Mutation of the IIB myosin heavy chain gene results in muscle fiber loss and compensatory hypertrophy

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Allen, David L., Brooke C. Harrison, Carol Sartorius, William C. Byrnes, and Leslie A. Leinwand. Mutation of the IIB myosin heavy chain gene results in muscle fiber loss and compensatory hypertrophy. Am J Physiol Cell Physiol 280: C637–C645, 2001.—The fast skeletal Iib gene is the source of most myosin heavy chain (MyHC) in adult mouse skeletal muscle. We have examined the effects of a null mutation in the Iib MyHC gene on the growth and morphology of mouse skeletal muscle. Loss in muscle mass of several head and hindlimb muscles correlated with amounts of Iib MyHC expressed in that muscle in wild types. Decreased mass was accompanied by decreases in mean fiber number, and immunological and ultrastructural studies revealed fiber pathology. However, mean cross-sectional area was increased in all fiber types, suggesting compensatory hypertrophy. Loss of muscle and body mass was not attributable to impaired chewing, and decreased food intake as a softer diet did not prevent the decrease in body mass. Thus loss of the major MyHC isoform produces fiber loss and fiber pathology reminiscent of muscle disease.

Myosin; muscle development; locomotion; motor behavior; dystrophy

Myosin heavy chain (MyHC) is both structurally and functionally one of the most important proteins expressed in skeletal muscle. Three adult fast isoforms of MyHC, along with type I or β-MHC, are expressed in different types of fibers that are named for the type of MyHC they express, i.e., IIB, IID, IIA, and type I (13). Fibers expressing these different isoforms of MyHC have been shown to have distinct contractile properties, sizes, and metabolic profiles (2, 3, 14). These studies have suggested that the different isoforms of MyHC are functionally distinct, despite the extremely high amino acid identity among the three adult fast genes (~93–94%; see Ref. 15). In adult murine skeletal muscle three fast isoforms, IIa, IID, and IIB, are expressed in varying percentages in different muscles. Type IIB MyHC, which accounts for ~70–80% of the total MyHC, is the predominant isoform expressed in adult mouse skeletal muscle (6). Previously, we reported the generation of mice containing a null mutation in either the IID or IIB MyHC gene (1). These mice are viable but demonstrate a number of morphological and physiological alterations, many of which are distinct between the two strains, despite the fact that total MyHC content was unchanged in either the IIB or IID null mice (1). Analysis of mice lacking expression of the IId MyHC gene revealed that the IId MyHC isoform is upregulated to compensate for the loss of IId MyHC, resulting in normal total MyHC content (12). Despite this upregulation of IId MyHC expression, IId null mice have considerable muscle pathology and spinal kyphosis (11). These studies have provided a convincing argument in favor of the hypothesis that different MyHC isoforms have unique roles in the growth and morphogenesis of skeletal muscle.

In the present study, we have analyzed mice in which the IIB MyHC isoform gene has been rendered null by homologous recombination to determine the morphological consequences of the loss of this gene. We demonstrate that mean body mass was significantly lower in mice lacking the IIB MyHC gene but that this was not a consequence of inability to chew hard rodent chow, because feeding Iib null mice a softer diet did not abrogate the loss in mean body mass over time. We also demonstrate that loss of the Iib MyHC gene results in a significant decrease in mean total fiber number in the gastrocnemius, tibialis anterior (TA), extensor digitorum longus (EDL), plantaris, and vastus muscles and a corresponding hypertrophy of the remaining fibers in an attempt to compensate for this fiber loss. Finally, we observed evidence of substantial fiber pathology, including degeneration and regeneration, in certain muscles of the hindlimb of Iib null mice. Together these data support a role for Iib MyHC in the maintenance of normal muscle growth and structure.

MATERIALS AND METHODS

Animals. The generation of Iib MyHC null mice has been described previously (1). Ten male wild-type mice and ten male mice homozygous for a null mutation in the Iib MyHC gene were weighed and killed at 6 wk of age by cervical dislocation. Muscles of the hindlimb (TA, EDL, gastroc-
micius, soleus, plantaris, quadriceps) and head (tongue, masseter) were dissected, weighed, and frozen in isopentane cooled in liquid nitrogen. Muscles were stored at −70°C until use.

Fiber percentages, fiber size, and fiber number. For immunohistochemical analysis, 10-μm sections were cut from the muscle midbelly using a cryomicrotome, placed on gelatin-coated slides, and stored at −70°C until use. Slides were air-dried for 30 min at room temperature followed by blocking for 1 h at room temperature in PBS containing 0.12% BSA, 0.1% nonfat dry milk, and 5 mg/ml purified Fab fragments (Jackson Immunoresearch, West Grove, PA). After several rinses with PBS, sections were incubated either at room temperature for 1 h or overnight at 4°C in primary antibody solution. The primary antibodies used were as follows and at the following dilutions: 1) MHCs, which recognizes type I/β MyHC (Novocastra, Newcastle-on-Tyne, UK), 1:20; 2) SC-71, which recognizes type IIa MyHC (5) 1:3; 3) BF-F3, which recognizes type IIb MyHC (5), 1:3; and 4) BF-35, which recognizes all but type IId MyHC, and thus pure IIb-expressing fibers are unstained (5). After primary antibody incubation, the sections were rinsed several times with PBS and then incubated for 1 h at room temperature in secondary antibody solution consisting of PBS plus secondary antibody diluted 1:100. The secondary antibody was either goat anti-mouse IgG-peroxidase conjugate (for MHCs, SC-71, and BF-35) or goat anti-mouse IgM-peroxidase conjugate (for BF-F3 and RT-D9), both from Jackson Immunoresearch. Sections were rinsed several times in PBS and visualized using a DAB reaction kit with nickel enhancement (Vector Laboratories, Burlingame, CA), dehydrated by serial washes with ethanol, and mounted in Permunt (Fisher Scientific, Pittsburgh, PA).

Fiber type percentages were assessed using immunohistochemically stained cross sections. For each MyHC isoform, a total of 5–10 regions/muscle were randomly chosen, and the number of positively stained vs. unstained fibers was counted for each region. Fiber cross-sectional area (CSA) was determined using a video camera (VideoScope International) mounted on a Zeiss bright-field microscope attached to a PowerBase 200 computer. A computer mouse was used to trace the outline of positively immunostained muscle fibers using NIH Image software. The analysis software was calibrated by outlining defined areas on a slide micrometer. A total of 50 fibers of each type per muscle was analyzed from 5–10 randomly chosen areas in each muscle; previous studies have demonstrated that this number is sufficient to establish stable fiber size means within a given population of fibers (9). For type IId fibers, the area of fibers not positively stained for the BF-35 antibody, which recognizes all MyHC isoforms except IId, was analyzed.

Total muscle fiber number was assessed as previously described (12). Briefly, total fiber number was calculated using the formula total fiber number = (muscle CSA − interfiber space)/mean fiber CSA. Muscle CSA was determined by circumscribing the entire muscle section using NIH Image software; interfiber space was determined by measuring fiber area for all muscle fibers within five different regions and then subtracting this value from the total screen area; mean fiber CSA was determined by the formula (percent type I/100 × mean I fiber CSA) + (percent type IIα/100 × mean IIα fiber CSA) + (percent IIb/100 × mean IIb fiber CSA) + (percent IId/100 × mean IId fiber CSA). In addition, total fiber number was determined for wild-type and IId null TA muscles (n = 3/group) by creating a photomosaic of the entire muscle cross section and counting each fiber by hand.

Histology. Muscle histology was assessed in the gastrocnemius muscle using immunohistochemical markers for muscle regeneration. Frozen sections were air-dried for 30 min and then rinsed in PBS for 5 min and stained with one of the following antibodies: 1) F1.652, which recognizes embryonic MyHC and 2) MCA-VIM, which recognizes the intermediate filament protein vimentin (Sigma Chemical, St. Louis, MO). After several rinses with PBS, sections were stained with the appropriate secondary antibody conjugated to peroxidase and visualized using either a DAB or an AEC reaction kit (Vector Laboratories).

Electron microscopy. From the histological studies mentioned above, it was evident that the gastrocnemius muscle demonstrated considerable histopathology. We therefore chose the gastrocnemius for ultrastructural analysis. Samples of gastrocnemius from wild-type and IId null mice (n = 2/group) were processed for electron microscopy following standard protocols. Small pieces from the superficial and deep portions of freshly isolated gastrocnemius muscles were placed in 2% glutaraldehyde for 2 h and then embedded and stained using standard electron microscopy protocols. All samples were processed by the Electron Microscopy Core of the University of Colorado.

Analysis of feeding behavior. Feeding behavior was studied in age-matched wild-type and IId null mice. Animals were divided into the following five different groups (n = 4/group): wild-type mice fed a standard hard rodent chow, wild-type mice fed a commercially available soft pet food, IId null mice fed hard rodent chow, IId null mice fed a powdered form of the standard hard rodent chow, and IId null mice fed soft pet food. Each day for 2 wk each animal was weighed. In a separate experiment, the amount of food consumed per 24 h was estimated by weighing the food each day for 1 wk.

Statistical analyses. All results are reported as means ± SD in Tables 1-3 and as means ± SE in Figs. 1–3. Statistical significance was assessed using one-way ANOVA combined with Fisher’s protected least significant difference post hoc test to compare wild-type with IId null parameters. P < 0.05 was used to indicate statistical significance.

RESULTS

Fiber percentages. Given that >70% of the skeletal MyHC in the mouse is IId, we determined the percentage of fibers expressing the different MyHC isoforms in these null mice. Staining with the BF-35 antibody, which recognizes all sarcomeric isoforms except IId, revealed an increase in the number of BF-35-negative fibers. In all muscles except the soleus and the vastus intermedius, the percentage of these BF-35-negative fibers, which we consider to be IID fibers, was significantly increased (Table 1). In the gastrocnemius muscle, the percentage of IID fibers increased from 11 to 82% in the IId null mice (Table 1). In addition, the percentage of fibers expressing IIa MyHC was also increased in most analyzed muscles, but to a lesser extent (Table 1). In the gastrocnemius muscle, the percentage of MyHC IIa-expressing fibers increased from 6 to 16%; similar increases in the percentage of IIa-expressing fibers occurred in most muscles of the hindlimb (Table 1). An even smaller increase of ~5% occurred in the percentage of fibers expressing type I MyHC in the hindlimb muscles of IId null mice (Table 1). Thus an increase in the number of IID fibers is the

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major adaptation to the loss of IIb MyHC gene expression.

**Mean body and muscle mass.** As previously described (1), mean absolute body mass was significantly smaller in age-matched IIb null mice compared with wild-type mice (Fig. 1A). In addition, the absolute masses of numerous muscles of IIb null mice were also significantly smaller than those of wild-type mice (Fig. 1A). The magnitude of this decrease appeared to depend on the amount of IIb MyHC normally expressed in a given muscle with the wild-type mouse. For example, muscles that contained a high percentage of type IIb MyHC such as the soleus or quadriceps experienced the greatest decrement in absolute muscle mass (up to 83%). Muscles containing a slightly smaller percentage of type IIb MyHC, such as TA and EDL demonstrated a smaller decrement in absolute muscle mass (~50–60%). Mean muscle mass for muscles that in the wild type express very low percentages of type IIb MyHC such as the soleus were not significantly different from the wild type (*P > 0.05; Fig. 1A). Examination of relative muscle mass revealed that only muscles containing a high percentage of IIb MyHC in the wild-type mouse, the TA, gastrocnemius, and quadriceps, had significantly lower relative mass compared with the wild type (Fig. 1B). Thus the elimination of IIb MyHC expression results in atrophy to specific muscles in the mouse hindlimb.

**Normal feeding and locomotor behavior of IIb null mice.** To examine the possibility that impairment in masticatory muscle function caused a reduction in food consumption, we examined the effect of feeding a softer food on the increase in body mass postweaning. Both wild-type and IIb null mice fed the soft chow increased in body mass to a greater extent than mice fed the standard hard rodent chow (Fig. 2A). However, IIb null mice fed soft food, powdered rodent chow, or rodent chow pellets all gained weight to a lesser extent than wild-type mice under all conditions (Fig. 2A). We also quantified the amount of food eaten per 24 h and found no significant difference between wild-type and IIb null mice (3.54 and 3.86 g·mouse⁻¹·day⁻¹, respectively; Fig. 2B). When food intake was normalized to body weight, IIb null mice actually consumed more food per day compared with the wild type (Fig. 2A) on standard chow. However, IIb null mice consumed less food compared with wild-type mice when fed the powdered rodent chow (Fig. 2B). Thus, mice fed the powdered rodent chow gained less weight than wild-type mice. The increase in body mass, IIb null mice actually consumed more food per

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**Table 1. Muscle fiber type percentages in adult wild-type and MyHC IIb null mice**

<table>
<thead>
<tr>
<th>MyHC</th>
<th>Tibialis Anterior</th>
<th>Extensor Digitorum Longus</th>
<th>Gastrocnemius</th>
<th>Plantaris</th>
<th>Soleus</th>
<th>Superficial Vastus</th>
<th>Vastus Intermedius</th>
<th>Rectus Femoris</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>I 3 ± 1</td>
<td>IIa 9 ± 2</td>
<td>IIB 67 ± 5</td>
<td>I 3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>IIb Null</td>
<td>9 ± 2</td>
<td>17 ± 3</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>IIb Null</td>
<td>IIb Null</td>
<td>IIb Null</td>
</tr>
<tr>
<td></td>
<td>9 ± 2</td>
<td>14 ± 3</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>IIb Null</td>
<td>IIb Null</td>
</tr>
<tr>
<td></td>
<td>15 ± 4</td>
<td>23 ± 4</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>&lt;1</td>
<td>14 ± 3</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>44 ± 4</td>
<td>51 ± 9</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
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</tr>
<tr>
<td></td>
<td>41 ± 3</td>
<td>58 ± 5</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>&lt;1</td>
<td>10 ± 3</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>24 ± 3</td>
<td>57 ± 5</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
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</tr>
<tr>
<td></td>
<td>32 ± 3</td>
<td>48 ± 6</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>&lt;1</td>
<td>7 ± 2</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>3 ± 1</td>
<td>10 ± 3</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>10 ± 4</td>
<td>16 ± 2</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
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<tr>
<td></td>
<td>89 ± 8†</td>
<td>20 ± 3</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>18 ± 2</td>
<td>87 ± 7†</td>
<td>67 ± 5</td>
<td>3 ± 1</td>
<td>&lt;1</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 4 mice/genotype. MyHC, myosin heavy chain; WT, wild type; IIb Null, myosin heavy chain IIb null. Less than 1% of all analyzed fibers were positive for alpha, embryonic, and perinatal MyHC in both wild-type and MyHC-IIb null mice. *P < 0.05 and †P < 0.001.

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**Fig. 1. A:** muscle mass in adult wild-type and IIb null mice. Absolute muscle mass is significantly decreased in various head and hindlimb muscles in IIb null mice compared with wild-type mice. Bars represent mean IIb null muscle mass expressed as a percentage of mean wild-type muscle mass. *Significantly different from wild type, *P < 0.05. Muscles are arranged from top to bottom in order of increasing amounts of IIb myosin heavy chain (MyHC) in the wild-type mouse as determined in Table 1. EDL, extensor digitorum longus; TA, tibialis anterior. **B:** relative muscle mass (mg muscle/g body mass) in IIb null mice is significantly decreased compared with wild-type mice in the TA, quadriceps, and gastrocnemius. Bars represent means ± SE. **Significantly different from wild type, *P < 0.001.**

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**Fig. 2. A:** body mass and muscle mass. As previously described (1), mean absolute body mass was significantly smaller in age-matched IIb null mice compared with wild-type mice (Fig. 1A). In addition, the absolute masses of numerous muscles of IIb null mice were also significantly smaller than those of wild-type mice (Fig. 1A). The magnitude of this decrease appeared to depend on the amount of IIb MyHC normally expressed in a given muscle with the wild-type mouse. For example, muscles that contained a high percentage of type IIb MyHC such as the soleus or quadriceps experienced the greatest decrement in absolute muscle mass (up to 83%). Muscles containing a slightly smaller percentage of type IIb MyHC, such as TA and EDL demonstrated a smaller decrement in absolute muscle mass (~50–60%). Mean muscle mass for muscles that in the wild type express very low percentages of type IIb MyHC such as the soleus were not significantly different from the wild type (*P > 0.05; Fig. 1A). Examination of relative muscle mass revealed that only muscles containing a high percentage of IIb MyHC in the wild-type mouse, the TA, gastrocnemius, and quadriceps, had significantly lower relative mass compared with the wild type (Fig. 1B). Thus the elimination of IIb MyHC expression results in atrophy to specific muscles in the mouse hindlimb.

**Normal feeding and locomotor behavior of IIb null mice.** To examine the possibility that impairment in masticatory muscle function caused a reduction in food consumption, we examined the effect of feeding a softer food on the increase in body mass postweaning. Both wild-type and IIb null mice fed the soft chow increased in body mass to a greater extent than mice fed the standard hard rodent chow (Fig. 2A). However, IIb null mice fed soft food, powdered rodent chow, or rodent chow pellets all gained weight to a lesser extent than wild-type mice under all conditions (Fig. 2A). We also quantified the amount of food eaten per 24 h and found no significant difference between wild-type and IIb null mice (3.54 and 3.86 g·mouse⁻¹·day⁻¹, respectively; Fig. 2B). When food intake was normalized to body weight, IIb null mice actually consumed more food per
body mass (0.159 vs. 0.239 g·mouse⁻¹·day⁻¹·g body mass⁻¹ for wild-type and IIb null mice, respectively).

In addition, we observed the locomotor behavior of IIb null mice to determine whether the elimination of the IIb MyHC gene resulted in any abnormalities in gait or motor behavior. We did not see any discernible difference in locomotor behavior between wild-type and IIb null mice. The pattern of gait and posture was unchanged, and there were not any overt differences in the quantity of cage locomotion (data not shown). Thus elimination of the IIb MyHC gene does not result in any pathological alterations to normal standing or walking in IIb null mice compared with the wild type, which might explain the specific atrophy of the hindlimb muscles.

**Fiber CSA is increased in the IIb null mice.** Given that muscle mass was significantly lower in IIb null mice compared with wild type mice, one logical hypothesis is that fiber CSA was reduced in the IIb null mice. However, mean fiber CSA was significantly larger in all fiber types in the IIb null mice (Table 2). For example, in the gastrocnemius muscle, the type I, IIA, and IID fibers in the IIb null mice were 20, 56, and 75% larger than the same fiber types in the wild type, respectively (Table 2). CSA of IID fibers of IIb null mice was larger than the same fiber types in the wild type, and IID fibers in the IIb null mice were 20, 56, and 75% larger than the same fiber types in the wild type, and IID fibers in the IIb null mice were 20, 56, and 75% larger than the same fiber types in the wild type, respectively (Table 2). CSA of IID fibers of IIb null mice was comparable to that of IIb MyHC-expressing fibers assuming that fiber CSA increased in the IIb null mice and wild-type mice, respectively. This was also true for the EDL, TA, plantaris, and quadriceps muscles (Table 2).

**Fiber number is decreased in IIb null mice.** To examine whether a decrease in fiber number was responsible for the decrease in mean muscle mass in the IIb null mice, estimates of total fiber number were made based on values for muscle CSA, interfiber space, mean fiber size, and mean fiber percentages. Total fiber number was significantly lower in all hindlimb muscles in the IIb null mice except the soleus and vastus intermedius muscles (Table 3). The percentage decrease in total estimated fiber number for the gastrocnemius muscle was extremely similar to the percentage decrease in mean muscle mass (66 vs. 64%, respectively), suggesting that the loss in fiber number could account for most of the loss in mean muscle mass. Moreover, direct counts of the number of fiber profiles in an entire cross section through the muscle midbelly of the TA also revealed a significant decrease in total fiber number (2,597 ± 135 vs. 1,919 ± 194 in the wild type and IIb null mice, respectively). Thus the decrease in mean muscle mass appears to be a consequence of a decrease in fiber number rather than fiber size.

A decrease in mean fiber number could come about either as a result of decreased fiber formation during development or by actual loss of fibers after myogenesis is completed. To address which of these was responsible for the decrease in mean fiber number observed in adult IIb null mouse muscles, we examined the hindlimb muscles of IIb null mice at various postnatal time points. Because the muscle fibers are very immature and difficult to distinguish from one another at very early time points, direct fiber counts were extremely difficult to obtain. Nonetheless, qualitative examinations of wild-type and IIb null hindlimb muscles revealed no overt evidence of insufficient fiber formation or muscle pathology at early postnatal time points (data not shown). In addition, we measured type I fiber CSA as an indirect measure of muscle fiber loss, assuming that fiber CSA increased in the IIb null animals as a compensatory hypertrophy as a result of fiber loss. Examination of muscle sections from mice at 1, 5, 10, and 20 days postnatal revealed that type I CSA was not significantly different during the early postnatal period (Fig. 3). Type I CSA was significantly increased compared with the wild type starting at 10 days postnatal (Fig. 3). This suggests that before this time there was no fiber hypertrophy because muscle fiber loss was probably minimal (see below).

**Ultrastructural and histological analyses reveal pathology in the IIb null mice.** Immunostaining of adult wild-type hindlimb muscles with antibodies to laminin revealed a regular pattern of normal-sized muscle fibers (Fig. 4A); however, in certain hindlimb muscles of
Table 2. Muscle fiber cross-sectional area in selected muscles of the hindlimb in adult wild-type and MyHC-IIb null mice

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Extensor Digitorum</th>
<th>Tibialis Anterior</th>
<th>Longus Gastrocnemius</th>
<th>Soleus</th>
<th>Superficial Vastus</th>
<th>Vastus Intermedius</th>
<th>Rectus Femoris</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>650 ± 100</td>
<td>390 ± 100</td>
<td>630 ± 100</td>
<td>605 ± 100</td>
<td>590 ± 100</td>
<td>575 ± 100</td>
<td>425 ± 100</td>
</tr>
<tr>
<td>IIb Null</td>
<td>590 ± 100</td>
<td>340 ± 100</td>
<td>600 ± 100</td>
<td>550 ± 100</td>
<td>550 ± 100</td>
<td>525 ± 100</td>
<td>395 ± 100</td>
</tr>
</tbody>
</table>

Values are means ± SEM; n = 4 animals/genotype and 50 fibers/animal for each fiber type. Units are μm².

DISCUSSION

In the present study, we examined the consequences of a null mutation in the IIb MyHC gene to elucidate the role of this isoform in the generation of normal muscle structure. In mice, the IIb MyHC accounts for ~70–80% of the total MyHC expressed in all muscles and is therefore quantitatively the most prominent of the adult fast isoforms. Our results demonstrate that the null mutation of the IIb MyHC gene results in a number of dramatic effects on skeletal muscle growth and morphology in the adult mouse.

Loss of IIb MyHC results in an increase in IId fibers. Immunohistochemical staining using the BF-35 antibody, which recognizes all except the IId MyHC isoform, demonstrated that the null mutation of the IIb MyHC gene resulted in a significant increase in the percentage of so-called “pure” fibers expressing only IId MyHC (Table 1). Given the lack of an antibody specific to just the IId MyHC, we were unable to evaluate the extent of coexpression of the IIb and IId isoforms in the wild-type mouse. This is important; one way an increase in the number of IId fibers could come about is...
if there is substantial coexpression of Iib and Iid MyHC in the wild type that is lost with loss of the Iib MyHC, resulting in Iid fibers by default without any change in Iid gene expression. However, this is probably not the case, since we previously showed using quantitative high-resolution gel electrophoresis that Iid MyHC levels are significantly increased in the Iib null mouse compared with wild-type mice (Allen and Leinwand, unpublished observation). Thus the increase in type IID fibers is a specific result of an increase in Iid MyHC protein expression compared with wild-type mice.

**Ilb null mice have normal feeding patterns.** Previously, we reported that the normal increase in mean body mass during postnatal growth is attenuated in Iib null mice. This is first seen around the age at which mice are weaned and begin to consume hard rodent chow. In the present study, analysis of body mass in Iib null mice fed softer food diets revealed that body mass was not rescued by these presumably more consumable diets, and mean body mass remained decreased compared with wild-type mice (Fig. 2). Moreover, the absolute amount of food consumed per 24 h was not significantly different between wild-type and Iib null mice (3.54 vs. 3.86 g mouse\(^{-1}\) day\(^{-1}\) for wild-type and Iib null mice, respectively; Fig. 2B). This was somewhat surprising considering the smaller overall body size of the Iib null mice. However, it is possible that Iib null mice may have a higher metabolic rate necessitated by the requirement to regenerate fibers lost to degeneration (see below). These data suggest that feeding ability is not overtly impaired by the null mutation in the Iib gene and that the decrement in mean body mass must arise as a more direct consequence of the mutation of this gene on the growth of the muscles themselves.

**The decrease in muscle mass is due to a loss of fibers in the Iib null mouse.** Mean absolute skeletal muscle mass was significantly lower in a large number of muscles in the Iib null mice compared with wild-type mice (Fig. 1A). Muscles such as the quadriceps and gastrocnemius demonstrated the greatest reduction in mean muscle mass, and muscles such as TA and EDL demonstrated a slightly lower decrease in mean absolute muscle mass compared with the wild type, whereas the soleus muscle was not significantly different from the wild type (Fig. 1A). Thus the magnitude of the decrease in mean absolute muscle mass for a given muscle appears to be related to the percentage of Iib MyHC that would ordinarily be expressed in that muscle. Moreover, relative muscle mass was significantly lower in only three hindlimb muscles, the TA, gastrocnemius, and quadriceps muscles (Fig. 1B). These muscles contain the highest percentage of Iib MyHC in the wild-type mouse and moreover were the muscles demonstrating the most pathology in the Iib null mice.

There are three ways in which a decrease in muscle mass could come about. First, all fiber types could be reduced in size in the Iib null mice compared with the wild type, resulting in decreased muscle mass. However, quantitative measurement of fiber CSA revealed that fiber size was actually increased in types I, IIA, and IID fibers in the Iib null mice compared with wild-type mice (Table 2), suggesting that reduced fiber size cannot account for the loss in muscle mass. Second, it is possible that the fibers that would ordinarily be IIB in the Iib null mice were unable to reach their normal size due to a compensatory shift in MyHC expression in the Iib null mice. This was a particularly attractive hypothesis given that the IIB fibers in the wild-type mouse are ordinarily the largest fibers in most muscles and were significantly larger than IID fibers (Table 2), which are the MyHC isoforms upregulated in the Iib null mice (Table 1). However, our results show that the IID fibers in the Iib null mice were significantly larger than IID fibers in the wild type and comparable in size to IIB fibers in the wild type.

The final possibility is that the decrease in mean muscle mass arose as a consequence of a decrease in the number of fibers, and the results of the present study are consistent with this hypothesis. Mean total

### Table 3. Estimated total muscle fiber number in selected muscles of the hindlimb in adult wild-type and MyHC-Iib null mice

<table>
<thead>
<tr>
<th>Muscle</th>
<th>WT</th>
<th>Iib Null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibialis Anterior</td>
<td>3,224 ± 188</td>
<td>1,499 ± 217*</td>
</tr>
<tr>
<td>Extensor Digitorum Longus</td>
<td>988 ± 174</td>
<td>707 ± 57†</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>5,448 ± 605</td>
<td>1,855 ± 221*</td>
</tr>
<tr>
<td>Plantaris</td>
<td>1,787 ± 322</td>
<td>862 ± 321*</td>
</tr>
<tr>
<td>Soleus</td>
<td>619 ± 72</td>
<td>509 ± 72</td>
</tr>
<tr>
<td>Superficial Vastus</td>
<td>4,621 ± 645</td>
<td>1,729 ± 367*</td>
</tr>
<tr>
<td>Vastus Intermedius</td>
<td>489 ± 80</td>
<td>427 ± 40</td>
</tr>
<tr>
<td>Rectus Femoris</td>
<td>3,781 ± 353</td>
<td>2,689 ± 610*</td>
</tr>
</tbody>
</table>

Values are means ± SD; *n = 4 animals/genotype. Values represent mean total fiber number as estimated by whole muscle cross-sectional analysis. *P < 0.01 and †P < 0.05.
fiber number was significantly lower in muscles from
the IIb null mice compared with wild-type mice. Fur-
thermore, the increase in fiber CSA in all fiber types in
the IIb null mice is consistent with a decrease in
muscle fiber number; the remaining fibers undergo a
compensatory hypertrophy, presumably as a conse-
quency of the increased activity of the remaining fibers
due to fiber loss. These data suggest that the compen-
satory hypertrophy of the remaining fibers is insuffi-
cient to prevent a decrease in mean muscle mass
brought about by the decrease in mean fiber number in
these animals.

The decrease in fiber number could conceivably come
about in one of two ways. First, it is possible that

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**Fig. 4.** Null mutation of the IIb MyHC isoform gene results in degeneration and regeneration in selected hindlimb
muscles in the adult mouse. Immunohistochemical staining for laminin (A and D) reveals the presence of small
fiber profiles indicative of fiber degeneration and regeneration. Immunostaining for embryonic MyHC (B and E)
and vimentin (C and F) reveals the presence of regenerating myoblasts and myotubes in the superficial gastro-
cnemius of IIb null but not wild-type animals. Arrows in E indicate embryonic MyHC-positive fibers. Arrows in F
indicate vimentin-positive fibers.

**Fig. 5.** Ultrastructural evidence of normal myofibril formation but muscle fiber degeneration in adult IIb null
mice compared with wild-type mice. Electron micrographs of the superficial gastrocnemius muscle from
wild-type (A–C) and IIb null (D–F) mice. A and D: longitudinal sections through wild-type (A) and IIb null (D)
mouse gastrocnemius showing normal sarcomere organization. B and E: transverse sections through wild-type
(B) and IIb null (E) mouse gastrocnemius showing normal alteration of thick and thin filaments. C and F:
transverse sections at lower magnification showing the presence of normal, large, rounded fiber profiles in the
wild-type mice (arrow in C) and the presence of small, angular fibers containing a degenerating nucleus in the
IIb null mice (arrow in F).
disruption of the IIb MyHC gene results in disregulation of normal fiber formation during prenatal and early postnatal development, resulting in fewer fibers formed a priori. Second, it is possible that the elimination of IIb MyHC results in some critical defect in fiber function that causes fibers to degenerate and die in the adult animal. Several lines of evidence support the latter conclusion. 1) As mentioned, the decrease in mean body mass (and presumably muscle mass as a result of fiber loss) is progressive, becomes more salient during late postnatal development, and is not evident at birth (1). 2) Histological examination of muscle from early postnatal development revealed no evidence of significant compensatory fiber hypertrophy until ~10–20 days of age (Fig. 3). 3) Immunohistochemical and ultrastructural analysis of muscle from adult IIb null and wild-type mice revealed substantial pathology in these muscles consistent with fiber loss (Figs. 4 and 5). Regarding this latter point, both anti-laminin immunostaining and electron microscopy revealed evidence of fiber pathology in the superficial aspects of limb extensors such as the gastrocnemius (Figs. 4 and 5). In addition, immunostaining with antibodies to vimentin and embryonic MyHC, developmental markers that are ordinarily not expressed in adult muscle except during periods of regeneration, revealed an increase in regenerating myotubes in the muscle of IIb null mice compared with control (Fig. 4).

The mechanism(s) responsible for producing this pathological response in the muscles of adult IIb null mice is not currently known. One possibility is that, in the IIb null mice, the muscle fibers that express type IId MyHC but would ordinarily express IIb MyHC are not able to withstand the mechanical loading normally experienced by these fibers. However, why these fibers are less able to withstand strain simply as a consequence of a shift in MyHC isoform expression is not clear, unless this shift is accompanied by a change in expression of other fiber-specific proteins involved in the generation and transmission of contractile force. Specifically, maximum force normalized to CSA tends to be lower in fibers expressing IId MyHC, whereas force normalized to MyHC content is not different between IId and IIb fibers (4). Another possibility is that the shift in MyHC isoform expression causes a mismatch between the contractile components of the muscle and the metabolic mechanisms that provide energy for muscle contraction; this in turn could cause excessive generation of reactive metabolic intermediates, which results in fiber damage and degeneration. We are currently testing both of these hypotheses to further elucidate the mechanisms responsible for this degenerative phenotype. Another intriguing possibility is that loss of the IIb MyHC results in disruption of the establishment and/or maintenance of normal innervation by the motoneurons. Motoneurons typically innervate fibers of the same type (7), and the loss of the predominant MyHC expressed in adult mouse muscle may result in an inability of the motoneurons innervating the IIb fiber pool to recognize the resulting IId MyHC-expressing fibers.

The presence of three adult fast MyHC isoforms in the adult mouse has prompted the question of whether these genes are functionally redundant, as is observed in some members of the myogenic regulatory factor family (11), or whether they are functionally distinct. Studies on isolated skeletal muscle fibers have demonstrated differences in contractile parameters in fibers containing different MyHC isoforms (2, 3, 10), suggesting that these fibers were functionally distinct. The present study on IIb null mice and studies on mice lacking the α-cardiac (8) and the IId adult skeletal MyHC gene (1, 12) have clearly demonstrated that MyHC isoforms have unique roles in the formation and/or maintenance of muscle structure in the mouse.

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