

# Desmin Cytoskeleton Linked to Muscle Mitochondrial Distribution and Respiratory Function

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**Abstract.** Ultrastructural studies have previously suggested potential association of intermediate filaments (IFs) with mitochondria. Thus, we have investigated mitochondrial distribution and function in muscle lacking the IF protein desmin. Immunostaining of skeletal muscle tissue sections, as well as histochemical staining for the mitochondrial marker enzymes cytochrome C oxidase and succinate dehydrogenase, demonstrate abnormal accumulation of subsarcolemmal clumps of mitochondria in predominantly slow twitch skeletal muscle of desmin-null mice. Ultrastructural observation of desmin-null cardiac muscle demonstrates in addition to clumping, extensive mitochondrial proliferation in a significant fraction of the myocytes, particularly after work overload. These alterations are frequently associated with swelling and degeneration of the mitochondrial matrix. Mitochondrial abnormalities can be detected very early, before other structural defects become obvious. To investigate related changes in mitochondrial function, we have analyzed ADP-stimulated respiration of isolated muscle mitochondria, and ADP-stimulated mitochondrial respiration in situ using sapo-

nin skinned muscle fibers. The in vitro maximal rates of respiration in isolated cardiac mitochondria from desmin-null and wild-type mice were similar. However, mitochondrial respiration in situ is significantly altered in desmin-null muscle. Both the maximal rate of ADP-stimulated oxygen consumption and the dissociation constant ( $K_m$ ) for ADP are significantly reduced in desmin-null cardiac and soleus muscle compared with controls. Respiratory parameters for desmin-null fast twitch gastrocnemius muscle were unaffected. Additionally, respiratory measurements in the presence of creatine indicate that coupling of creatine kinase and the adenine translocator is lost in desmin-null soleus muscle. This coupling is unaffected in cardiac muscle from desmin-null animals. All of these studies indicate that desmin IFs play a significant role in mitochondrial positioning and respiratory function in cardiac and skeletal muscle.

**Key words:** intermediate filaments • mitochondrial proliferation • cardiomyopathy • respiration • desmin

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## Introduction

The role of mitochondria in cellular energy production and metabolism has been well established, and the function of mitochondria in these important areas of cellular physiology has been investigated for decades. Much less understood are the mechanisms controlling and influencing mitochondrial biogenesis, and the mechanisms determining mitochondrial localization within the cell and the influence of mitochondrial localization upon metabolic function. The cytoskeletal networks are intimately involved in transport and localization of organelles, including mitochondria. The association of mitochondria with the cytoskeleton has been noted for many years (Heggeness et al., 1978; Martz et al., 1984; Drubin et al., 1993; for

reviews see Bereiter-Hahn and Voth, 1994; Rappaport et al., 1998; Yaffe, 1999), and numerous studies have suggested that, as with other organelles and cellular components, the cytoskeleton is involved in movement and localization of mitochondria (Bereiter-Hahn and Voth, 1994; Morris and Hollenbeck, 1995; Yaffe, 1999; Suelmann and Fischer, 2000). However, the role the cytoskeleton plays, if any, in regulating the metabolic function of mitochondria is not clear.

Striated muscle tissue provides an excellent system in which to study mitochondrial function, as muscles require high amounts of energy for contractile activity to take place, and thus have high mitochondrial content. Additionally, different classes of striated muscle differ with respect to mitochondrial content. Cardiac and slow twitch skeletal muscles have high mitochondrial content, fast glycolytic skeletal muscle fibers have low mitochondrial con-

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tent, but fast oxidative fibers have intermediate mitochondrial content. In all striated muscle types, however, the mitochondria are intimately associated with myofibrils. In skeletal muscle fibers, mitochondria are usually localized in pairs at the Z disc of the sarcomere throughout the length of the myofibril, whereas in cardiac muscle the mitochondria are usually arranged in packed strands of mitochondria running between the myofibrils. The appearance of mitochondria in pairs is most likely the result of cross-sectioned mitochondria side branches wound around muscle fibers at the level of the I bands, as reported previously (Rambourg et al., 1980; Ogata et al., 1997; Reipert et al., 1999). The proteins responsible for mediating the association of mitochondria with myofibrils are currently unknown. However, the muscle-specific intermediate filament (IF)<sup>1</sup> protein desmin is an attractive candidate, since as with microtubules, IFs have also been suggested to associate with mitochondria. Ultrastructural studies in several cell types have demonstrated numerous times that there is an apparent connection between mitochondria and the IF cytoskeleton (Stromer and Bendayan, 1990; Almahbobi et al., 1993; for reviews see Georgatos and Maison, 1996; Rappaport et al., 1998). It has also been recently proposed that this association could be mediated by the cytolinker protein plectin (Reipert et al., 1999). However, there is no functional evidence to support any association of mitochondria with IF proteins.

The IF protein desmin, encoded by a single copy gene (Capetanaki et al., 1984), is expressed in all striated and smooth muscle tissues (for reviews see Lazarides, 1982; Lazarides and Capetanaki, 1986). It is one of the earliest known muscle-specific genes to be expressed during cardiac and skeletal muscle development (Herrmann et al., 1989; Schaart et al., 1989). The distribution of desmin within striated muscle suggests that it could function as a linkage between mitochondria and myofibrils. In mature striated muscle, desmin IFs surround the Z disc and link adjacent myofibrils at the level of the Z disc, and link myofibrils to the sarcolemma at the level of the costamere (Granger and Lazarides, 1978, 1980; for review see Capetanaki and Milner, 1998), possibly through plectin (Hijikata et al., 1999; Schroder et al., 1999). This allows the formation of a continuous cytoskeletal network that could be involved in several diverse functions, including among others, maintenance of cellular integrity, force transmission, and mechanochemical signaling. Ultrastructural studies have indicated that desmin IFs can extend from the Z disc and form connections with mitochondria and other organelles, including the nucleus (Tokuyasu et al., 1983a,b; Lockard and Bloom, 1993; Reipert et al., 1999). Thus, desmin IFs could potentially function to link mitochondria to myofibrils and possibly provide a mechanism by which contractile activity could influence the metabolic function of mitochondria.

Recent ablation of desmin expression in mice by gene targeting (Li et al., 1996; Milner et al., 1996) demonstrates that desmin expression is crucial for maintaining architectural and functional integrity of striated muscle. Mice lack-

ing desmin develop defects in all muscle tissue. These defects are most severe in the heart, where cardiomyocyte hypertrophy and chamber dilation characterized by extensive myocyte cell death lead to heart failure (Li et al., 1996; Milner et al., 1996, 1999). The mechanism by which the absence of desmin leads to myocyte death and heart failure is still not clear. Mice lacking desmin demonstrate numerous muscle architectural and ultrastructural defects. These structural abnormalities include loss of lateral alignment of myofibrils, perturbation of myofibril anchorage to the sarcolemma, and loss of nuclear shape and positioning. Loose cell adhesion and increased intercellular space are also prominent defects (Capetanaki et al., 1997; Capetanaki and Milner, 1998).

In addition to these structural defects, abnormalities in mitochondrial appearance were also observed. Considering the potential association of IFs with mitochondria described above and the suggestions for their possible involvement in mitochondria function (Kay et al., 1997), we have further investigated the consequences of the absence of desmin on mitochondrial behavior in cardiac and skeletal muscle. We observe severe changes in mitochondrial number and distribution in cardiomyocytes and in slow twitch skeletal muscle. These changes are seen before any other structural defects become obvious. Additionally, we observe changes in mitochondrial function in muscle lacking desmin. Although respiratory function of isolated cardiac mitochondria from desmin-null animals is unaltered, measurement of respiration in situ using skinned fibers shows impaired mitochondrial function in the absence of desmin in cardiac and slow twitch skeletal muscle. These studies suggest that desmin IFs play a role, either directly or indirectly, in mitochondrial positioning and respiratory function in cardiac and skeletal muscle, and perturbation of mitochondrial function might be the root of muscle degeneration in desmin-null animals

## Materials and Methods

### Electron Microscopy

For cardiac muscle, animals were heparinized 30 min before killing, with 5 U/g body wt heparin. Mice were killed by cervical dislocation and the chest cavity was opened. The heart was then perfused with cold fixative solution (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) by injection into the left ventricle with a small needle and syringe. A slit was cut in the right atrium to provide drainage. Approximately 4 ml of fixative was perfused by slow, gradual injection. If perfusion was done properly, the myocardium gradually turned light to golden brown in color.

After perfusion, the heart was excised and placed in a small dish of cold fixative solution. Using a razor blade or scalpel, unwanted portions of the heart were trimmed away and segments of the myocardium to be used were cut and separated. These areas were further cut into small cubes with dimensions of 1 mm and no greater than 3 mm in any dimension. These small cubes were placed in a vial of fixative solution and fixed further overnight at 4°C.

The next day, the fixative was removed and the fixed tissue cubes were washed with 0.1 M phosphate buffer, pH 7.4, at 4°C. After washing, the tissue cubes were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature. The tissue cubes were then dehydrated in a graded series of ethanol solutions. After dehydration, the tissue cubes were infiltrated with the transitional solvent propylene oxide, followed by infiltration with Embed 812 resin (Electron Microscopy Sciences). The tissue cubes were then placed in fresh resin in block molds and oriented. The blocks were then cured at 60–70°C for 48 h. After curing, the blocks were trimmed and sectioned. 80-nm sections were cut on

<sup>1</sup>Abbreviations used in this paper: ANT, adenine nucleotide translocator; IF, intermediate filament; SDH, succinate dehydrogenase.

an ultramicrotome, picked up on grids, and stained with a solution of lead citrate and uranyl acetate. The sections were then observed, and photographs were taken on a Hitachi 7100 electron microscope.

For skeletal muscle, the procedure used was the same except for fixation. For small muscles that are 1–3 mm in diameter, the muscles were excised, pinned to cork at the ends (to prevent shortening during fixation), and fixed by immersion overnight at 4°C. For larger muscles, the muscles were excised, placed in a dish of cold fixative, trimmed into 1–3-mm cubes as for cardiac muscle, and then fixed overnight.

For all EM studies, a random sampling procedure was used with numerous sections of three to four different individuals for each case.

The swimming work overload protocol used has been described previously (Milner et al., 1999). In brief, mice were exercised twice a day, beginning at 10 min per session on the first day. The duration of swimming was increased by 10 min per day to a final duration of 90 min per session, which was maintained for the duration of the experiment. The total duration of each experiment was 8 wk. Up to 50% of the desmin-null mice died before the end of this protocol.

### **Histochemical Staining and Western Blotting for Mitochondrial Markers**

The enzymes cytochrome C oxidase and succinate dehydrogenase (SDH) were used as mitochondrial markers for visual inspection of mitochondrial distribution in skeletal and cardiac muscle. Frozen sections of cardiac and skeletal muscle were cut 10–8  $\mu\text{m}$  thick and picked up onto silane-coated slides. For histochemical staining for SDH, tissue sections were incubated for 20 min at 37°C in a solution comprised of 5 mM EDTA, 1 mM KCN, 0.2 mM phenazine methosulfate, 50 mM succinic acid, 1.5 mM nitro blue tetrazolium, and 5 mM phosphate buffer, pH 7.6. The slides were then washed three times for 5 min each in ddH<sub>2</sub>O before sealing under a coverslip with glycerin gel. For histochemical staining for cytochrome C oxidase activity, tissue sections were incubated for 1 h in a solution comprised of 5 mM phosphate buffer, 0.1% 3'3'-diaminobenzidine, 0.1% cytochrome C, and 0.02% catalase, pH 7.4. The slides were then washed and sealed as the slides stained for SDH. For all histochemical staining studies, a random sampling procedure was used with numerous sections of three to four different individuals for each case.

For Western blot analysis, protein samples were prepared by pulverizing the cardiac tissue with liquid nitrogen and extracting it with SDS-PAGE sample buffer. Proteins were separated in 9% nonreducing SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride membranes (Immun-Blot; Bio-Rad Laboratories). The membranes were probed with antiporin antibody (cat no. 529536; Calbiochem) and with anti-cytochrome oxidase I antibody (cat no. A-6403; Molecular Probes). Proteins were visualized using chemiluminescence (ECL kit; Amersham Pharmacia Biotech). Images were scanned and quantified using a densitometer and Image QuaNT software (Molecular Dynamics). Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories).

### **Isolation of Cardiac Mitochondria and Assay of Respiration**

Isolation of cardiac mitochondria was performed by the method of Mela and Seitz (1982). At least three hearts were excised from mice and placed in ice-cold MSE solution (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, and 1 mM Tris, pH 7.4). The hearts were minced into small pieces and homogenized using a teflon homogenizer at 500 rpm for four to five pestle strokes. The homogenate was then centrifuged at 700  $g$  for 5 min at 4°C. The supernatant was collected and centrifuged at 7,700  $g$  for 10 min at 4°C. The supernatant was then discarded, and the pellet was washed gently with 1 ml of MSE. After washing, the pellet was resuspended in 100  $\mu\text{l}$  of MSE and centrifuged again at 7,700  $g$  for 10 min at 4°C. The final pellet was resuspended in 200  $\mu\text{l}$  MSE. A small aliquot of the final suspension was taken and used for protein determination by the Bradford protein assay (Bio-Rad Laboratories).

Measurement of mitochondrial respiration was carried out essentially as described by Trounce et al. (1996). Respiration of isolated mitochondria was assessed using a Clark electrode (Yellow Springs Instruments) in a chamber containing 2 ml of mitochondrial respiration buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 2 mg/ml BSA, and 5 mM Hepes, pH 7.2) containing 0.1 mg/ml mitochondrial protein. State IV respiration was recorded by the addition of respiratory substrates glutamate plus

malate, pyruvate plus malate, or succinate. State III respiration was then measured after the addition of 100 mM ADP.

### **Assessment of Mitochondrial Respiration In Situ**

Respiratory function of the entire muscle fiber mitochondrial population in situ was done using the saponin skinned fiber technique described by Veksler et al. (1987, 1995). Thin fiber bundles measuring between 0.25 and 0.5 mm in diameter and 2–5 mm in length were excised from ventricular free wall muscle and from soleus, plantaris, and gastrocnemius skeletal muscle from wild-type and desmin-null mice. The muscles were dissected at 4°C in relaxing solution, which consisted of 2.77 mM CaK<sub>2</sub>EGTA, 7.23 mM K<sub>2</sub>EGTA (free Ca<sup>2+</sup> concentration of 100 nM), 6.56 mM MgCl<sub>2</sub>, 5.7 mM ATP, 15 mM phosphocreatine, 0.5 mM DTT, 50 mM K-MES buffer, 20 mM imidazole, and 20 mM taurine, pH 7.1.

After dissection, the fiber bundles were transferred into relaxing solution containing 50  $\mu\text{g/ml}$  saponin and rocked gently at 4°C for 40 min to perforate the sarcolemma. At the end of this incubation, the fibers were transferred into respiration buffer, which consisted of 2.77 mM CaK<sub>2</sub>EGTA, 7.23 mM K<sub>2</sub>EGTA (free Ca<sup>2+</sup> concentration of 100 nM), 1.38 mM MgCl<sub>2</sub>, 0.5 mM DTT, 100 mM K-MES buffer, 20 mM imidazole, 20 mM taurine, 3 mM K<sub>2</sub>HPO<sub>4</sub>, and 2 mg/ml BSA, pH 7.1. The fibers were rocked gently at 4°C with two changes of buffer for 10 min each to ensure removal of all high-energy phosphates.

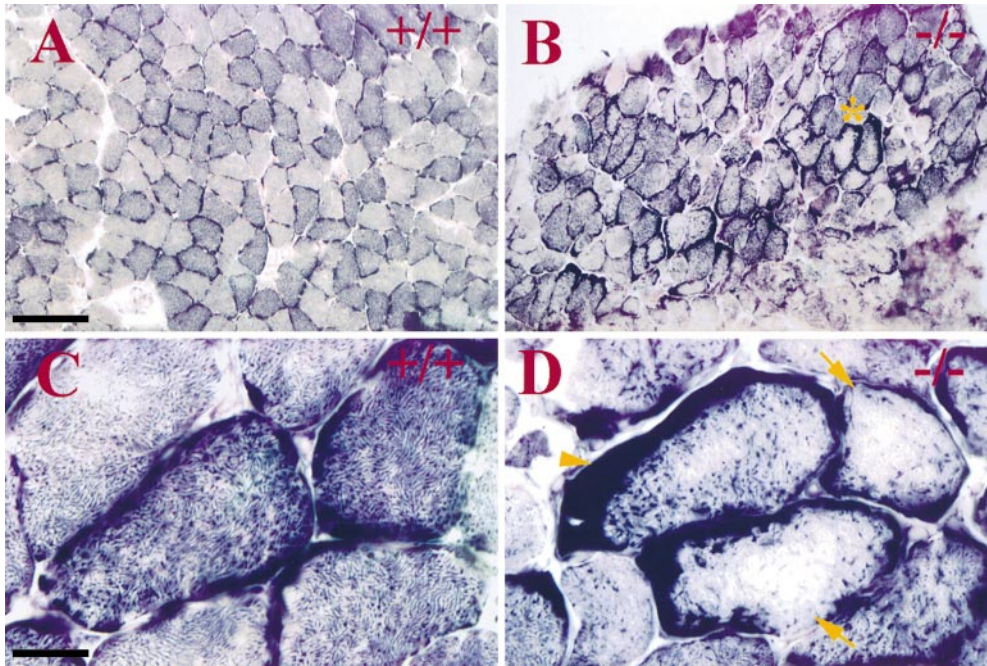
ADP-stimulated respiration of the fiber bundles was measured by a Clark electrode (Yellow Springs Instruments) in a chamber containing 4 ml of respiration solution supplemented with 5 mM pyruvate and 2 mM malate. Measurements were made at 25°C with constant stirring. At this temperature, the solubility of oxygen was taken to be 430 ng atoms O/ml. After completion of measurements, the fiber bundles were removed and dried overnight. Respiration rates were expressed as nanograms of atoms of oxygen per minute per milligram of dry weight. To determine respiratory parameters, respiration rates were plotted as a function of ADP concentration, and the dissociation constant ( $K_m$ ) and maximal rate of oxygen consumption ( $V_{\text{max}}$ ) were calculated using either Cornish-Bowden and Eisenthal (1974), or SigmaPlot (Jandel Scientific).

For respiration measurements in the presence of creatine, creatine was added to the respiration buffer at a final concentration of 20 mM. For assessing outer mitochondrial membrane damage using the cytochrome C test, a modified buffer was used that consisted of 125 mM KCl, 20 mM Hepes, 3 mM magnesium acetate, 5 mM K<sub>2</sub>HPO<sub>4</sub>, 0.4 mM EGTA, 0.3 mM DTT, and 2 mg/ml BSA, pH 7.1. Cytochrome C was added at a final concentration of 8  $\mu\text{M}$ .

## **Results**

### **Alteration of Mitochondrial Positioning in Muscle Lacking Desmin**

As mitochondrial abnormalities were observed during the initial characterization of the desmin-null mouse, we first looked at mitochondrial distribution within muscle cells using enzyme histochemistry and immunofluorescent staining. SDH is a mitochondrial matrix enzyme that is frequently used as a histochemical marker for mitochondria in tissue sections. In sections of normal mouse muscle, the intensity of staining corresponds to the oxidative capacity, and thus the mitochondrial content, of the muscle fibers. In sections of normal mouse soleus muscle (Fig. 1, A and C), a muscle consisting mostly of highly oxidative slow twitch fibers, a checkerboard pattern of intensely staining fibers is observed. The staining is mostly granular throughout the fibers, with slight enhancement of subsarcolemmal staining in a few fiber sections. In soleus sections from corresponding desmin-null animals (Fig. 1, B and D), this discernible checkerboard pattern is mostly lost. Large clumps of subsarcolemmal staining appear in a majority of the fibers, and the interior of fibers frequently appears almost devoid of staining. In muscle such as the gastrocnemius, the fast glycolytic fibers normally show only faint SDH



**Figure 1.** Alteration of mitochondrial distribution in desmin-null soleus skeletal muscle. SDH histochemical staining (A–D) demonstrates alteration of mitochondrial distribution in desmin-null (–/–) soleus muscle. In sections of normal (+/+) 10-mo-old mouse soleus muscle (A), a muscle consisting mostly of highly oxidative slow twitch fibers, a checkerboard pattern of intensely staining fibers is observed, with a variable amount of fibers staining with less intensity. The staining is mostly granular throughout the fibers, with slight enhancement of sub-sarcolemmal staining in a few fiber sections (C). In soleus sections from corresponding desmin-null animals (B, same scale as A), this discernible

checkerboard pattern is partially lost. Large clumps of sub-sarcolemmal staining appear in a majority of the fibers (arrowhead), and the interior of fibers frequently show reduced SDH staining (arrows) (C and D, higher magnification view of A and B accordingly).

staining, and there is no apparent difference in staining between wild-type and desmin-null tissue (Fig. 2, C and D). However, in areas of the gastrocnemius that normally harbor fast fibers and slow oxidative fibers, sub-sarcolemmal clumping and loss of mitochondrial staining from the interior are observed, similar to the results observed in sections from the soleus (Fig. 2 B). Histochemical staining for cytochrome C oxidase, another mitochondrial marker enzyme, yields results identical to the SDH staining (data not shown). Changes in mitochondrial distribution in the soleus can also be confirmed at the ultrastructural level (Fig. 3).

Observation of abnormal sub-sarcolemmal clumps of mitochondrial staining could be made in sections of muscle from both old (8 mo–1 yr) and young (1 mo) mice. However, in the younger mice, the clumping was usually less severe, although the interior of the fibers usually showed decreased SDH staining compared with young normal mice (Fig. 2, E and F). Thus, it appears that the sub-sarcolemmal aggregation of mitochondria intensifies in desmin-null muscle with aging.

#### **Ultrastructural Alteration of Mitochondrial in Cardiac Muscle Lacking Desmin**

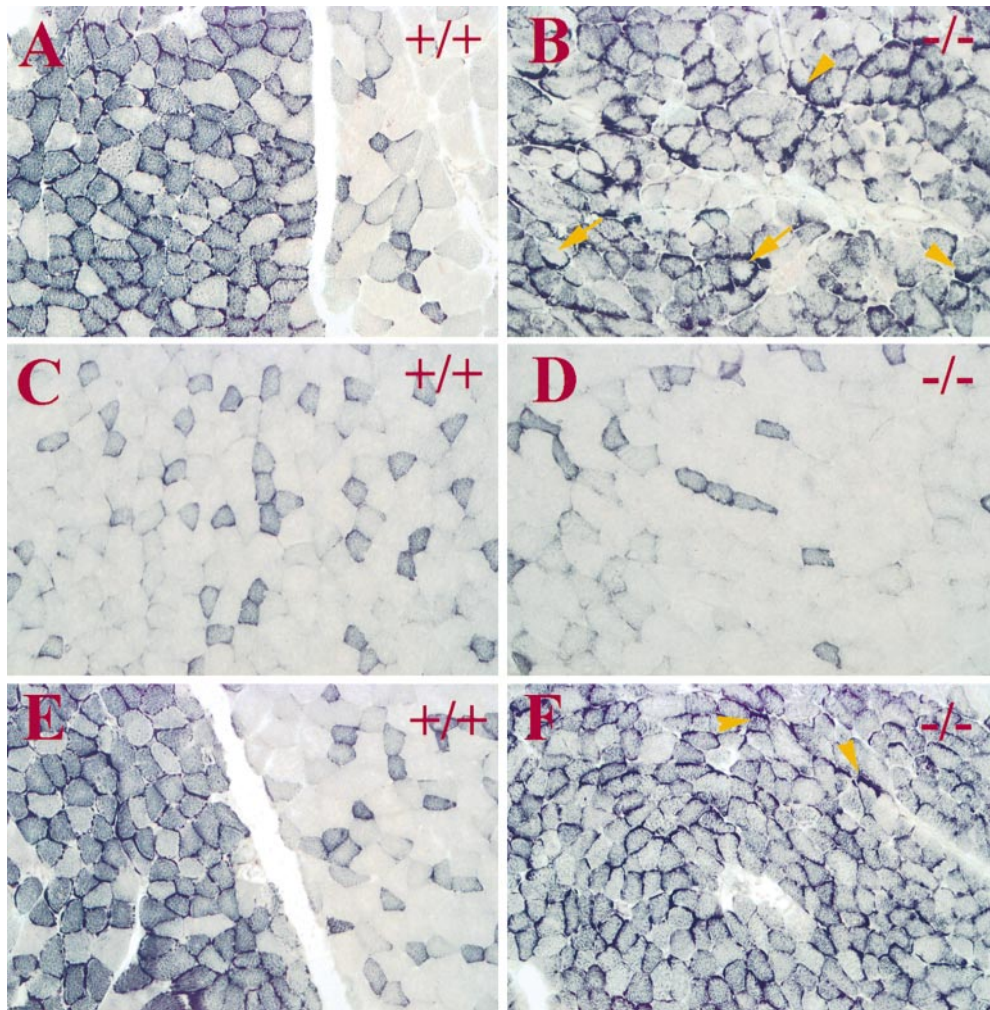
As staining of cardiac muscle for mitochondrial markers is frequently difficult to analyze due to the normally high mitochondrial content of this tissue, we further analyzed mitochondrial distribution in cardiac muscle using electron microscopy. In normal cardiac muscle (Fig. 4 A), mitochondria are usually packed tightly in strands running between myofibrils, with some mitochondria found beneath the sarcolemma, and some aggregation at the ends of the

nucleus. Some small areas of sub-sarcolemmal clumping can be observed as well.

Ultrastructural examination of desmin-null cardiac muscle frequently reveals abnormalities in mitochondrial positioning and appearance. Observations of mitochondrial proliferation, sub-sarcolemmal clumping of mitochondria, and swelling of the mitochondrial matrix are frequently seen (Fig. 4, B–D). These abnormalities increase in severity in muscle from mice that have been exercised (Fig. 4 C). In these extreme cases, higher numbers of myocytes appear to be almost completely filled with swollen mitochondria, and occasionally cristae appear fragmented (Fig. 4 C).

The apparent hyperproliferation of mitochondria in work overload-stressed hearts of desmin-null mice was confirmed by evaluating changes in total mitochondrial mass. Western blot analysis of two mitochondrial proteins, cytochrome C oxidase and porin (Fig. 5), and densitometric evaluation revealed a 1.5–2-fold increase in the abundance of these two proteins in total desmin-null heart extracts compared with wild-type. Considering that only ~20% of the cardiomyocytes display extensive obvious hyperproliferation, it could be estimated that the maximum increase in mitochondria number could be ~7.5–10-fold. This would be the case if it is assumed that the remaining 80% of the cells show no change in mitochondria number. However, this has not been determined. On the contrary, electron microscopy observations suggest a very low to moderate increase in mitochondria number throughout the myocardium. Therefore, the maximum increase in mitochondria number must be lower than estimated above.

In some fibers, clusters of mitochondria appear to be sequestered in membrane-bound structures (Fig. 4 D, en-



**Figure 2.** Alteration of mitochondrial distribution occurs in other muscles. SDH histochemical staining (A–F) demonstrates alteration of mitochondrial distribution in the red gastrocnemius (A and E, left), an area comprised mostly of slow twitch fibers. Control wild-type muscle (+/+; A) shows granular staining with some slight subsarcolemmal clumping, whereas subsarcolemmal clumps are pronounced in desmin null (–/–) sections (B, arrowheads), and some fibers appear to have lost mitochondrial staining from the fiber interior altogether (B, arrows). Sections of fast twitch skeletal muscle of the gastrocnemius (+/+ and –/– in C and D, respectively; and A and E, right) do not show any changes in SDH staining patterns. When young (1 mo) mouse muscle is stained for SDH, some slight enhancement of clumping is seen in desmin-null sections (F, arrowheads), and the staining of the interior of the fibers is slightly reduced compared with wild-type (E) fibers.

larged in E), suggesting that they have been taken up by some autophagic process. There are occasionally huge empty membrane-bound spaces within myocytes that seem to have been generated by fusion of large numbers of degenerating mitochondria. Often these structures are surrounded by intact mitochondria (not shown). In areas where subsarcolemmal clumps of mitochondria can be observed, clusters of mitochondria within membrane-bound structures can be found in the extracellular space, suggesting that they have been extruded from the myocyte (not shown).

In most cases, myocytes displaying mitochondrial abnormalities are prevalent in areas surrounding or near the calcified, fibrous lesions which are characteristic of desmin-null myocardium (Milner et al., 1996). As these lesions tend to be more prevalent in the right ventricle, mitochondrial abnormalities are prevalent there as well. Nonetheless, myocytes displaying mitochondrial abnormalities can be found throughout the myocardium, even in areas where calcified lesions are absent. To determine if abnormalities in mitochondria comprise one of the first obvious defects in desmin-null cardiac muscle, similar studies were performed with cardiac tissue from 2-wk-old mice (Fig. 6). At this age, mitochondrial abnormalities can be observed, although they are considerably more mild than the defects

seen in adults. The most striking difference between desmin-null and wild-type cardiac tissue is the degree of mitochondrial degeneration observed. Mitochondrial density is only slightly increased in a small fraction of desmin-null muscle. Although both tissue types contain big clusters of mitochondria, not common in adult tissue, in the null heart they are often degenerating and occasionally abnormally clumped (Fig. 6, A and C). Although mitochondrial swelling is not common, it can be occasionally seen and it is often accompanied by more extensive degenerating regions (Fig. 6, D and E). Moreover, while some mitochondrial abnormalities can be observed in 2-wk-old null animals, other abnormalities commonly seen in adult desmin-null myocytes are not yet observed. For instance, myofibrils are aligned properly in young desmin-null myocytes (Fig. 6 B). Thus, it appears that the earliest observable structural changes in desmin-null muscle are changes in mitochondria.

#### *Respiratory Function of Isolated Mitochondria*

The observation of mitochondrial abnormalities in muscle sections from desmin-null animals suggests that mitochondrial function may also be impaired. To assess mitochondrial function in the absence of desmin, we first investi-

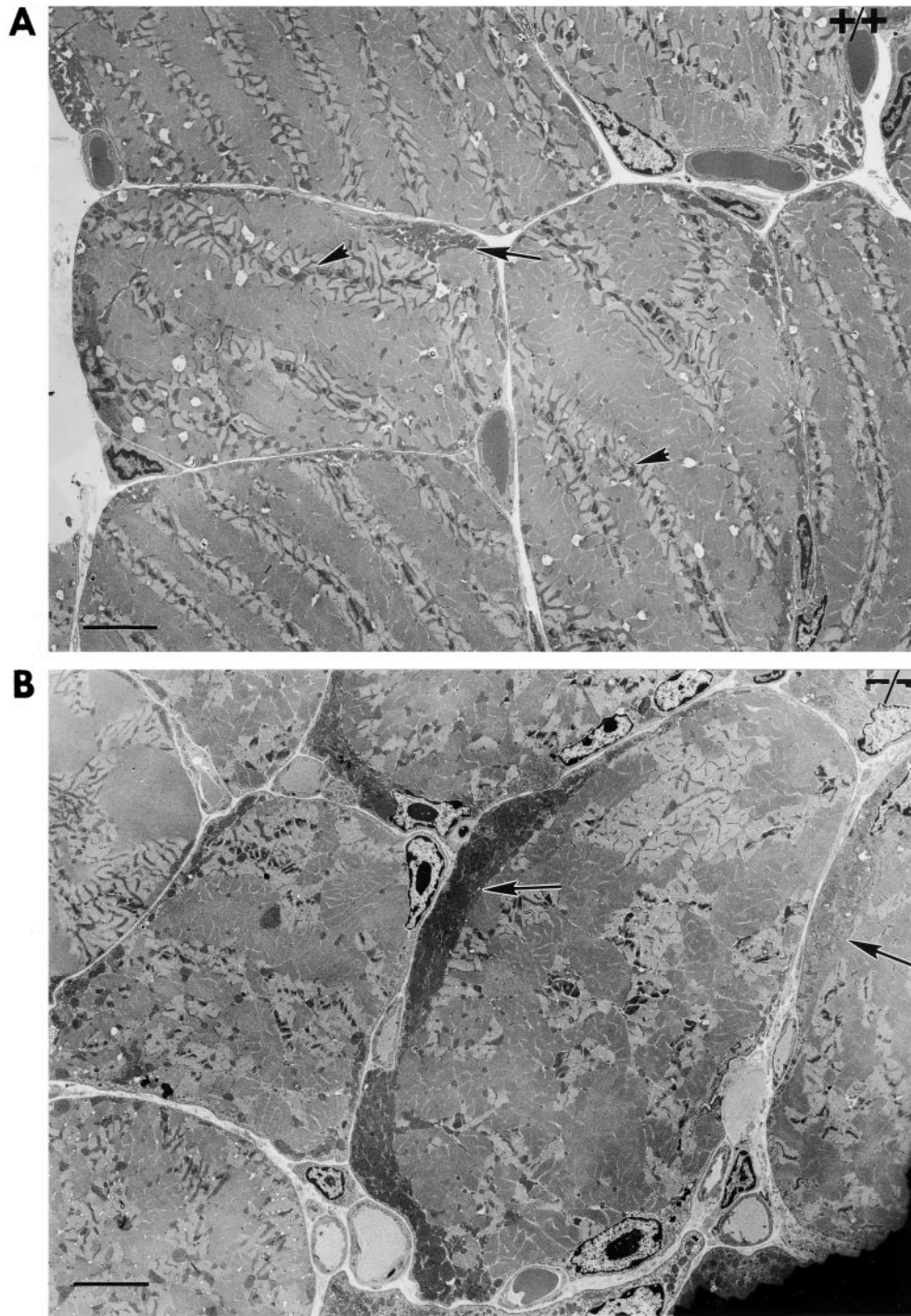
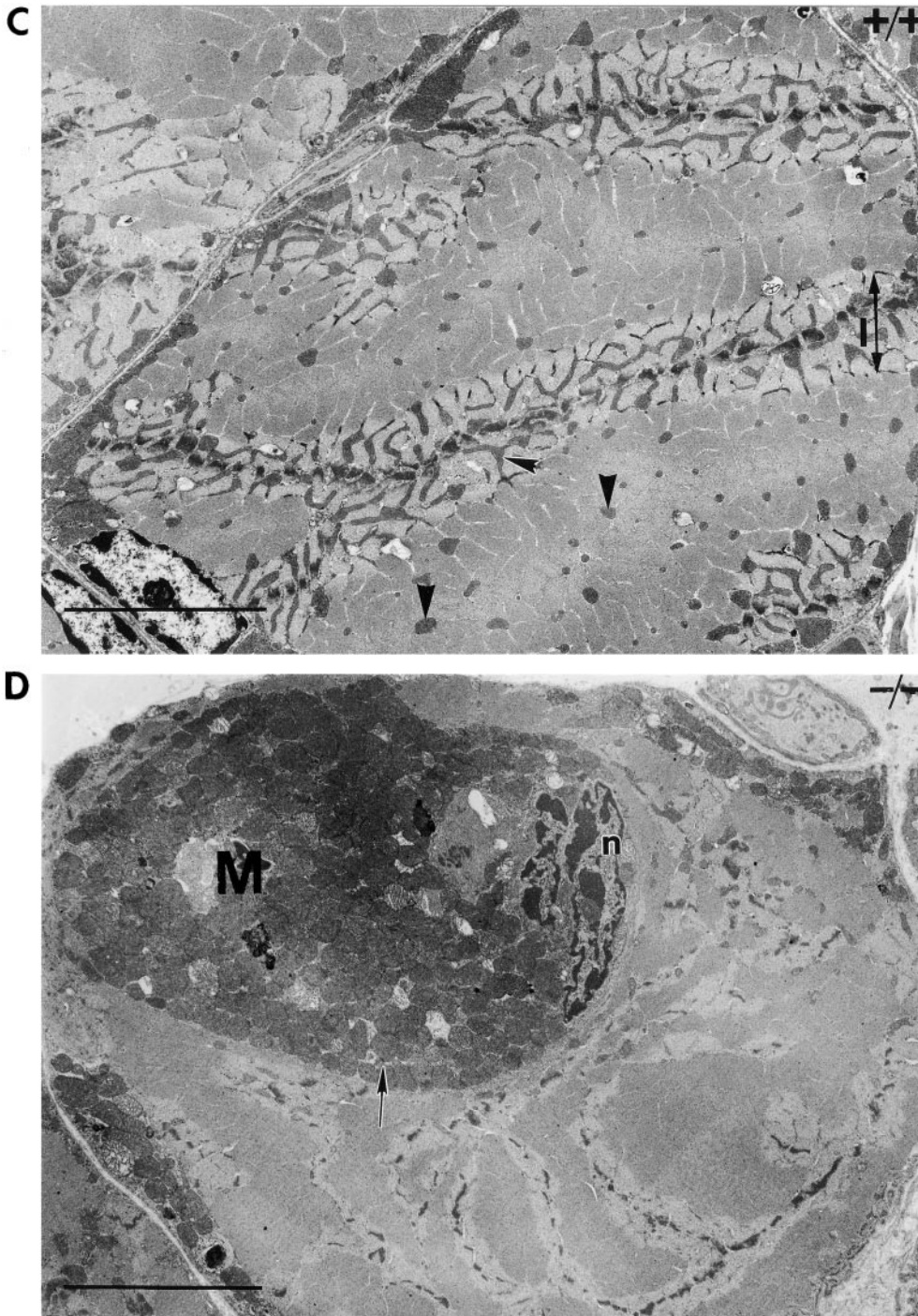


Figure 3 (continues on facing page).

gated respiratory function in mitochondria isolated from cardiac muscle. As expected from our ultrastructural observations in desmin-null cardiac muscle, we were typically able to isolate more mitochondrial protein from desmin-null cardiac muscle than from wild-type cardiac muscle, thus confirming the increase in mitochondrial number in desmin-null cardiac muscle (data not shown). Measurement of oxygen consumption of isolated mitochondria from control and desmin-null animals, using pyruvate plus malate as respiratory substrates, did not show any variance in the state III (ADP-stimulated), state IV (ADP-limited),

or uncoupled respiration rates, nor was there any alteration of the respiratory control ratio (Table I). There was also no difference in respiratory activity between the mitochondrial samples when respiratory substrates succinate or glutamate plus malate were used. Thus, there did not seem to be any effect on specific respiratory complexes in mitochondria from desmin-null mice. Titration of oxygen consumption with varying amounts of ADP gave a  $K_m$  for ADP of  $22.79 \mu\text{M}$  in wild-type cardiac mitochondria and  $20.52 \mu\text{M}$  in desmin-null mitochondria, indicating no significant difference in affinity for ADP in the mitochon-



**Figure 3.** Ultrastructural observation of mitochondrial distribution in skeletal muscle. Electron microscopic analysis of oblique sections from soleus skeletal muscle confirm the change in mitochondrial distribution in desmin null ( $-/-$ ) skeletal muscle demonstrated by SDH staining in Figs. 1 and 2. Wild-type ( $+/+$ , A and C) soleus muscle displays regularly arrayed mitochondria (arrowheads) throughout the interior of the fiber visible at the level of the lighter color I band (I in C) and interspersed between the myofibrils (arrowheads). Only slight subsarcolemmal clumping can be observed (arrow). A desmin-null fiber (B) showing increased subsarcolemmal clumping (arrow) and decrease in intermyofibrillar mitochondria. (D) A severe case of mitochondrial (M) clumping in a desmin-null fiber (arrow). Note the internal nucleus (n) and the lack of intermyofibrillar mitochondria compared with the wild-type section (C). Bars, 5  $\mu\text{m}$ .

drial samples (Table I). Thus, there is no discernible difference in respiratory activity in isolated mitochondria from desmin-null cardiac muscle compared with mitochondria isolated from normal mouse myocardium.

### **Mitochondrial Respiration In Situ**

Numerous studies have demonstrated that isolated mitochondria behave differently from mitochondria in situ with respect to respiratory activity (for review see Saks et al., 1998). Isolated mitochondria from desmin-null and

wild-type animals, as described above, did not display differences in ADP-stimulated respiration. We then decided to investigate mitochondrial respiratory activity in situ using the skinned fiber technique (Saks et al., 1991, 1998; Veksler et al., 1995), which allows analysis of mitochondrial activity as it occurs in the muscle cell. Thus, this technique can be used for the investigation of the effect of positioning and sarcoplasmic connections of mitochondria with other structures on mitochondrial activity.

We analyzed ADP-stimulated respiration in situ using saponin skinned muscle fibers. The results are shown in

Table I. Respiration of Isolated Cardiac Mitochondria from Wild-Type (*Des<sup>+/+</sup>*) and Desmin-null (*Des<sup>-/-</sup>*) Mice

Substrates		III	IV	RCR	UC
Pyruvate + malate	<i>Des<sup>+/+</sup></i>	210.55 ± 16.80	67.54 ± 4.21	3.13 ± 0.33	254.28 ± 38.27
	<i>Des<sup>-/-</sup></i>	211.95 ± 18.65	67.89 ± 10.41	3.19 ± 0.47	281.48 ± 12.67
Glutamate + malate	<i>Des<sup>+/+</sup></i>	206.60 ± 1.15	72.88 ± 5.65	2.86 ± 0.21	ND
	<i>Des<sup>-/-</sup></i>	204.55 ± 2.50	76.38 ± 3.98	2.69 ± 0.11	ND
Succinate	<i>Des<sup>+/+</sup></i>	461.45 ± 9.90	208.98 ± 15.85	2.22 ± 0.13	644.25 ± 30.75
	<i>Des<sup>-/-</sup></i>	472.41 ± 27.44	216.76 ± 18.74	2.19 ± 0.12	647.00 ± 20.50
		<i>K<sub>m</sub></i> (ADP)			
	<i>Des<sup>+/+</sup></i>	22.79 ± 3.95			
	<i>Des<sup>-/-</sup></i>	20.52 ± 1.16			

III, state III (ADP-stimulated respiration); IV, state IV (ADP-limited respiration); RCR, respiratory control ratio (III/IV); UC, uncoupled respiration. Data for III, IV, and UC are shown in ng atom O/min/mg mitochondrial protein. *K<sub>m</sub>* is given in μM ADP.

Table II. As expected, no ADP stimulation of oxygen consumption was observed in fibers analyzed without treatment with saponin (data not shown). Fibers analyzed from the gastrocnemius, containing mostly fast glycolytic fibers, showed no difference in ADP-stimulated oxygen consumption. Both the *K<sub>m</sub>* for ADP and *V<sub>max</sub>* of oxygen consumption were statistically identical in wild-type and desmin-null fibers. However, in fibers prepared from both the soleus and ventricular muscle of the heart, alterations in respiratory activity were observed in desmin-null samples (Table II). In both tissues, the *V<sub>max</sub>* was significantly lower in desmin-null fibers. The apparent *K<sub>m</sub>* for ADP was also significantly lower in soleus and cardiac fiber samples from desmin-null animals. Thus, desmin-null cardiac and soleus muscle have an increased affinity for ADP (decreased *K<sub>m</sub>*), but do not consume oxygen as effectively (decreased *V<sub>max</sub>*).

To ensure that the lower values in *V<sub>max</sub>* in the soleus were not due to decreased mitochondrial content in desmin-null muscle fibers, suggested by the loss of mitochondrial staining in the interior of soleus myofibers (Fig. 1, B and D), activity of the mitochondrial marker enzyme citrate synthase was assayed. There was no difference in activity in both gastrocnemius and soleus muscle between wild-type and knockout mice, whereas desmin knockout cardiac muscle displayed slightly elevated citrate synthase levels (data not shown). Thus, the decrease of *V<sub>max</sub>* in desmin-null soleus muscle is not due to a decrease in mitochondrial content.

If desmin filaments associate with mitochondrial mem-

branes either directly or indirectly (see Reipert et al., 1999), the absence of desmin may perturb mitochondrial outer membrane integrity, and the differences in respiration in situ may be explained by outer mitochondrial membrane damage. To assess if this is the case, respiration of cardiac and soleus muscle bundles was measured in KCl media with the addition of exogenous cytochrome C. If it is simply outer mitochondrial membrane damage that is responsible for changes in respiration, then the addition of exogenous cytochrome C should restore respiratory activity to normal levels (Saks et al., 1995; 1998). Addition of exogenous cytochrome C did not restore respiratory activity in desmin-null soleus or cardiac fiber bundles to wild-type levels, nor did it enhance respiratory activity (not shown). Thus, the differences in respiratory activity are not due simply to outer membrane damage.

A phenomenon observed in mitochondria of slow twitch skeletal and cardiac muscle in situ is the enhanced stimulation of respiration by creatine in the presence of submaximal levels of ADP, due to the coupling of mitochondrial creatine kinase and the adenine nucleotide translocator (ANT) (Veksler et al., 1995; Saks et al., 1996, 1998). Thus, the presence of elevated levels of creatine has the effect of lowering the *K<sub>m</sub>* for ADP when this coupling is intact. If mitochondria in situ have changes in membrane morphology, this coupling is lost, and the presence of creatine does not enhance ADP-stimulated oxygen consumption. Isolated mitochondria do not display this effect due to loss of coupling (Saks et al., 1995). In wild-type cardiac and soleus muscle, and in desmin-null cardiac muscle, respiration in

Table II. Respiration of Wild-Type (*Des<sup>+/+</sup>*) and Desmin-null (*Des<sup>-/-</sup>*) Muscle Fiber Mitochondria In Situ

	<i>K<sub>m</sub></i>	<i>V<sub>max</sub></i>	A/C	Plus creatine		
				<i>K<sub>m</sub></i>	<i>V<sub>max</sub></i>	A/C
Soleus +/+	214.56 ± 2.56	35.15 ± 2.35	5.79 ± 1.79	99.71 ± 13.88	37.61 ± 2.87	6.67 ± 1.79
Soleus -/-	159.14 ± 7.05*	21.73 ± 0.20*	5.26 ± 0.19	153.31 ± 7.30**	28.94 ± 1.03**	5.76 ± 0.83
Cardiac +/+	157.77 ± 5.32	47.46 ± 2.00	4.75 ± 0.52	79.05 ± 5.68	47.27 ± 3.08	4.79 ± 0.79
Cardiac -/-	118.59 ± 9.34**	29.35 ± 5.31**	3.67 ± 0.66	61.09 ± 4.32***	34.13 ± 1.63*	4.03 ± 1.00
Gastrocnemius +/+	12.80 ± 2.80	9.45 ± 1.13	4.34 ± 0.43	NA	NA	NA
Gastrocnemius -/-	15.43 ± 1.16	9.49 ± 0.42	5.19 ± 0.53	NA	NA	NA

*K<sub>m</sub>* is given as μM ADP; *V<sub>max</sub>* as ng atom O/mg min. A/C is calculated as *V<sub>max</sub>*/*V<sub>basal</sub>*.

\**P* < 0.005.

\*\**P* < 0.010.

\*\*\**P* < 0.05.



the presence of creatine is enhanced at submaximal ADP levels, and the  $K_m$  for ADP is lowered (Table II). However, in desmin-null soleus fibers, creatine has no effect on ADP-stimulated respiration, indicating that coupling of creatine kinase and ANT is somehow lost in this tissue in the absence of desmin.

## Discussion

We have demonstrated that lack of desmin leads to loss of proper mitochondria distribution, number, morphology, and function. This is indeed in agreement with previous ultrastructural observations which have suggested potential physical association of mitochondria with intermediate filaments in both muscle (Tokuyasu et al., 1983a,b; Stromer and Bendayan, 1990; Lockard and Bloom, 1993; Reipert et al., 1999) and other tissues (Bereiter-Hahn and Voth, 1994; Letierrier et al., 1994; Hollenbeck, 1996; Rappaport et al., 1998; Yaffe, 1999). Whether this association is direct or indirect through associated proteins remains elusive. In any case, loss of this association could explain the abnormal mitochondrial distribution in desmin-null muscle. Recent studies in yeast have functionally linked *mdm1*, a protein with some similarity to mammalian IF proteins, with proper mitochondria transport (Fisk and Yaffe, 1997). Though *mdm1* is not considered an IF protein, this correlation could assist in the identification of IF-mitochondrial association. At present it is unknown why the mitochondrial aggregates tend to localize beneath the sarcolemma. It should be noted that subsarcolemmal clumping occurs normally to a much lower extent in wild-type mouse muscle, especially in sarcolemmal areas adjacent to capillaries, than in desmin-null animals, in which it is often observed in areas away from capillaries. Furthermore, subsarcolemmal localization of mitochondria in wild-type animals increases with age. A possible correlation among this age-dependent behavior of mitochondria, the desmin-null mitochondrial abnormalities, and the shortening of life span in desmin-null mice remains to be determined. The distribution of mitochondria in some desmin-null fibers appears relatively normal, suggesting that other cellular structures also contribute to proper mitochondrial positioning. As microtubules and F-actin are also found in striated muscle, these cytoskeletal systems may play a role in mitochondrial positioning, and thus counteract the loss of desmin. It should also be pointed out that there are other IFs known to be present in adult striated muscle, including synemin, paranemin (Hemken et al., 1997; Bilak et al., 1998), and now apparently nestin as well (Carlsson et al., 1999). These IF molecules could also play critical roles in mitochondrial positioning.

Severe mitochondrial proliferation can be observed in some fibers of the desmin-null heart, particularly under work overload (Fig. 4 C). The mechanism by which the absence of desmin leads to an increase in mitochondrial number remains elusive. It is unknown how cells control the number of mitochondria they contain. Increased oxidative demand or mitochondrial energy deficiency is generally believed to trigger a compensatory induction of mitochondrial proliferation. This hypothesis is strongly supported by a mouse model for mitochondrial myopathy generated by knockout inactivation of the heart/muscle

isoform of the ANT (Graham et al., 1997; Wallace, 1999). It seems possible that compromised mitochondrial function in desmin-null muscle might be the trigger for the mitochondrial proliferation that is observed. In addition to triggering proliferation, there must also be a mechanism by which transcription of both nuclear and mitochondrial oxphos genes are coordinately induced to promote mitochondria proliferation. Communication between the mitochondria and the nucleus is probably very important for mitochondria biogenesis, but how it takes place is not clear. The participation of IFs in such processes is unclear, but it has not been excluded. Recent studies have revealed some regulatory factors associated with mitochondrial proliferation, such as nuclear respiratory factor 1, nuclear respiratory factor 2, and mitochondrial transcription factor A (Wu et al., 1999). It will be of interest to ascertain if muscle from desmin-null animals displays elevated levels of these regulatory factors. In the case of skeletal muscle, it is not clear if the subsarcolemmal aggregates of mitochondria that can be found in most desmin-null slow oxidative skeletal muscle fibers arise from migration of pre-existing myofibrillar mitochondria or from proliferation of subsarcolemmal mitochondria. A lack of increase in citrate synthase levels in leg skeletal muscles from desmin-null mice, coupled with loss of mitochondria from the interior of some fibers, suggests that migration of mitochondria is possible.

In addition to regulating mitochondrial positioning, it has been postulated that interactions of the cytoskeleton with mitochondria may modulate mitochondrial function. Mitochondrial function could be influenced by changes in mitochondrial shape, by stretching and by contraction of the mitochondrial membrane, which could be directed via the cytoskeleton (Rappaport et al., 1998). Additionally, mitochondrial function could also depend on defined interactions of outer mitochondrial membrane proteins with specific cytoskeletal proteins or cytoskeleton-associated proteins. Specifically, it has been postulated that the cytoskeleton somehow plays a role in the affinity of mitochondria for ADP (Letierrier et al., 1994; Saks et al., 1995, 1998). Previous studies have shown that mitochondrial respiration and affinity for ADP *in vivo* are not influenced by the presence of drugs that disrupt microfilaments and microtubules (Saks et al., 1996). This study and a related study (Kay et al., 1997), however, demonstrate that the absence of the IF protein desmin does influence mitochondrial respiration and affinity for ADP in cardiac muscle and certain skeletal muscle fibers.

In our work, we demonstrate that mitochondria in both cardiac and soleus muscles from desmin-null mice display increased affinity for ADP (lowered  $K_m$ ) and decreased maximal respiration *in vivo*. These results are not caused by damage to the outer mitochondrial membrane, as indicated by results of the cytochrome C test. Nor in the case of maximal respiration rate are they explained by a decrease in mitochondrial content. Isolated mitochondria from desmin-null animals display normal respiratory function typical of cardiac mitochondria *in vitro*. Thus, the alterations in mitochondrial respiration are only observed *in vivo*, and are displayed in fibers lacking desmin. Moreover, defects are not observed in fast glycolytic fibers of the gastrocnemius in desmin-null animals, muscle fibers

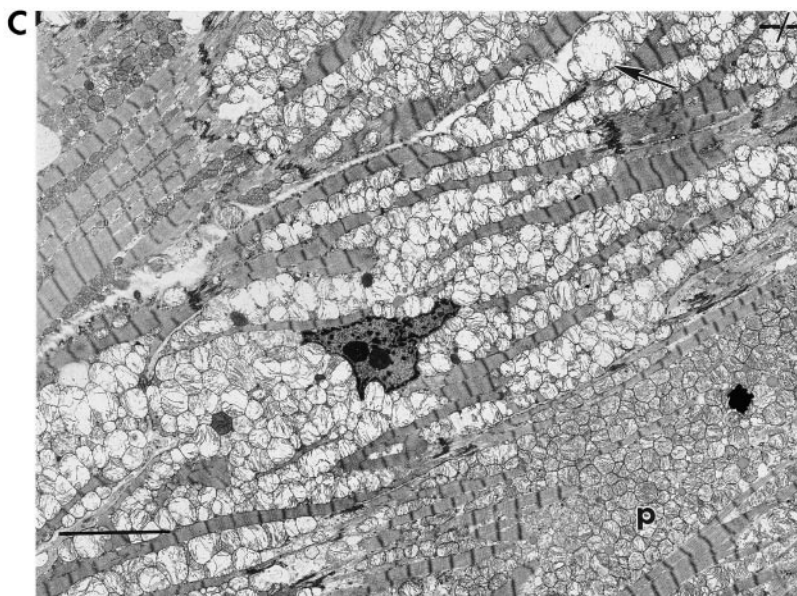
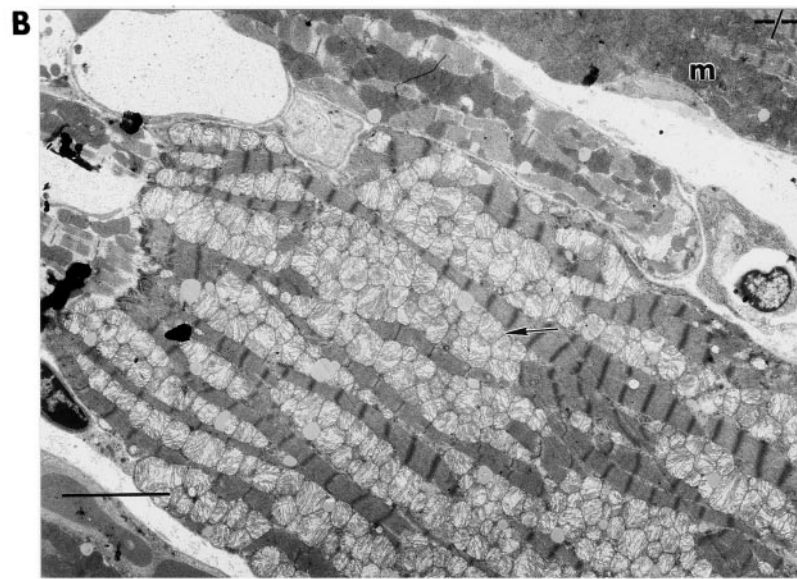
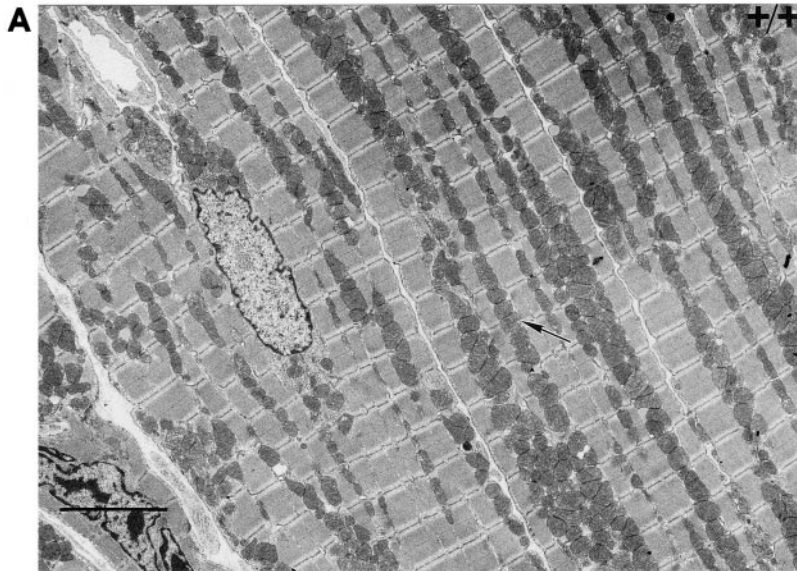


Figure 4 (continues on facing page).

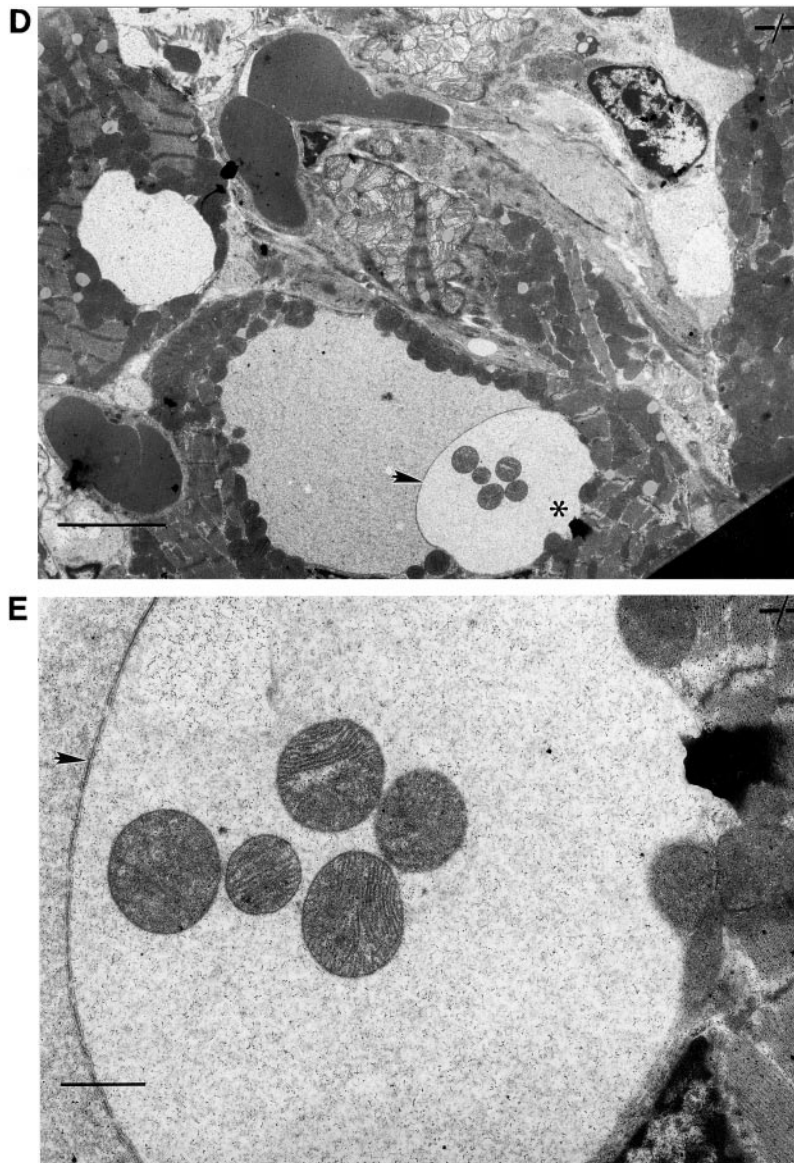


Figure 4. Ultrastructural observations of mitochondrial alterations in cardiac muscle. (A) Wild type (+/+) cardiac muscle showing normal cardiomyocyte ultrastructural appearance with laterally aligned myofibrils and strands of mitochondria (arrow) between the myofibrils. (B) Desmin-null (-/-) cardiac muscle demonstrating proliferation and swelling of mitochondria (arrow) between myofibrils and subsarcolemmal clumping of mitochondria (m). (C) Desmin-null cardiac muscle from exercised mice exhibiting severe mitochondrial swelling, including mitochondria with broken cristae (arrow). An area of severe mitochondrial proliferation is also observed (p). (D) Desmin-null cardiac muscle showing severe architectural disruption, large empty spaces surrounded by mitochondria, and mitochondria sequestered within a membrane-limited structure (arrowhead). (E) High magnification view of area in D (asterisk) showing mitochondria enclosed in a membrane-limited structure (arrowhead) within a myocyte. Note the mitochondria display normal condensed morphology. Bars: (A–D) 5  $\mu\text{m}$ ; (E) 1  $\mu\text{m}$ .

that have very low mitochondrial content. Thus, it appears that the desmin cytoskeleton influences mitochondrial respiratory activity *in vivo* in cardiac and slow oxidative skeletal muscle.

The results of this study are generally in agreement with *in situ* respiratory measurements reported previously (Kay et al., 1997). Both studies demonstrated no effect on *in situ* respiration in skinned fibers from desmin-null gastrocnemius muscle, and the presence of creatine lowered the apparent  $K_m$  for ADP in skinned cardiac muscle fibers from both desmin-null and wild-type mice. The Kay group was able to identify two populations of mitochondria in skinned desmin-null cardiac and soleus fibers by kinetic analysis, one population with normal affinity for ADP, and another with dramatically increased affinity for ADP (decreased  $K_m$ ). Although the kinetic programs used in our study could not calculate  $K_m$  values of two distinct populations of mitochondria, the  $K_m$  values obtained in this study could be considered as the average  $K_m$  of two mitochondrial populations. The presence of two popu-

lations of mitochondria could reflect the observations obtained by enzyme histochemistry and electron microscopy, which demonstrated considerable increase of subsarcolemmal mitochondrial population in both cardiac and soleus skeletal muscle.

It is not entirely clear how the cytoskeleton in general, or desmin in particular, may influence mitochondrial function. While ultrastructural studies have observed association of desmin IFs with mitochondria, direct biochemical data demonstrating desmin binding to mitochondria or to a specific mitochondrial outer membrane protein(s) are lacking. One possibility proposed by Saks and colleagues (Saks et al., 1995; 1998; Kay et al., 1997) is that the disorganization or absence of the IF cytoskeleton results in the release of a cytoskeletal-associated mitochondrial binding factor, which normally regulates the permeability of the outer membrane for adenine nucleotides. It is distinctly possible that a desmin binding protein, such as plectin (Reipert et al., 1999), may interact with a mitochondrial membrane protein, thus serving as a cross-linker of the

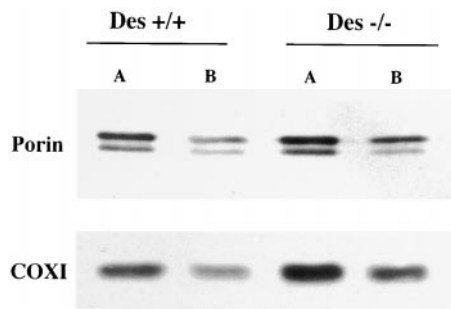


Figure 5. Western blot analysis of total protein extracts from myocardial tissue of desmin null (Des<sup>-/-</sup>) and wild-type (Des<sup>+/+</sup>) mice for the mitochondrial proteins porin and cytochrome oxidase (COXI). The membranes were probed with the corresponding antibodies. The lower porin band is a commonly obtained porin degradation product. A: twice the total protein of B.

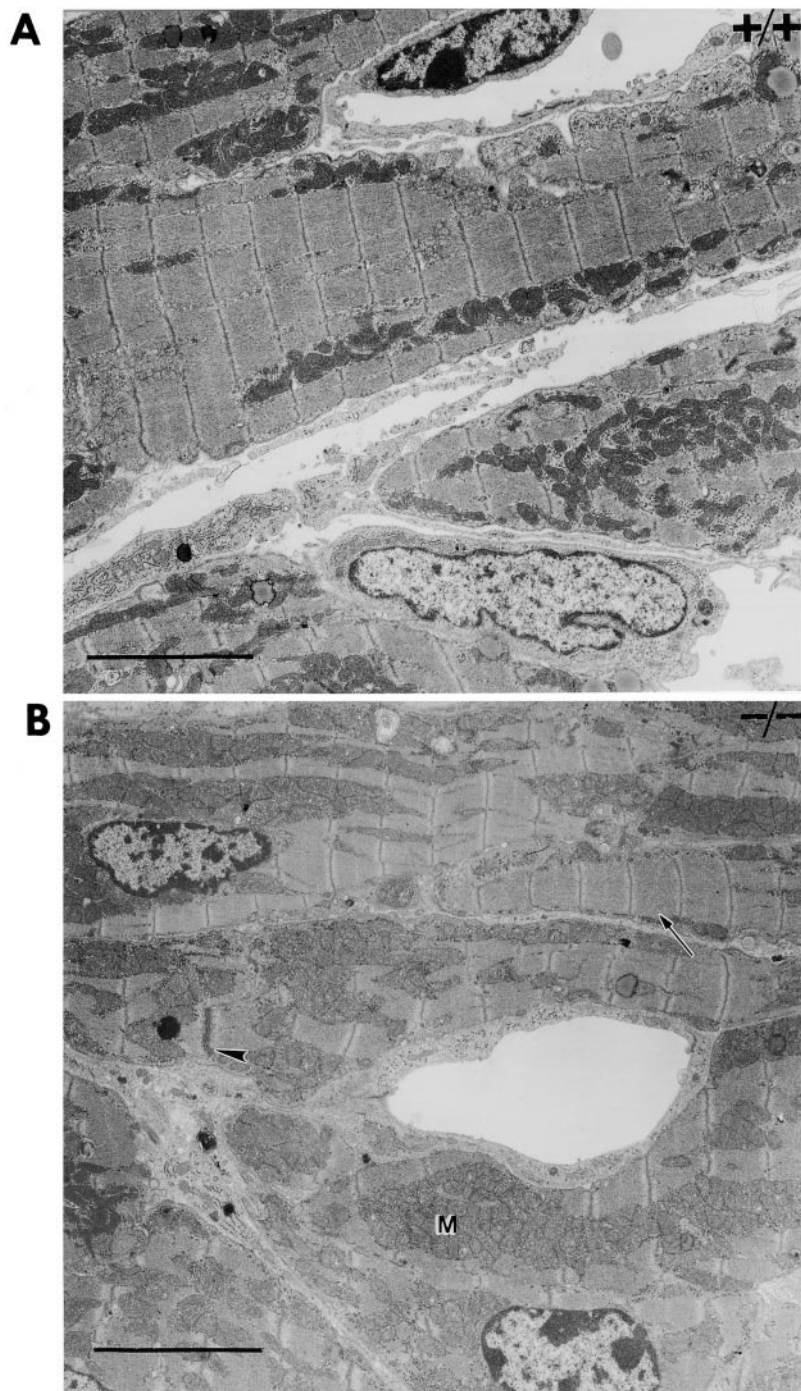
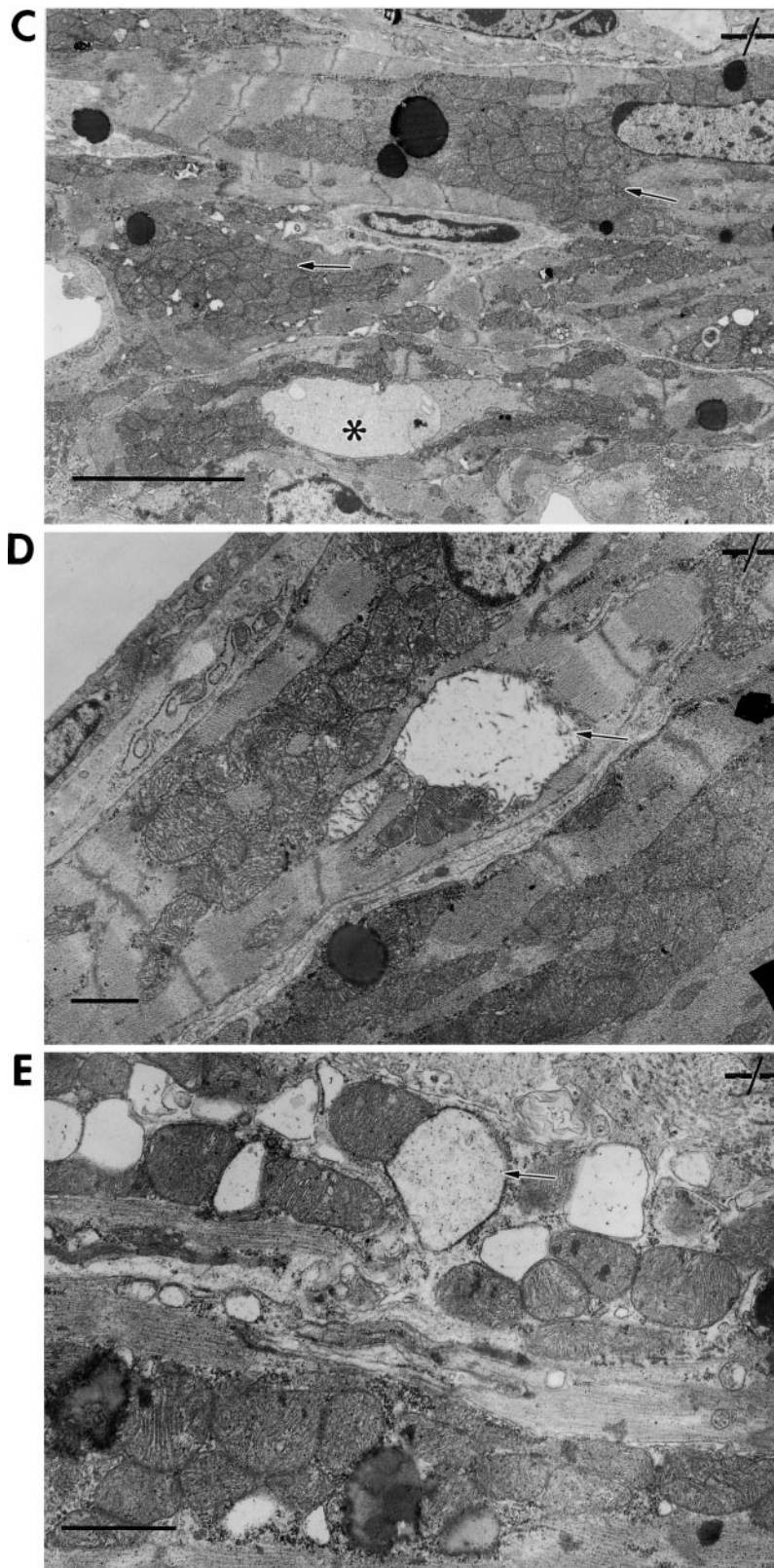


Figure 6 (continues on facing page).

desmin IF cytoskeleton to the mitochondrial outer membrane. This possibility is also supported by the demonstration that the microtubule binding protein MAP2 binds to the mitochondrial porin voltage-dependent anion channel (Linden and Karlsson, 1996). More and more IF-interact-

ing proteins are being identified that serve as cross-linkers of the IF cytoskeleton to the other cytoskeletal networks (Yang et al., 1996, 1999; Cleveland and Fuchs, 1998). With this in mind, it is interesting to note that mice lacking dystonin (BPAGn1), a known IF-microfilament cross-linker,



*Figure 6.* Mitochondrial changes begin before other ultrastructural changes in young desmin-null mice. Sections of cardiac muscle from 2-wk-old desmin-null ( $-/-$ ) animals (B–E) display mitochondrial changes similar to but less severe than those seen in adults, including occasional clumping and slight increase in mitochondria number (C, arrow), swelling, and degeneration (D and E, arrows) compared with wild-type ( $+/+$ ) sections (A). Other ultrastructural changes seen in adult desmin-null animals are not observed in 2-wk-old desmin-null sections, as myofibrils (arrow) and intercalated disks (arrowhead) appear normal (B). Also note the large membrane-limited structure (C, asterisk) inside a cardiomyocyte that is similar to the structures seen in adult null cardiomyocytes (Fig. 4 D). M, mitochondria. Bars: (A–C) 5  $\mu$ m; (D and E) 1  $\mu$ m.

also display perturbations of mitochondrial positioning in skeletal muscle (Dalpe et al., 1999).

Whether the influence of desmin on mitochondrial activity occurs via direct interaction or interaction mediated via a linker, it is interesting to note that the linkage of desmin IFs to mitochondria might provide a means by which contractile activity could mechanically influence mitochondrial activity, as desmin IFs seem to link mitochondria to the myofibril at the Z disc. This could also explain why there is no observable effect in fast glycolytic fibers, as these fibers have only sparse mitochondria and the energy for contractile activity is generated almost exclusively by glycolysis, whereas cardiac and slow oxidative muscles rely overwhelmingly on oxidative phosphorylation. Thus, increased contractile activity could result in increased mechanical perturbation of mitochondria, and could stimulate respiratory activity.

In addition to the mitochondrial defects, several other defects can be seen in desmin-null muscle. At the ultrastructural level, loss of contractile material, loss of proper myofibrillar alignment, and separation of myofibrils from the intercalated disk can be observed (Li et al., 1996; Milner et al., 1996), in addition to mitochondrial abnormalities. However, it is not clear which defects arise first. To address this, we examined ultrastructurally samples from 2-wk-old mouse cardiac muscle. Between birth and 2 wk of age, degenerative lesions are rarely observed, but such are later found in every desmin-null animal examined between 2 and 3 wk of age (Milner, D.J., and Y. Capetanaki, unpublished results). While not as severe as the observations in adult animals, we observe mitochondrial abnormalities and degeneration in the absence of any other noticeable structural defect in desmin-null cardiac muscle from 2-wk-old animals. Thus, mitochondrial alterations are the earliest observable differences we can detect in desmin-null cardiac tissue.

In conclusion, it is suggested that the absence of desmin leads to loss of proper mitochondrial positioning which might be the cause of the observed compromised mitochondrial function. As a consequence, cell death follows either directly due to energy deprivation or/and indirectly due to oxidative stress caused by mitochondrial proliferation and eventually degeneration and direct release of death triggering factors.

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