NF90 regulates cell cycle exit and terminal myogenic differentiation by direct binding to the 3’-untranslated region of MyoD and p21WAF1/CIP1 mRNAs

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Running title: Diaphragmatic respiratory failure in NF90(-/-) mice

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NF90 and splice variant NF110/ILF3/NFAR are double-stranded RNA-binding proteins that regulate gene expression. Mice with targeted disruption of NF90 were engineered: NF90(-/-) mice were born small and weak and succumbed to perinatal death within 12 h due to neuromuscular respiratory failure. Lung inflation and morphology were normal in NF90(-/-) mice. The diaphragm and other skeletal muscles in NF90(-/-) mice demonstrated disorganized arrangement and paucity of myofibers, evidence of myocyte degeneration and increased apoptosis. The expression of myogenic regulators, MyoD, myogenin and p21WAF1/CIP1, was severely decreased in NF90(-/-) mice. These myogenic transcription factors and cell cycle inhibitor are regulated in part through posttranscriptional mRNA stabilization. Northwestern blotting revealed that NF90 is the principal and specific p21WAF1/CIP1 and MyoD 3’-untranslated region RNA-binding protein in developing skeletal muscles. NF90 regulates transcription factors and a cell-cycle inhibitor essential for skeletal muscle differentiation and for survival.

Keywords: Transcription, NF45, RNA stability, skeletal muscle

Introduction

Proteins that contain double-stranded RNA binding motifs (dsRBM) contribute to gene regulation at multiple levels. The dsRBM consists of 65-75 amino acids that adopt an α−β−β−β−α conformation that confers the ability to bind structured RNAs. The dsRBM is evolutionarily conserved from E.coli (RNAse III) through Drosophila (Staufen), to humans (NF90, PKR, ADAR). Proteins with dsRBMs contribute to RNA metabolism at multiple levels including transcription (NF90 and RNA helicase A), RNA splicing (NF90), RNA editing (ADAR), RNA export (NF90), subcellular RNA localization (NF90, Staufen) and translation initiation (NF90, PKR) (reviewed in (1-3)). Posttranscriptional gene silencing through RNA interference involves enzymatic cleavage of RNA by the dsRBM protein, DICER (4).

NF90 and its splice variant, NF110, are proteins with 2 dsRBMs, which are localized predominantly in the nucleus of diverse cells and tissues (5). Alternative names conferred on the NF90 family include human mitotic phase phosphoprotein 4 (MPP4) (6), Xenopus 4F.1 and 4F.2 (7), murine interleukin enhancer binding factor (ILF)-3 (8), translation control protein (TCP)-80 (9), double-stranded RNA-binding protein (DRBP)-76 (10), and nuclear factor
associated with RNA (NFAR)-1 and 2 (11). We originally purified NF90 and NF45 in a complex with lupus autoantigens Ku70 and Ku80 from the nucleus of activated Jurkat T-cells (12). The NF90/NF45/Ku complex bound specifically to the antigen receptor response element/nuclear factor of activated T-cells (NF-AT) binding site in the human IL-2 promoter (5,12,13). NF90 and NF45 stabilize the association between the catalytic subunit of DNA-dependent protein kinase, DNA-PKcs and DNA-targeting subunits, Ku70 and Ku80 (14). NF90 and NF110 activate or repress transcription depending on the promoter context (11,15). NF90 and NF45 specifically transactivate the IL-2 promoter in activated T-cells (16)(Zhao and Kao, unpublished observations). The arginine-glycine rich C-terminal domain of NF110/ILF3 is methylated in vitro by protein arginine methyltransferase 1 (17), and in vivo methylation may modulate transcriptional regulation and chromatin reorganization (18).

The Xenopus homolog of NF90 and NF110 is termed CCAAT-box transcription factor (CBTF) and regulates the transcriptional activation of the hematopoietic regulatory factor, GATA-2 (19). In early development, CBTF is retained in the cytoplasm through interactions with maternal RNA. At the midblastula transition, degradation of maternal RNA releases CBTF for translocation into the nucleus, followed 4h later by development of CCATT DNA-binding activity and transcriptional activation of GATA-2 (20). The dsRBMs of CBTF are capable of binding both DNA and RNA (21), and intact dsRBMs are required for transcriptional control (15).

In addition to roles in transcriptional regulation, NF90 contributes to RNA splicing as a component of the spliceosome (11,22). NF90 specifically binds to AU-rich elements in the 3’ UTR of IL-2 mRNA, stabilizing IL-2 mRNA against rapid degradation, and contributing to the upregulation of IL-2 gene expression in activated T-cells (23). NF90 mediates nuclear export of IL-2 mRNA to the cytoplasm through the interaction of NF90’s nuclear export signal with the nuclear export karyopherin, exportin-5 (24). Redistribution of NF90/MPP4 from the nucleus to the cytoplasm during mitosis is associated with increased phosphorylation (6,13).

In the cytoplasm, NF90 is associated both with translationally quiescent ribonucleoprotein (RNP) complexes and with ribosomes (25). Like many other mRNP components, NF90 is an autoantigen in human and experimental systemic lupus erythematosus (26). DsRBM proteins such as NF90/ILF3 are highly expressed in the testis, where they contribute to gene regulation by repressing the translation of nascent mRNAs (8). The mRNA encoding acid β-glucosidase is specifically translationally repressed by NF90/TCP80 (9). A search for other liver mRNAs specifically bound and translationally repressed by NF90/TCP80 identified aldolase B, complement protein 8 and fibronectin receptor β1 (9). The prototypic dsRBM protein, staufen, contributes to localized translation of specific mRNAs in mammalian neurons (27). Tau is a microtubule-associated protein that is preferentially localized in axons, and abnormal accumulations of tau form neurofibrillary tangles that contribute to the pathology of Alzheimer’s dementia (28). The 3’ UTR of tau mRNA contains AU-rich elements that contribute to axonal targeting; the axonal targeting element is specifically bound by NF90 and NF110/ILF3 (29). The interactions between cytoplasmic NF90/TCP80, translation elongation initiation factors α, β and γ (14) and PKR (10,11,25,30) are likely to regulate translation of specifically-bound mRNAs.

NF90 has been implicated in host antiviral responses (25,30) (31,32). Virally encoded structured RNAs interact specifically with NF90 dsRBMs, including the HIV TAR loop, the adenovirus VA2 RNA (30), hepatitis B RNA (33) and the 5’ and 3’ UTRs of the bovine virus diarrhea RNA virus (32). Specific binding of NF90 and NF45 to both the 5’ and 3’ UTRs of BVDV RNA virus was proposed to regulate the balance between viral replication and translation (32).
To further characterize the role of NF90 in regulation of gene expression and development, we have generated mice with targeted disruption of NF90. Our observation of perinatal lethality associated with muscle weakness and respiratory failure in NF90(-/-) mice revealed an unexpected contribution of NF90 to myogenic differentiation. Muscle development is regulated through the coordinated expression of myogenic regulatory factors that include MyoD, Myf5, myogenin and Mrf4, and cyclin-dependent kinase inhibitors of the p21WAF1/CIP1 family (34,35). MyoD and Myf5 direct primary myocyte differentiation, myogenin directs secondary differentiation, and p21WAF1/CIP1 expression promotes cell cycle exit and terminal differentiation. In differentiating myoblasts and regenerating adult muscle, upregulation of MyoD, myogenin and p21WAF1/CIP1 expression occurs concurrently through transcriptional mechanisms and posttranscriptional mRNA stabilization by specific RNA-binding proteins (35,36). Here, we demonstrate that MyoD, myogenin and p21WAF1/CIP1 are novel target genes essentially regulated by NF90. Our results lead us to propose that the phenotype of muscle weakness and increased apoptosis in NF90(-/-) mice is explained by defects in skeletal myocyte differentiation arising from insufficient expression of myogenic regulatory factors.

**Experimental Procedures**

**Genomic cloning and targeting vector generation:** The murine genomic NF90/ILF3 was isolated from a 129SvJ library using the human NF90 cDNA probe (performed at Incyte Genomics). The nucleotide sequence and intron-exon structure of the 29085 bp gene was determined and deposited in GenBank (AF506968).

A targeting vector was designed for constitutive disruption of NF90 exons 2-4 (Figure 1A). The 5’ targeting arm was a 6.75 kB BamH1 restriction fragment digested from a genomic NF90 subclone, and the 3’ targeting arm was 2.8 kB generated with PCR primers that introduced flanking EcoR1 restriction sites (sense primer b3: 5'-GGAGTGCGGTCTTCTGCGCCAGTTGCG-3’ and anti-sense primer c3: 5’-GGCAGATTCTCTCAGCTATCAAGGA-3’). These targeting arms were cloned into pKO Scrambler NKTV-1903 targeting vector, which contains a positive selection neomycin resistance gene and a herpes thymidine kinase negative selection gene (Stratagene). Embryonic stem cells (R1) were transfected by electroporation and selected for resistance to G418 and ganciclovir.

**Genotype analysis by Southern hybridization and PCR:** Genomic DNA was isolated from ES cells or tail biopsy specimens, digested with HindIII (3’ analysis) or EcoR1 (5’ analysis), and separated on a 0.7% agarose gel followed by transfer to nitrocellulose membrane. Southern hybridization of the specific wild type and targeted genomic fragments was performed using randomly-primed 32P-labeled probes outside the targeting arms (Figure 1a), or an internal probe that hybridized to the neomycin gene, according to standard methods.

Genotyping was also performed by PCR across the 3’ targeting arm, using LA Taq™(Takara Bio) with locus primers, b3-5’-GGAGTGCGGTCTTCTGCGCCAGTTGCG-3’ & C4-4, 5’-GGTCCAGCCTCGACTGAGTAAGCTAA GAGGGC-3’, or target primers, Neo-1179, 5’-TATCAGGACATAGCGTTGGCTACCCGTGA TATT-3’ & C4-4. PCR amplification was conducted according to the manufacturer’s directions consisting of an initial incubation at 94°C for 2 min, followed by 35 cycles at 98°C for 20 s, 68°C for 3 min.

**Expression analysis by Northern and Western hybridizations:** Total RNA was extracted from newborn mice using Trizol reagent (Invitrogen), and 20 µg was fractionated in a 1% denaturing agarose gel then transferred to nitrocellulose. Northern hybridization was performed using a randomly primed 32P-labeled probe for NF90.
Extracted total proteins (20 µg) from newborn mice were fractionated by SDS-PAGE (8% separating gel) and transferred to nitrocellulose membranes. Western immunoblotting was performed using rabbit polyclonal antisera (1:1000 dilution) generated against recombinant NF90 protein (5), followed by secondary goat anti-rabbit antibody (1:3000 dilution) and detection using enhanced chemiluminescence (Amersham). Immunoblotting analysis of wild type murine tissue extracts was performed using a monoclonal antibody that recognizes NF90 (anti-DRBP76. Transduction Laboratories 612154), followed by a goat anti-mouse secondary antibody (Santa Cruz SC-2031).

Histology, immunohistochemistry and in situ TUNEL analysis: Whole embryos or newborn mice with skin removed were fixed in 10% neutral buffered formalin followed by paraffin-embedding and tissue sectioning at 4-µm thickness. Staining with hematoxylin and eosin was performed according to standard procedures.

For immunodetection of NF90, sections were deparaffinized and rehydrated, then boiled for 20 min in 10 mM citric acid in a microwave oven. Blocking of nonspecific mouse antigens was performed by incubation with 3% H2O2 for 5 mins followed by incubation with the Vector M.O.M. immunodetection kit (Vectorlabs) for 1 h. Anti-DRBP76 monoclonal antibody specific for NF90/ILF3/DRBP76 (Transduction Laboratories) was incubated at 1:100 dilution for 35 min at room temperature. The signal was amplified by using VECTASTAIN Elite ABC Reagent (Vector labs) and visualized by a HRP reaction using stable diaminobenzidine (Invitrogen).

In situ apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL; TACS 2 TdT, Trevigen).

Reverse Transcription Polymerase Chain Reactions. RT-PCR analysis was performed to detect mRNA expression of myogenic regulatory factors and cell cycle control protein p21\textsuperscript{WAF1/CIP1}. Total RNA from skeletal muscles of newborn mice (2-5 µg) was used as template for first strand cDNA synthesis using Superscript reverse transcriptase (Invitrogen), according to the manufacturer’s directions. PCR amplifications were subsequently performed using 20% of the first-strand cDNA mixture, and specific primers for p21\textsuperscript{WAF1/CIP1} (NM_007669) 511+ 5’-GCCAGACCAGCAGCAGATTTT (with one mismatched base from human p21\textsuperscript{WAF1/CIP1} underlined) and 883- 5’-CCCTCCCAAACCAAAGCTCTG (372bp), myogenin (BC048683) 5’-GAGGCCGATCTCCGCTACAGAGG and 5’-CTGGCTTGTGGCCAGCCAG (380bp), and MyoD (NM_010866) 5’-AGGCTCTGTGCACGCAGGCC and 5’-TGCAGTCATCTCTCAAGCACC (489bp). Control amplification of murine β-actin mRNA was performed using primers 5’-AGACCGGGGTCACCCACACTGTGCCCATCT and 5’-CTAGAAGCACTTGGCAGCATGGAA GGG (671bp). The PCR cycling conditions were 24 cycles of denaturation at 95°C for 30”, annealing at 55°C for 30” and extension at 72°C.

Northwestern Blotting. Partial cDNAs of murine MyoD, myogenin and p21\textsuperscript{WAF1/CIP1} were amplified by RT-PCR from skeletal muscle RNA. Nested amplifications were performed with a forward primer containing a T7 RNA polymerase recognition sequence, T7, CCAAGCTTCTAATACGACTCACTATAGGG AGA. Primers to generate DNA templates for T7-directed \textit{in vitro} transcription of the 3’ UTRs were: MyoD forward (T7-1241+), 5’ T7-GAGACTCTTCCAACACTGCTTCC and MyoD reverse (1762 -), 5’ -GCACTACACAGCATGCT (36), p21\textsuperscript{WAF1/CIP1} forward, T7-511+ and 883- (above). Radiolabeled RNA probes were generated by \textit{in vitro} transcription using T7 RNA polymerase, in the presence of \textsuperscript{32}P-labeled UTP (36), according to the manufacturer’s directions (Promega).
Murine tissue extracts (testis, brain, skeletal muscle, 80 µg each) or recombinant NF90 and NF45 proteins (5) (10µg) were fractionated by 10% SDS-PAGE, and transferred electrophoretically (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol) to nitrocellulose membranes. The proteins were renatured by incubating the membranes overnight at room temperature in Northwestern buffer (10 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM MgCl2 0.1% gelatin, 0.1% Tween20, containing salmon sperm DNA (50µg/ml). The membranes were hybridized with the radiolabeled RNA probes (2-4 x 10^7 cpm) for 2 h at room temperature in the presence of yeast tRNA (50µg/ml) (29,37), washed twice for 5 min at room temperature and once at 45˚C for 2 h, followed by autoradiography.

Results

**Generation of mice with targeted disruption of NF90 gene.** The targeting strategy for NF90 was to disrupt Exons 2-4, eliminating the ATG translation initiation codon and introducing a translational frameshift that disrupts the open reading frame (Figure 1A). The targeting vector was introduced into R1 embryonic stem cells by electroporation and transfected ES cells were subjected to positive and negative selection. Twelve out of 110 G418/gancyclovir-resistant ES cell clones showed appropriate gene targeting. The targeted allele shows a Southern hybridization signal at 9 kB, in addition to the wild type allele with a hybridization signal of 7.4 kB (Figure 1B, lane 4). Targeted ES cell clones were further analyzed for correct integration using an internal probe for the neomycin resistance gene (data not shown). Three independently derived ES cell lines were injected into blastocysts (C57BL/6), and chimeric mice were derived. Two separate NF90-targeted ES cell lines transmitted through the germline. In mice examined through 18 months of age, NF90(+/-) heterozygote mice demonstrate no abnormal phenotypes.

**Gene expression analysis in NF90(-/-) mice.** We analyzed the expression of NF90 mRNA and protein in Day 1 NF90 homozygote, heterozygote and wild type littermates. The Northern analysis shows that wild type mice express a single NF90 mRNA migrating at 3.4 kB (Figure 2A, lanes 2, 4, 5), and heterozygote mice express two isoforms of NF90 mRNA, migrating at 3.4 and 3.1 kB (Figure 2A, lanes 1, 3). These mRNA sizes are consistent with transcription of both wild type and targeted NF90 alleles. The NF90(-/-) mice demonstrate expression of a single isoform of NF90 mRNA migrating at 3.1 kB (Figure 2A, lanes 6, 7), and this size is consistent with transcription just of targeted alleles, which lack exons 2-4. The prominent intensity of the truncated 3.1 kB targeted NF90 mRNA in NF90(-/-) mice indicates that the endogenous NF90 promoter is functional following deletion of Exons 2-4. Furthermore, the increase in intensity of the 3.1 kB truncated form of NF90 indicates...
M4:11034R2 Shi et al, Diaphragmatic respiratory failure in NF90(-/-) mice

mRNA compared to the 3.4 kB wild type form (Figure 2A, lanes 6, 7 vs. 2, 4, 5) suggests that the null phenotype (absent NF90 protein, Figure 2B) is associated with increased transcription of the NF90 gene. Because the targeting strategy introduced a translational frameshift downstream of exon 4, there is no NF90 protein synthesized in NF90(-/-) mice (see below and Figure 2B). Our prior studies demonstrated that transgenic expression of histidine-tagged NF90 protein potently suppressed the expression of endogenous NF90 protein (5). Here, the absence of NF90 protein in NF90(-/-) mice is associated with increased mRNA expression of the 3.1 kB targeted NF90 allele, through transcriptional or posttranscriptional upregulation.

We analyzed protein expression of NF90 in extracts prepared from newborn wild type, heterozygote and homozygote NF90 mice by Western immunoblotting (Figure 2B). In NF90 wild type and heterozygote mice we observed a prominent immunoreactive band at 90 kDa, and a fainter band at 110 kDa, similar to the principal size observed in human Jurkat T-cell nuclear extracts (5) (Figure 2B, lanes 1-5). The level of NF90 protein expression in heterozygote NF90 mice was slightly decreased from that in wild type mice (Figure 2B, lanes 1 and 3 vs. 2, 4, 6). The NF90(-/-) mice showed complete absence of the 90 and 110 kDa bands (Figure 2B, lanes 6, 7 vs. 1-5), demonstrating that the translational frameshift in the knockout phenotype was associated with complete absence of NF90 and NF110 protein expression. Examination of the tissue-specific expression of NF90 and NF110 proteins in adult wild type mice showed the greatest expression in testis, brain and skeletal muscles (Figure 2C, lanes 8, 2, 6), and moderate expression in heart, spleen, lung, liver and kidney (Figure 2C, lanes 1, 3, 4, 5 and 7).

Histological analysis of NF90(-/-) mice. We performed careful histology on hematoxylin and eosin-stained sections of developing and newborn wild type, heterozygote and homozygote NF90 mice and did not discern any dramatic organ-specific developmental abnormalities. Consistent with the overall size reduction of the homozygote NF90-deficient mice, we observed a proportionate reduction in the size of all the internal organs, compared to wild type and heterozygote littermates.

We characterized the tissue-specific expression and subcellular localization of NF90 protein by immunohistochemistry with a monoclonal antibody that specifically recognizes NF90 (Figure 3). Wild-type mice show widespread expression of NF90 protein, predominantly in the nucleus of cells, including cardiomyocytes (Figure 3A), lung epithelial and endothelial cells (Figure 3B), certain hepatocytes (Figure 3C), and most epithelial cells lining the stomach (Figure 3D). We observed the strongest expression of NF90 in cerebral (Figure 3E) and cerebellar (Figure 3F) neurons, and in skeletal muscles of the back (Figure 3G) and diaphragm (Figure 3H) in wild type mice. Skeletal muscles (Figure 3G), certain neurons (Figure 3F) and epithelial cells (Figure 3B, D) of NF90(+/+) mice revealed moderate NF90 immunostaining in the cytoplasm. For comparison, NF90 immunostaining was completely absent in NF90(-/-) mice in cerebral (Figure 3I) and cerebellar (Figure 3J) neurons, and in skeletal muscles of the back (Figure 3K) and diaphragm (Figure 3L). These results support the specificity of the monoclonal antibody in recognizing NF90, and confirm that NF90(-/-) mice express no detectable NF90 protein.

As the cause of death of the homozygote NF90-deficient mice appeared to be respiratory failure, we examined the lung histology in detail. We found no evidence of lung developmental abnormalities, insufficient lung inflation, pulmonary edema or hyaline membrane formation in the NF90(-/-) mice that might explain perinatal death due to respiratory failure. We detected no differences in the expression of lung surfactant genes SP-A, B and C, and a 50% decrease in SP-D expression by RT-PCR in NF90(-/-) mice compared to wild-type littermates (data not shown). Surfactant protein-D functions
principally in lung host defense, and mice lacking SP-D are viable after birth (38).

**Blood, serum and lymphocytes subset analyses of NF90(-/-) mice.** Complete blood count analyses of the neonatal mice were difficult to perform due to the low amount of blood recoverable at sacrifice. However, there was a consistent suggestion of anemia in the NF90(-/-) mice (data not shown). White blood cell counts and differentials were similar between NF90(-/-) mice and wild-type littermates. There were no significant differences in the clinical chemistry measurements of hepatic or renal function between homozygote, heterozygote and wild type mice NF90 mice (data not shown).

Flow cytometry analyses of thymic and splenic lymphocyte subsets from neonatal NF90(-/-) and heterozygous littermates revealed no differences in the expression of CD4 and CD8 double positive and single positive thymocytes between NF90(+/-) and NF90(-/-) mice (data not shown).

**NF90(-/-) mice show pathological maturation of skeletal muscles.** Searching for a potential cause of perinatal respiratory failure that would be independent of the lung parenchyma, we carefully examined the skeletal muscle fibers of the diaphragm and discovered that NF90(-/-) mice exhibited a relative paucity of muscle fibers and generally thinner diaphragm muscles than heterozygote or wild type littermates (Figure 4B vs. A, and Figure 3L vs. H). The abnormal features present in the diaphragm were also present in intercostal and back muscles of homozygote NF90(-/-) mice, including overall paucity of muscle fibers, wider variation of muscle fiber sizes, and some evidence of myofibers with strongly eosinophilic and homogeneous cytoplasm (Figure 4B vs. A). These abnormal fibers were likely hypercontracted, possibly as a consequence of disrupted integrity of the sacrolemma inducing unstable ion fluxes. Nuclei within skeletal myofibers of NF90(-/-) mice appeared larger with more granular chromatin visible compared to nuclei from myofibers of heterozygote and wild type littermates. We observed greater numbers of multinucleated giant fusing myocytes in the NF90(-/-) mice (Figure 4D vs. C), suggesting that skeletal muscles in the NF90(-/-) mice are developmentally delayed, or that ongoing muscle degeneration may be triggering efforts at regeneration.

**TUNEL analysis of NF90-deficient mice.** The small size of the NF90(-/-) mice and their internal organs may be a consequence of a combination of generalized growth retardation, developmental delay and/or increased apoptosis during development. To further understand the causes of small size and perinatal death in NF90-deficient mice, we performed in situ TUNEL staining (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling). TUNEL-positive cells were more prominent in E19 and newborn NF90(-/-) mice compared to wild-type or heterozygote littermates, throughout multiple organs. The greatest numbers of TUNEL-positive cells were present in cortical neurons and skeletal muscles of the diaphragm in NF90(-/-) animals (Figure 5B, D vs. 5A, C). The increased presence of TUNEL-positive cells in NF90(-/-) animals correlated with the observed reduction in size and weight.

**Reduced expression of myogenic regulatory factors, MyoD, myogenin and p21WAF1/CIP in NF90(-/-) mice.** We analyzed neonatal skeletal muscle expression of MyoD, myogenin and p21WAF1/CIP by semi-quantitative RT-PCR (Figure 6A). Expressio of MyoD mRNA expression was greatly inhibited, and myogenin and p21WAF1/CIP expression was reduced approximately 50% in NF90(-/-) compared to wild-type and heterozygote littermates (Figure 6A, lanes 4-6 vs. 2, 3 and 1). We discerned slight decreases in the myogenin and p21WAF1/CIP RT-PCR products in the heterozygote compared to the wild-type littermates (Figure 6A, lane 1 vs. 2, 3). We confirmed the reduction in myogenic regulatory factors in NF90(-/-) mice by Western analysis of skeletal muscle extracts (Figure 6B). Protein expression of MyoD and p21WAF1/CIP1 is markedly reduced in NF90(-/-) mice (Figure 6B, lanes 4-6.
vs. 2, 3), which corresponds well with the results of mRNA expression (Figure 6A).

*p21\textsuperscript{WAF1/CIP1} and MyoD 3’ UTR RNAs interact specifically with NF90*. The 3’-UTRs of the murine and human p21\textsuperscript{WAF1/CIP1} mRNA sequences show significant conservation in the proximal 350 nt region that contains multiple AU-rich binding elements (39). This proximal 3’ UTR is predicted to adopt secondary structures that includes regions of double-stranded RNA and hairpin loops (Figure 7A) (40). The MyoD 3’ UTR is also predicted to adopt extensive secondary structure (Figure 7B). These RNA secondary structures are binding targets for specific regulatory RNA-binding proteins such as HuR (36) and NF90 (this work). We used Northwestern blotting to characterize specific interactions between a radioactively labeled murine p21\textsuperscript{WAF1/CIP1} 3’-UTR probe and testis, brain and muscle extracts from adult wild type mice, as well as brain and muscle extracts from neonatal NF90(-/-) mice (Figure 7C). The p21\textsuperscript{WAF1/CIP1} 3’-UTR RNA probe specifically labels a 90 kDa protein that is present in wild type tissues (Figure 7C, lanes 1, 2, 3), is completely absent in extracts of NF90(-/-) mice (Figure 7C, lanes 4, 5), and that comigrates with labeled, recombinant NF90 protein (Figure 7C, lane 6). The MyoD 3’ UTR probe also specifically labels a 90 kDa protein in wild-type tissues (Figure 7D, lanes 1, 3) and recombinant NF90 protein (Figure 7D, lane 6), and shows no labeling in extracts of NF90(-/-) mice (Figure 7D, lanes 4, 5). Both 3’ UTR probes showed no labeling of recombinant NF45 protein (Figure 7C, D, lanes 7). Taken together, these results identify NF90 as the principal specific p21\textsuperscript{WAF1/CIP1} and MyoD 3’-UTR RNA binding protein present in skeletal muscles.

**Discussion**

Our study is the first to establish that NF90, a dsRNA-binding protein, is essential for perinatal survival in mice. The gross phenotype of NF90(-/-) mice is small size, weak cry, lack of spontaneous movements and death due to rapidly progressive respiratory failure. The relative contributions of diaphragmatic weakness, neurologic abnormalities or metabolic derangements to perinatal death are difficult to distinguish. Nevertheless, the immediate cause of death less than 12 h after delivery appears to be diaphragm muscle weakness and respiratory failure. We observed morphological correlations with respiratory failure of disorganized diaphragm architecture and muscle fibers with inclusions suggestive of apoptosis in the NF90(-/-) mice. A similar phenotype of perinatal death due to respiratory failure and decreased mass of diaphragm and intercostals muscles was observed in mice deficient in the activin- and transforming growth factor β-modulating protein, follistatin (41).

Targeted disruptions of other dsRBM-binding proteins in mice have been associated with varying phenotypes. RNA helicase is a nuclear transcriptional regulator that is the mammalian homolog of the Drosophila X-chromosome dosage-compensating factor, maleless. Targeted disruption of RNA helicase resulted in embryonic lethality before gastrulation, and was associated with enhanced TUNEL-positive staining (42). Zfr is a nuclear zinc finger RNA-binding protein with homology to NF90, expressed at highest levels in testis, ovary and brain (43). Targeted disruption of Zfr resulted in early embryonic lethality at day 8 - 9 as a consequence of developmental delay and enhanced apoptosis (44). Spnr is a cytoplasmic dsRNA-binding protein localized to microtubules in the testis, brain and ovary that may contribute to translational activation (45). A hypomorphic allele of Spnr produced by a gene trap strategy resulted in small size, increased lethality and neurological and reproductive defects (46). Two independent knockouts of the cytoplasmic dsRNA-activated protein kinase, PKR, resulted in no obvious phenotype, and this may reflect the finding that each represents only a partial knockout (47). Near complete targeted disruption of the nuclear ADAR2 RNA-editing enzyme
resulted in perinatal lethality by P12, due to increased seizure activity (48). Targeted disruption of nuclear ADAR1 resulted in embryonic lethality before E12, associated with widespread apoptosis (49). Together, these results suggest that nuclear dsRNA-binding proteins serve essential roles such as transcriptional regulation and RNA splicing. Cytoplasmic dsRNA binding proteins contribute to mRNA stabilization, subcellular localization and regulation of translation.

Essential and redundant contributions of myogenic regulatory factors (MRFs), MyoD, Myf5 and myogenin, and p21\textsuperscript{WAF1/CIP1} family members to embryonic muscle development have been elucidated through gene targeting studies. Either MyoD or Myf5 can direct myogenic specification (50), whereas myogenin (51), and either p21\textsuperscript{WAF1/CIP1} or p57\textsuperscript{KIP2} are essential for normal myogenic differentiation (52). An essential role for MyoD in the development of the skeletal muscle of the diaphragm was revealed by the failure to obtain viable mice during backcrossing with mdx mice (lacking dystrophin) (53). Reductions in myogenin gene expression in mice resulted in embryonic or neonatal lethality characterized by severe reductions in skeletal muscles (51,54,55). Recent evidence suggests that Mrf4, an MRF previously considered to be involved in myogenic differentiation, may in fact be an early myogenic specification factor, serving a redundant function to that of MyoD and Myf5 (56). Upregulation of p21\textsuperscript{WAF1/CIP1} promotes cell cycle exit and terminal differentiation in skeletal muscles, characterized by the fusion of myoblasts into myotubes and increased synthesis of muscle contractile proteins (57). P21\textsuperscript{WAF1/CIP1}(−/−) mice develop normally however (58), and this was proposed to be due to redundant cell cycle regulation by p57\textsuperscript{KIP2} (52). Mice that lacked both p21\textsuperscript{WAF1/CIP1} and p57\textsuperscript{KIP2} were not viable due to severely compromised muscle development as manifested by diminished diaphragm muscles. The absence of both cell cycle inhibitors resulted in overproliferation and increased apoptosis leading to a marked decrease in mature skeletal muscle fibers \textit{in vivo}. The absence of p21\textsuperscript{WAF1/CIP1} was associated with enhanced programmed cell death in differentiating myoblasts \textit{in vitro} (59).

Mechanisms of posttranscriptional mRNA stabilization of MyoD, myogenin and p21\textsuperscript{WAF1/CIP1} have been studied in differentiating C2C12 myoblasts (36,60). Ultraviolet crosslinking of radiolabeled 3′UTR RNA probes to cytosolic extracts of C2C12 cells labeled proteins of 37, 86 and 116 kDa (36). The 37 kDa protein was identified as the embryonic lethal abnormal vision (\textit{elav}) protein, HuR, that binds AU-rich elements (AREs) and mediates RNA-stabilization and nuclear-to-cytoplasmic export. Knockdown of HuR protein expression by RNA interference in C2C12 cells inhibited myogenesis \textit{in vitro} (60). However, in MDA-468 human breast cancer cells, although HuR bound p21\textsuperscript{WAF1/CIP1} RNA, it was not a major modulator p21\textsuperscript{WAF1/CIP1} expression or growth inhibition (39). HuR protein expression in young adult mice was prominent in intestine, thymus and spleen, followed by the liver, and was almost undetectable in skeletal muscle, brain, kidney, lung and heart (61). This low to absent expression of HuR in skeletal muscles raises a question about its contributions to posttranscriptional stabilization of MyoD, myogenin and p21\textsuperscript{WAF1/CIP1} during myogenesis \textit{in vivo}.

Here, we demonstrate that NF90 is the principal and specific RNA-binding protein in skeletal muscles for MyoD and p21\textsuperscript{WAF1/CIP1}. Gene-targeted NF90(−/−) mice, completely lacking NF90 protein, demonstrate severely reduced expression of MyoD, myogenin and p21\textsuperscript{WAF1/CIP1}. We propose that NF90(−/−) mice are deficient in posttranscriptional stabilization of MyoD, myogenin and p21\textsuperscript{WAF1/CIP1} mRNAs. The phenotype of impaired muscle formation associated with increased apoptotic myonuclei is likely the direct result of this coordinated downregulation of multiple myogenic regulators, including those involved both in myogenic specification, cell cycle withdrawal, and differentiation. Because MyoD(−/−)(50) and
p21\textsuperscript{WAF1/CIP1}(-/-)(58) mice are each viable through compensatory expression of myogenic regulators, we reasoned that our phenotype of perinatal death due to diaphragmatic respiratory failure must arise from effects on more than one of these myogenic regulators. Mice deficient in MyoD (62), or in cell cycle inhibitors p21\textsuperscript{WAF1/CIP1} and p57\textsuperscript{KIP2} (52), exhibited overproliferation and increased programmed cell death. This is consistent with our observations of increased apoptosis in NF90(-/-) mice, especially prominent in skeletal muscles and brain. The overall growth stunting of NF90(-/-) mice (40% lower weight than littermates) is likely a consequence of abnormal myogenesis and organogenesis related to defects in exiting the cell cycle.

Future studies will be directed at identifying other structured RNA targets involved in differentiation that are critically regulated through specific binding to NF90.

**Acknowledgements**

We thank Tushar Desai and Mark Krasnow for helpful discussions. Supported by NIH R01-AI39624 and R01-HL62588 and the Donald E. and Delia B. Baxter Foundation.

**References**

Genotype analysis of progeny from NF90+/− heterozygous intercrosses

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Figure Legends

**Figure 1.** Targeted disruption of the NF90 gene results in growth retardation and perinatal lethality. (A) Genomic organization of the NF90 locus, and targeting strategy to delete exons 2 – 4 by homologous recombination. Restriction sites are shown for BamH1 (B1), EcoRI (E1), HindIII (H3), NotI (N1). (B) Southern blot analysis of Hind III restricted R1 ES cell DNA. The 7.44-kb fragment represents the wild-type allele, and the 9.05-kb fragment represents the targeted allele (due to elimination of the Hind III site downstream of Exon 4). (C) Photograph of newborn mice from F1 intercross of NF90-targeted heterozygotes. (D) Genotype analysis of NF90-targeted E19 embryos by Southern hybridization.

**Figure 2.** Expression analysis of NF90 and NF110 in gene-targeted mice. (A) Northern hybridization of NF90 RNA expression in gene-targeted littermates. NF90(+/+) mice show expression of a single transcript of 3.4 kB, NF90(-/-) mice show prominent expression of a single targeted transcript of 3.1 kB and NF90(+/-) mice show mRNA transcription of both sizes. Constant expression of glyceraldehyde phosphate dehydrogenase (GAPDH) is shown in the lower panel. (B) Western immunoblot of NF90 protein expression in gene-targeted littermates. Equal amounts of extracted total embryonic proteins (20 µg) were analyzed. (C) Tissue expression of NF90 and NF110 in wild type mice. Equal amounts of total protein were analyzed (80 µg) by immunoblotting.

**Figure 3.** Immunohistochemical expression analysis of NF90 in wild-type NF90(+/+) and NF90(-/-) mice. NF90(+/+) staining is shown in (A – H) and NF90(-/-) staining is shown in panels (I – L). (A) Heart, (B) lung, (C) liver, (D) stomach; (E and I) cerebral cortex, (F and J) cerebellum, (G and K) diaphragm muscle, and (H and L) skeletal muscle (latissimus).

**Figure 4.** Skeletal muscle histopathology. Comparative histology by hematoxylin and eosin staining of diaphragm muscle (A, B) and skeletal muscle (latissimus)(C, D) between NF90(+/+) mice (A, C) and NF90(-/-) mice (B, D).

**Figure 5.** TUNEL analysis of apoptosis in NF90(-/-) mice. TUNEL-positive nuclear staining predominated in cerebral and muscle tissues of NF90(-/-) mice compared to wild-type mice. Sections for TUNEL analysis were prepared from E19 specimens.

**Figure 6.** Myogenic Regulatory Factor expression is reduced in NF90(-/-) mice. (A) MyoD, myogenin and p21<sup>WAF1/CIP1</sup> and b-actin mRNA were detected in neonatal skeletal muscle by reverse-transcription PCR. (B) Western immunoblot of p21<sup>WAF1/CIP1</sup> expression; actin expression is shown as a loading control.

**Figure 7.** Northwestern analysis reveals that p21<sup>WAF1/CIP1</sup> and MyoD 3’ UTR RNAs interact specifically with NF90. RNA secondary structure predictions were performed using Zucker’s mfold program (40) for (A) p21<sup>WAF1/CIP1</sup> 3’ UTR (511 – 883nt), and (B) MyoD (1241-1762 nt). Northwestern blots were performed using in vitro transcribed, radiolabeled 3’-UTR RNA probes for murine p21<sup>WAF1/CIP1</sup> (C) or MyoD (D). Murine tissue extracts (80 µg each) (lanes 1, 2, 3: adult NF90(+/-)), lanes 5, 6: neonatal NF90(-/-)) and recombinant histidine-tagged NF90 and NF45 proteins (lanes 7, 8: 10 µg each) (5) were fractionated by SDS-PAGE, transferred to nitrocellulose and hybridized with probed 32P-labeled 3’-UTR probes.
Figure 1
Figure 2

[Image of gel electrophoresis with bands labeled as NF90 and G3PDH, showing WT and MU variants.]

A
WT 3.4kb--
MU 3.1kb--

B
NF110/ILF3
90 kDa
110 kDa

C
NF110/ILF3
90 kDa
110 kDa

Heart
Brain
Spleen
Lung
Liver
Skel Muscle
Kidney
Testis
Figure 4
Figure 5
Figure 6

A

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Figure 6
**Figure 7**

**A**
Murine p21^{WAF1/CIP1} 3'-UTR
Free energy = -72 kJ/mol

**B**
MyoD 3' UTR
Free energy = -156 kJ/mol

**C**

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Probe: p21 3'-UTR

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Probe: MyoD 3'-UTR

90 kDa