibilities. First, the transgenic dystrophin may saturate the sarcolemmal membrane, displacing or out-competing the endogenous normal and revertant dystrophin. This competition may be less pronounced in newborn mice since neonatal muscles are undergoing a rapid phase of growth and myocyte fusion that is producing a large surface area of new sarcolemma that has not yet become saturated with dystrophin. There is also the possibility that transgenic Δ71–78 and ΔH2–R19 protein is more functional or stable than smaller, internally truncated, revertant dystrophins. Therefore, these revertant proteins might be unable to compete with the highly functional transgenic proteins. Indeed, the precise structure of different mini- and micro-dystrophins has an enormous impact on the relative functional capacity of the truncated proteins (23,27; unpublished data).

Our data suggest that multiple events influence revertant fiber formation. The initial appearance of revertant fibers results from events early in development that are independent of dystrophin or the dystrophic pathology. The apparent clonal origin of revertant fiber clusters (18) suggests that the initiating events occur before muscle precursor cells differentiate into myocytes. The dystrophin gene is not transcribed in muscle precursor cells, so it is not surprising that dystrophin expression has no influence on initial revertant fiber appearance. In contrast, maintenance and expansion of revertant fibers occurs only in the absence of a functional dystrophin. We suggest that revertant fibers are not maintained in adult transgenic mice due to a competitive advantage of the transgenic versus the revertant dystrophin. Dp71 lacks both the rod and actin-binding domains, and may not effectively compete with nearly full-length 'revertant' dystrophin. However, since the ΔH2–R19 protein lacks the rod domain but is able to suppress reversion, effective competition between exogenous and endogenous dystrophins appears to require the N-terminal actin-binding domain. Alternatively, the failure to detect expanded clusters of revertant fibers in old transgenic/*mdx* mice might be due to a lack of ongoing necrosis and regeneration in these protected muscles, which also eliminates any selective advantage for revertant fibers.

The natural occurrence of revertant fibers in DMD patients and animal models for the disease has potential implications

for therapy (16). Although individual revertant fibers appear to be protected from dystrophic degeneration, they are not present in sufficient numbers to be clinically significant. Developing a better understanding of the events underlying the initiation of revertant fiber formation could facilitate the development of therapeutic strategies aimed at increasing endogenous dystrophin expression in the muscles of DMD patients (28–30).

MATERIALS AND METHODS

Transgenic mice and immunofluorescence

Mice expressing dystrophin deleted for exons 71–78 (Δ71–78) or for hinge 2 to spectrin-like repeat 19 (ΔH2–R19), or for spectrin-like repeats 2–21 [but including hinge 3 (ΔR2–21+H3)] were generated as described by Crawford *et al.* (22). Unless explicitly stated otherwise, all transgenic strains were carried on the *mdx* dystrophin deficient background. To analyze revertant fibers, muscles were isolated and frozen in liquid nitrogen cooled OCT (Tissue Tek). Serial sections (50–100), 10 µm thick, were analyzed for each mouse strain. Tissue sections from the Δ71–78 mice were stained with a rabbit antibody specific for the C-terminal domain of dystrophin (26). This antibody does not recognize the Δ71–78 dystrophin protein (Figs 1 and 2). Dp71 mice express only the Dp71 isoform of dystrophin, encoded by exons 63–79, and are dystrophic (26). To identify revertant fibers in Dp71 mice, a rabbit antibody specific for the dystrophin N-terminal domain was used (31). After incubation with antiserum for 2 h, sections were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies. Images were collected on a Nikon E1000 microscope using fixed exposure times. Muscles from ΔH2–R19 mice were incubated with a monoclonal antibody specific for the rod domain. Muscle fibers that were dystrophin positive were scored as revertant. Every fiber within each muscle cross section was scored for the presence of revertant dystrophin, and similar numbers of fibers were counted from each test group. Revertant fiber counts were performed as a blind study and the mean average number of revertant fibers per serial section was determined. Individual revertant fibers were followed through multiple sections to determine their approximate length.

RT–PCR

To estimate the relative ratios of endogenous dystrophin from wild-type, *mdx* and Δ71–78 (on the *mdx* background) transcripts, RT–PCR was performed as described previously (32). Briefly, 1 µg of total RNA from 1-year-old mice was used in a reverse transcription reaction, using either an exon 77-specific reverse primer (5′-GGGAAGGAGTTGTTGAGTTGCTC-3′) for dystrophin or an oligo(dT) primer for HPRT. PCRs used either HPRT primers (5′-GCTGGTGAAAAGGACCTCT-3′ and 5′-CACAGGACTAGAACACCTGC-3′) or dystrophin primers specific for exons 74–76 (5′-GAGAATCCTAGCA-GATCTTGAGG-3′ and 5′-GAAGTTTGACTGCCAACCAC-3′). Note that these latter primers do not amplify the Δ71–78 transcript. Products were separated after 40 cycles of PCR by electrophoresis on 1% agarose gels and the approximate levels of the dystrophin products were determined relative to the HPRT products by densitometry using Molecular Analyst software (Bio-Rad).

Western analysis

Protein was extracted from mouse quadriceps as described previously (33). Briefly, tissue was ground to a fine powder in a liquid nitrogen cooled mortar and pestle. Frozen tissue was boiled and vortexed in homogenate solution (1% SDS, 5 mM EGTA) plus protease inhibitor Complete (Boehringer). Protein concentrations were determined by Bradford assay and confirmed by Coomassie blue staining of SDS–PAGE separated samples. Forty micrograms of protein was separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in TBST (10 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.05% Tween 20) and incubated with primary Dys1 or Dys2 monoclonal antibodies (Novacastra) for 3 h at room temperature. After three washes in TBST, the membranes were incubated for an additional 2 h in secondary goat anti-mouse antibody conjugated to horseradish peroxidase (HRP; Jackson Labs) and washed again three times in TBST. Immunoreactive proteins were visualized using an ECL kit (Amersham).

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