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MrgX Is Not Essential for Cell Growth and Development in the Mouse

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MRGX is one of the members of MORF4/MRG family of transcriptional regulators, which are involved in cell growth regulation and cellular senescence. We have shown that MRGX and MRG15 associate with Rb in nucleoprotein complexes and regulate B-*myb* promoter activity. To elucidate the functions of MRGX and to explore its potential role in modulating cell growth in vivo, we have generated *MrgX*-deficient mice. Characterization of the expression pattern of mouse *MrgX* demonstrated it was ubiquitously expressed in all tissues of adult mice and also during embryogenesis and overlapped with its homolog *Mrg15*. MRGX and MRG15 proteins localize predominantly to the chromatin fraction in the nucleus, although a small amount of both proteins localized to the nuclear matrix. Whereas disruption of *Mrg15* results in embryonic lethality, absence of *MrgX* did not impair mouse development and *MrgX* null mice are healthy and fertile. *MrgX*-deficient and wild-type mouse embryonic fibroblasts (MEFs) also had similar growth rates and showed no differences in cell cycle-related gene expression in response to serum stimulation. *Mrg15* expression in *MrgX*-deficient tissues and MEFs was not upregulated compared with wild-type tissues and MEFs. MRG15 is highly conserved with orthologs present from humans to yeast and is essential for survival of mice. In contrast, MRGX, which evolved later, is expressed only in vertebrates, suggesting that the lack of phenotype of *MrgX*-deficient mice is secondary to a compensatory effect by the evolutionarily conserved MRG15 protein but not vice versa.

Normal human fibroblasts do not divide indefinitely in vitro but finally enter a terminally nondividing state after undergoing several rounds of division (11). This process is referred to as cellular or replicative senescence and is believed to be an antitumor mechanism (32, 35). In studies to identify cellular senescence-related genes, we isolated a novel gene family, of which three members, MORF4 (mortality factor on human chromosome 4), MRG15 (MORF4-related gene on human chromosome 15), and MRGX (MORF4-related gene on human X chromosome), are expressed (2). MORF4 induces senescence in a subset of human tumor cell lines, whereas MRGX and MRG15 do not induce senescence. These three proteins share common motifs which are seen in many transcriptional regulators such as helix-loop-helix (HLH) and leucine zipper (LZ) regions. MRG15 has an N-terminal chromodomain region (2, 3) that has been identified in other chromatin-related proteins and is involved in recognition of methylated histone H3 and RNA binding (1, 4, 7, 16–18). MRGX has a unique N-terminal sequence which has no homology to other proteins. MORF4 lacks the N-terminal regions of both MRG15 and MRGX. We have speculated that the differences in the N-terminal sequences of these three proteins may confer functional differences. Both MRGX and MRG15 interact with Rb and a novel protein, PAM14, and activate or repress the B-*myb* (*Mybl2*) promoter in a cell type-dependent manner (34). The HLH and LZ regions are important for both the activation and repression activities and therefore require the common C-terminal motif in both proteins. However, in the case of MRG15, the N-terminal chromodomain-containing region of the protein is also required for activation of the B-*myb* promoter and it activates versus represses, as does MRGX, this promoter in EJ cells (20, 29). MRG15 has been shown to be one of the components of the human NuA4 histone acetyltransferase multiprotein complex that includes TIP60, which is the catalytic subunit in the complex (5, 6, 8, 9, 15). Although Cai et al. have identified MRGX as a component of the human NuA4 complex (5, 6), other groups have not and additional biochemical analysis is needed to verify this.

Since MRGX is present only in vertebrates, whereas MRG15 is a highly conserved protein with orthologs in yeast to humans (3), MRGX may be involved in higher-order functions in mammalian cells whereas MRG15 is required for more fundamental processes. In fact, the *Mrg15*-deficient phenotype is embryonic lethal in mice (33), with null embryos exhibiting a runted phenotype and *Mrg15* null mouse embryonic fibroblasts (MEFs) displaying a clear growth deficit (33).

In this study, we have generated *MrgX*-deficient mice to study the function of *MrgX* and to explore its possible role in modulating cell growth in vivo. We demonstrate that *MrgX* is expressed ubiquitously in adult mouse tissues and during embryogenesis and its expression pattern is similar to that of *Mrg15*. We have also found that *MrgX* null mice ($MrgX^{-/-}$ female and $MrgX^{-}$ male) are viable and healthy and appear to have normal fertility. Analysis of MEFs has demonstrated that *MrgX*-deficient cells have the same growth rate and express genes related to cell cycle initiation and progression in a manner equivalent to control littermates. The results suggest that MRGX is not essential for either development or cell prolif-

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eration and that its function is likely compensated for by MRG15.

MATERIALS AND METHODS

Northern blot analysis. Mouse *MrgX* cDNA was PCR amplified from a brain cDNA library of adult C57BL/6J mice using the primers MMRGX-5′ (5′-GGC TTTCTATGGCGGTTGGAGGAG-3′) and MMRGX-3′ (5′-AGACAATAGT GAGCGGTCAGTAGA-3′). The amplified fragment was subcloned into the EcoRV site of pBluescript II and the sequence confirmed. A mouse RNA Master Blot (no. 7771-1; Clontech, Palo Alto, CA) was hybridized using as probe a fragment of the mouse *MrgX*-specific sequence amplified by PCR using the plasmid mentioned above as a template. The primers were MMRGX-5′ and MMRGX-9 (5′-CTTAAAGTTGTCTTCTTCAGCAGA-3′), and the probe DNA contained a 188-bp 5′ untranslated region and a 66-bp coding sequence.

RNA was isolated from tissues or MEFs using the Trizol reagent (Invitrogen). Frozen tissue samples were homogenized with a 1.5-ml pellet pestle, 1 ml of Trizol was added to the tissue sample, and the suspension was passed thorough a 21-gauge needle to shear the DNA. RNA was extracted according to the manufacturer's instructions. Total RNA (20 μg) was resolved on a 1% agarose gel and then transferred to Hybond-N^+ nylon membrane. Blots were hybridized in NorthernMax hybridization buffer (Ambion, Austin, TX) at 42°C overnight with an *MrgX*- or *Mrg15* (as control)-specific probe (36). The plasmids which contain mouse cyclin E1 (*Ccne1*), cyclin D1 (*Ccnd1*), *Mybl2*, and *Myc* fragment were kindly provided by Nicholas J. Dyson. The blot was washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C twice for 10 min and then washed with 0.2× SSC–0.1% SDS at 65°C twice for 15 min.

Nuclear protein fractionation. HeLa cells (9.4×10^6) were harvested by trypsin treatment and washed with phosphate-buffered saline (PBS). Protein fractionation was performed by a previously published method (31). In brief, pelleted cells were suspended in 800 μl of RSB buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 1× Protease Inhibitor Cocktail Set I [no. 539131; Calbiochem]), and the cytoplasmic membrane of the cells was disrupted by being passed through a 25-gauge needle 20 times. We confirmed microscopically that over 95% of the cells were effectively disrupted by this treatment. The nuclei were collected by centrifugation at 6,800 × *g* for 3 min and washed twice with RSB buffer. The pelleted nuclei were suspended in DNase I buffer (10 mM Tris-HCl [pH 7.6], 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5% Triton X-100, 1× Protease Inhibitor Cocktail Set I) supplemented with 4 mM vanadyl ribonucleoside complex (RNase inhibitor; Fluka no. 94742) and 100 U of DNase I (New England BioLabs no. M0303S) and incubated at 30°C for 50 min. After incubation, 100 μl of 1 M ammonium sulfate (final concentration, 0.25 M) was added, and the lysate was centrifuged at 6,800 × *g* for 3 min and collected (nuclear fraction 1). The pellet was suspended in DNase I buffer supplemented with 2 M NaCl, incubated on ice for 10 min and centrifuged at 6,800 × *g* for 3 min, and the lysate was collected (nuclear fraction 2). The pellet was suspended in DNase I buffer, RNase A (100 μg/ml) and RNase T1 (40 U/ml) were added, and the mixture was incubated at room temperature for 1 h. The lysate was centrifuged at 6,800 × *g* for 3 min and collected (nuclear fraction 3). The pellet was dissolved in 1× SDS sample buffer (nuclear fraction 4). We adjusted loading amounts by cell number (corresponding to 5×10^5 cells). Nuclear proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Bio-Rad).

Construction of the targeting vector. The intronic fragment of the mouse *MrgX* gene was amplified by PCR using 129S6/SvEv tail DNA as a template. The primers for PCR were MMRGX-1 (5′-TGGAAGGGAAAGAAGGAACATTG T-3′) and MMRGX-2 (5′-TCAGCCCGTGCCCTTTTCTTCCG-3′). The amplified fragment (1.1 kb) was subcloned into the EcoRV site of pBluescript II (Stratagene), confirmed by sequencing, and used as a probe for screening of genomic clones. Three independent *MrgX* genomic clones were isolated from a 129S6/SvEv mouse embryonic stem (ES) cell genomic library (Stratagene, La Jolla, CA).

The targeting vector to inactivate *MrgX* contained a 3.4-kb EcoRI-ClaI fragment of the *MrgX* gene for the 5′ homology arm, a *loxP* site, a 2.2-kb ClaI-HindIII fragment of the *MrgX* gene, a PGK-*neo* selectable marker cassette floxed by *loxP* sites, a 3.0-kb HindIII-BamHI fragment of the *MrgX* gene for the 3′ homology arm, and an MC1*tk* expression cassette for negative selection. Twenty-five micrograms of KpnI-linearized targeting vector was electroporated into 10^7 AB2.2 ES cells. ES clones were selected in culture medium containing G418 and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5′-iodouracil. DNA from the clones was analyzed by Southern blotting, targeted ES cell clones were expanded and injected into C57BL/6J blastocysts, and chimeric males were mated with C57BL/6J females as previously described (24). Germ line transmission was obtained from chimeras derived from two independent ES cell clones (*MrgX*-126-B7 and *MrgX*-126-D5).

To produce a *MrgX*-deficient mouse line, mice which had a recombinant floxed allele of the *MrgX* gene were mated with EIIa-*cre* transgenic mice which express Cre recombinase under the control of the adenovirus EIIa promoter (19).

The generation of $Mrg15^{+/-}$ mice, which were used for double-knockout (DKO) analysis, has been previously reported by our group (33).

Genotyping. Either tail DNA, DNA from the yolk sac, or DNA from a part of the embryo digested with EcoRV or EcoRI (for *MrgX*) or BamHI (for *Mrg15*) at 37°C overnight was submitted to Southern analysis to determine the genotype of the embryos or mice. The digested DNAs were separated on 0.7% agarose gel and transferred to Hybond-N^+ (Amersham Pharmacia) using an alkaline transfer method. The membrane was probed with the 3′ external or 5′ internal probe (for *MrgX*) using Rapid Hybridization solution (Amersham Pharmacia). The *Mrg15* probe for genotyping has been described previously (33). Genotyping of the EIIa-*cre* transgene was performed by PCR using 5′-CCGGGCTGCCACG ACCAA-3′ and 5′-GGCGCGGCAACACCATTTTT-3′ primers.

Western blot analysis. Tissues from adult mice were lysed with TNESV buffer (20 mM Tris-HCl [pH 7.5], 1% NP-40, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1× Protease Inhibitor Cocktail Set I [Calbiochem]). The lysates were kept on ice for 30 min and centrifuged at 20,000 × *g* for 15 min. The protein concentration of the supernatants was determined by the Bradford method (Bio-Rad). The total proteins (100 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. MRG15 protein was detected using rabbit anti-MRG15 C-terminal antibody raised by our laboratory. Mouse anti-β-actin antibody (Abcam AC-15) was used as a loading control.

Antibodies used to detect MRG15, MRGX, and lamin A/C in nuclear protein fractionated preparations were rabbit anti-MRG15 C-terminal antibody (1:1,000; antigen, SASDYEVAPPEYHRKAV), rabbit anti-MRGX antibody (1:1,000; antigen, KQGSQPRGQQSA), and goat anti-lamin A/C antibody (1:500; Santa Cruz, sc-6215). The anti-MRG15 and anti-MRGX antibodies were affinity purified using an antigen column, and their specificity was confirmed by Western blotting using lysates of cells expressing hemagglutinin-tagged MRG15 or MRGX. Anti-MRG15 or anti-MRGX and anti-hemagglutinin antibodies detected the same protein band.

Generation of MEFs and analysis of growth properties. We bred $MrgX^-$ males with $MrgX^{+/-}$ females to isolate13.5-day-old (E13.5) embryos. After removal of the head and abdominal organs, each embryo was washed with PBS and minced, and the tissue was placed in a 15-ml conical tube. After centrifugation, 1 ml of trypsin solution (0.25% trypsin–0.005% EDTA in PBS) was added to the pelleted tissue, which was digested on ice overnight. Trypsin was inactivated by the addition of Eagle's minimum essential medium (Invitrogen) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM nonessential amino acids, and 50 μg of gentamicin per ml. After pipetting several times, the cells were plated into one T75 tissue culture flask and incubated at 37°C until confluent. We designated this culture PD 0.

For the growth study, equal numbers of cells (1×10^5) from PD 0 MEFs were plated into 60-mm-diameter tissue culture dishes and maintained in 5% CO_2 at 37°C and counted every 24 h (in triplicate) using a Coulter Counter. We tested at least two independent MEF lines for each genotype for the growth study.

For gene expression analysis, PD 0 MEFs (1×10^6) were plated into 100-mm-diameter tissue culture dishes and maintained at 37°C for 2 days. The cells were washed twice with PBS, 0.1% FBS-containing medium was added, and the cells were maintained at 37°C for 3 days. The cells were then stimulated by the addition of 10% FBS-containing medium, and total RNA was isolated at various points thereafter.

RESULTS AND DISCUSSION

***MrgX* mRNA expression during mouse development and in adult mice.** To determine expression of mouse *MrgX*, we performed dot blot and Northern blot analyses of embryonic and adult tissues. *MrgX* mRNA was ubiquitously expressed in embryos beginning at 7 days and in adult tissues (Fig. 1A), and the expression pattern was very similar to that of *Mrg15* mRNA (36). *Mrg15* mRNA appears to be more highly expressed in adult testis based on the hybridization signal.

Next, we analyzed *MrgX* versus *Mrg15* mRNA expression in

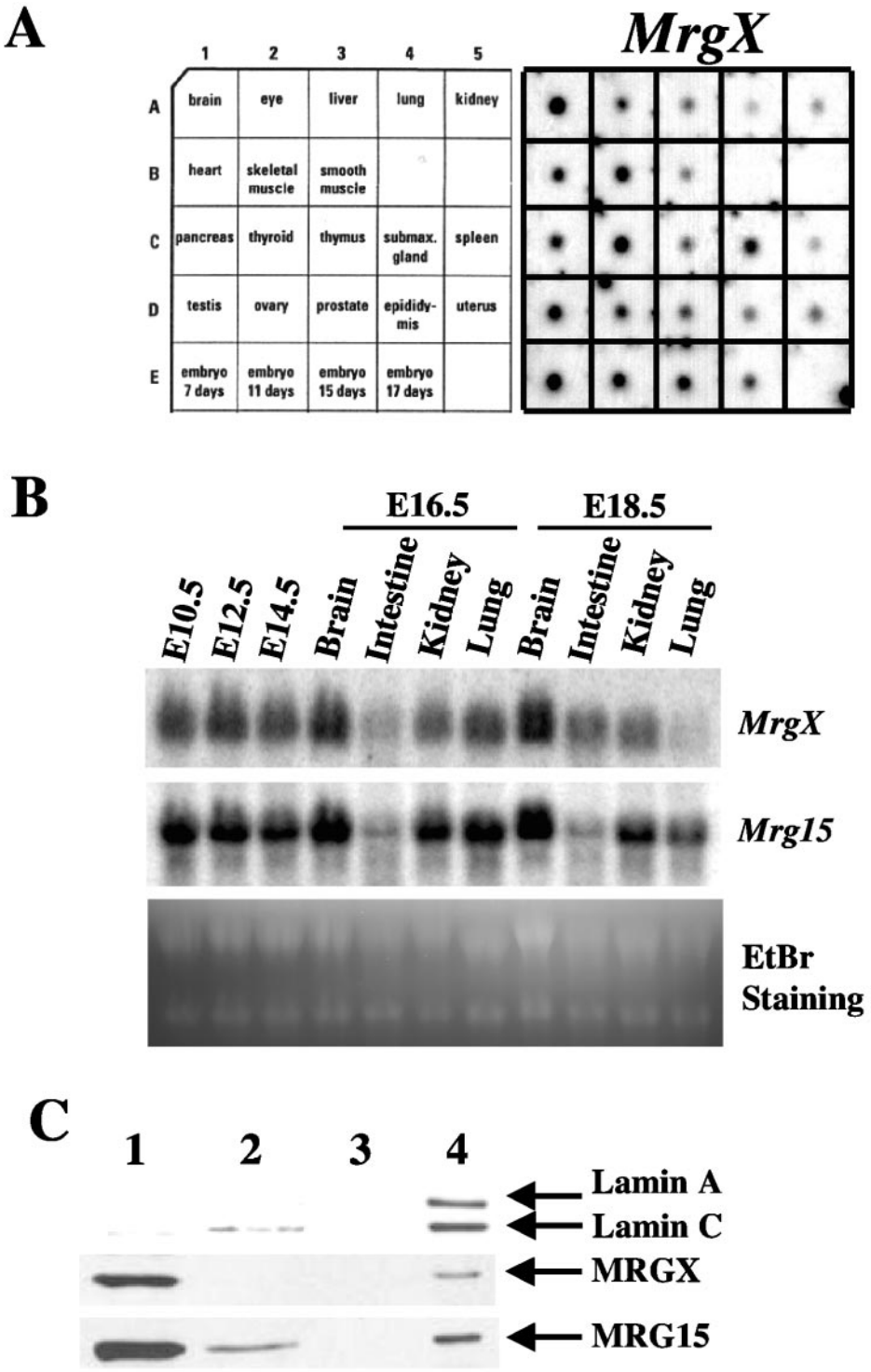
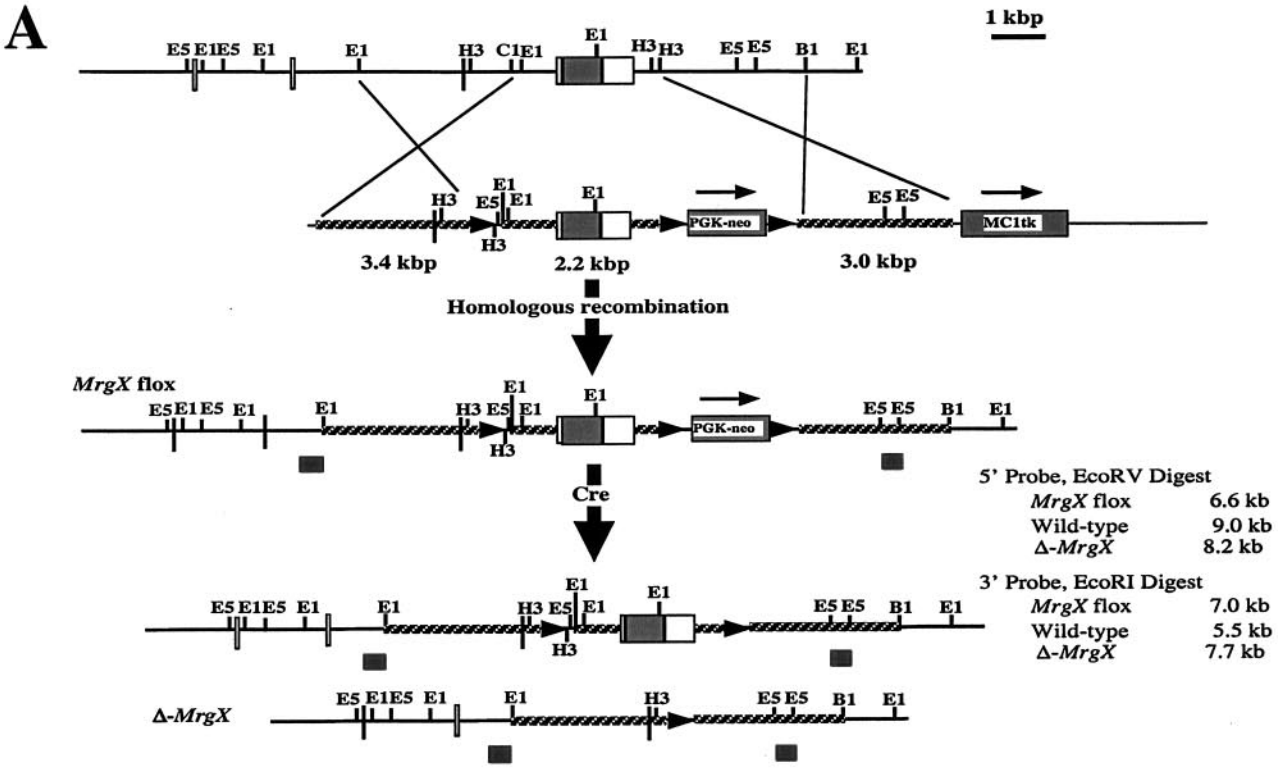


FIG. 1. Expression of *MrgX* mRNA and protein. (A) *MrgX* mRNA expression in adult tissues and embryos. A mouse RNA Master Blot was hybridized using an *MrgX*-specific probe. (B) *MrgX* and *Mrg15* mRNA expression during mouse development. *MrgX* and *Mrg15* mRNA expression in embryos and embryonic tissues was determined by Northern blotting. EtBr staining of RNAs (28S and 18S) indicated an equal amount of loading of the various samples. (C) Subnuclear fractionation analysis of MRGX and MRG15 proteins in HeLa cells. Nuclear proteins from HeLa cells were fractionated as reported previously (31). The amount of sample loaded in each lane was based on cell number (protein from 5×10^5 cells per lane). MRGX and MRG15 proteins were detected by Western blotting. The presence of lamins A and C served as a marker for the nuclear matrix fraction. Lanes: 1, 0.25 M $(NH_4)_2SO_4$ extracted fraction after DNase I treatment; 2, 2 M NaCl extracted fraction; 3, extracted fraction after RNase A plus RNase T1 treatment; 4, nuclear matrix (pelleted) fraction.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Homologous recombination
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MrgX flox 6.6 kb
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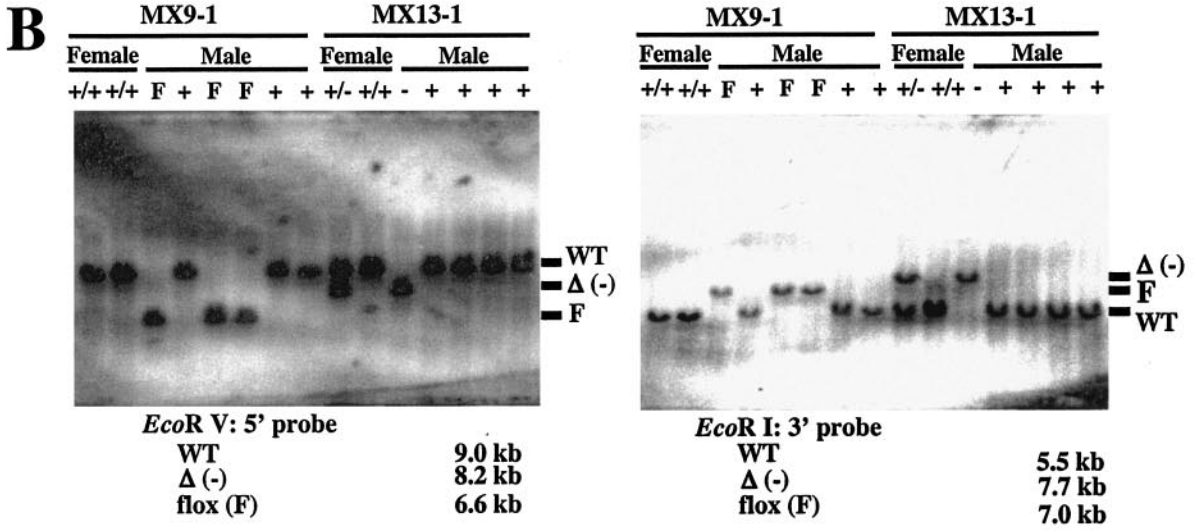
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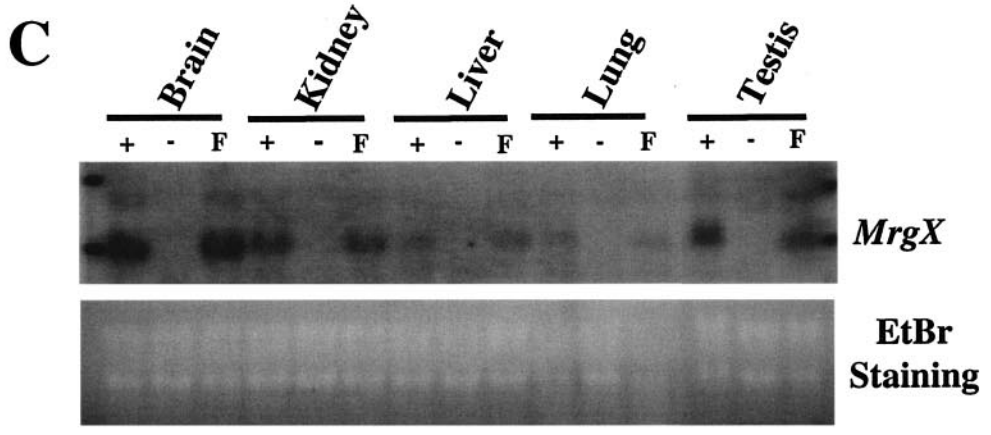
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embryos and embryonic tissues at later stages of embryogenesis (Fig. 1B) and determined that *MrgX*, as well as *Mrg15*, is highly expressed in early stages of embryos similar to that observed from dot blot analyses. *MrgX* and *Mrg15* mRNA expression in embryonic brain was high, and this did not change at later stages (compare E16.5 and E18.5). Expression of *MrgX* and *Mrg15* in intestine and kidney was moderate, and there was no major change in the expression pattern between E16.5 and E18.5. Both *MrgX* and *Mrg15* were moderately expressed in lung at E16.5, but expression decreased slightly at E18.5. These results suggest that the differential expression of *MrgX* and *Mrg15* in different tissues at various stages of embryogenesis may reflect gene expression regulation by these proteins and impact on development. The most interesting fact is that the expression pattern of *MrgX* in embryonic tissues is generally similar to that of *Mrg15*.

Nuclear protein fractionation analysis of MRGX and MRG15. To determine the localization of MRGX in cells, we transfected a plasmid construct which encoded MRGX fused with enhanced green fluorescent protein into HeLa cells. Enhanced green fluorescent protein-MRGX localized to the nucleus, similar to MRG15, as described before (data not shown) (36). Next, we determined MRGX and MRG15 protein localization in the nucleus following biochemical protein fractionation. Most of the MRGX and MRG15 protein was eluted by 0.25 M $(NH_4)_2SO_4$ solution following DNase I treatment (Fig. 1C, fraction 1), which causes removal of chromatin from nuclei together with many other nuclear proteins (over 80% of nuclear proteins) (12). Thus, most of the MRGX and MRG15 protein appears to be present in the nucleoplasm and/or weakly attached to chromatin. Interestingly, a small amount of MRGX and MRG15 localized to the nuclear matrix fraction, in which lamins A and C were localized (Fig. 1C, fraction 4). Recently, it has become evident that the nuclear lamina, which is a filamentous nuclear structure involved in many nuclear activities including transcription, RNA splicing, DNA replication, chromatin organization, cell cycle regulation, cell differentiation, and apoptosis (23, 28, 38), is dysregulated or mutated in genes in a wide range of human diseases such as cancer (26, 27) and Hutchinson-Gilford progeria syndrome (10, 30). The latter premature-aging syndrome is also observed in mutant mice with genetically modified lamin A (25). We do not know the physiological implications of the nuclear matrix localization of MRGX and MRG15 proteins at this time. We have reported that MRGX and MRG15 are both associated with Rb (20, 29, 34) and some fraction of Rb is known to be associated with the nuclear matrix (21). The hypophosphorylated form of Rb binds to lamin A/C and is anchored by binding to lamina-associated polypeptide 2α-lamin A/C complexes (22). It has been speculated that the lamin/lamina-associated polypeptide 2α complex may tether Rb molecules to a promoter and form an effective silencing complex. Thus, one possible explanation for the nuclear matrix localization of MRGX and MRG15 is that they are tethered with Rb in the nuclear matrix and are involved in Rb-related functions.

Disruption of mouse *MrgX* by gene targeting. The genomic structure of the mouse *MrgX* gene is very similar to that of the human *MrgX* that is deposited in GenBank (accession no. AL049610). The 5′ untranslated region is divided by at least three exons, and the coding sequence of both the mouse and human *MrgX* is encoded by only one exon. We decided to use a conditional-knockout system for the generation of *MrgX*-deficient ES lines because the ES cell line (AB2.2) that we use is of male origin and has just one X chromosome. If *MrgX* was essential for cell growth, and we used a conventional knockout method, we would obtain no positive ES clones from screening. The strategy we used is depicted in Fig. 2A. The coding exon of the mouse *MrgX* gene was flanked by two *loxP* sites so that Cre recombinase-mediated excision of this entire region would result in loss of production of MRGX. The linearized targeting construct was electroporated into ES cells, followed by drug selection. We screened 93 drug-resistant clones by Southern blotting using external 5′ and internal 3′ probes, and 35 clones (37.6%) displayed the correct targeting pattern on both sides. ES cells from two independent clones were injected into blastocysts to generate mice bearing the *MrgX* recombinant floxed allele. This allele was successfully germ line transmitted (Fig. 2B, MX9-1). The resulting $MrgX^{Flox/Flox}$ females and $MrgX^{Flox}$ males were healthy and fertile, indicating that the recombinant allele had no overt effects on the mice, and *MrgX* mRNA was expressed at the same level as in the wild type in $MrgX^{Flox}$ male mice (Fig. 2C).

To generate *MrgX*-deficient mice, we crossed $MrgX^{Flox/+}$ females with adenovirus EIIa promoter-driven deleter (EIIa-*cre*) males (19). Cre recombinase effectively deleted the coding exon of the mouse *MrgX* gene from the *MrgX* recombinant allele and produced an *MrgX* deletion (Δ) allele (Fig. 2B, MX13-1). We obtained *MrgX*-deficient mice ($MrgX^{-/-}$ females and $MrgX^{-}$ males) at the expected ratio from a combination of mating pairs, indicating no impairment in mouse development.

We confirmed that there was no expression of *MrgX* in tissues from adult $MrgX^{-}$ males (Fig. 2C) and $MrgX^{-/-}$ females (data not shown) by Northern blotting. The *MrgX*-deficient mice were healthy and fertile, and we did not observe any obvious abnormalities after 1 year. This suggests that *MrgX* is not required during embryogenesis or in adult mice under normal conditions.

Cell growth and expression of cell cycle-related genes in *MrgX*-deficient MEF in response to serum stimulation. We have found that *Mrg15*-deficient MEFs have a growth defect

FIG. 2. Disruption of the mouse *MrgX* locus by gene targeting. (A) Genomic structure of the mouse *MrgX* gene and the targeting vector used to generate the conditionally targeted allele of *MrgX*. The targeting vector was constructed by flanking a 2.2-kb region containing the *MrgX* coding exon by two *loxP* sites with a PGK-*neo* cassette. The regions of homology consist of a 3.4-kb region for the 5′ arm and a 3-kb region for the 3′ arm, respectively. The MC1*tk* cassette was located next to the 3′ homology arm. The probe fragments for genotyping are indicated as shaded boxes at the bottom. Abbreviations: E5, EcoRV; E1, EcoRI; H3, HindIII; C1, ClaI; B1, BamHI. (B) Southern blot analysis of tail DNA from pups at weaning from breeding pairs containing the recombinant (floxed) allele (MX9-1) and the *MrgX*-deleted [Δ (−)] allele (MX13-1). Genomic DNAs were digested with EcoRV or EcoRI and hybridized with 5′ or 3′ probes, respectively. WT, wild type. (C) Northern blot analysis of *MrgX* expression in wild-type (+), $MrgX^{-}$ (−), and $MrgX^{Flox}$ (floxed allele, F) males. EtBr staining of RNAs (28S and 18S) was used to demonstrate equal loading of samples.

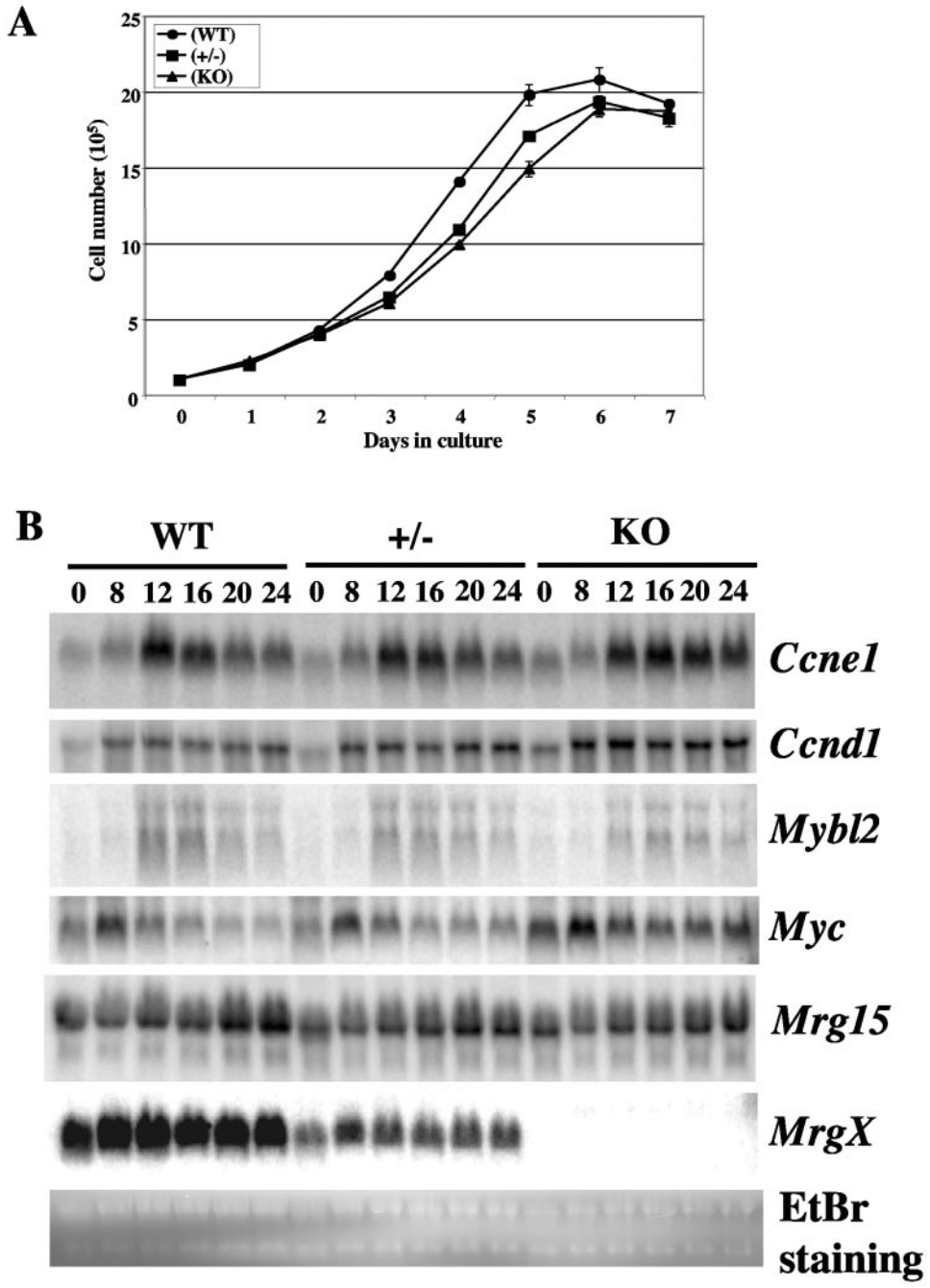

FIG. 3. MEF analyses. (A) Growth kinetics of MEFs. MEFs (1×10^5) were plated in 60-mm-diameter dishes and counted every 24 h. This result is representative of two independent experiments. (B) Expression patterns of cell cycle-regulated genes by Northern blot analysis. Quiescent MEFs were stimulated by adding 10% FBS-containing medium, and total RNA was isolated at the indicated time points (hours after stimulation). The expression levels of cyclin E1 (*Ccne1*), cyclin D1 (*Ccnd1*), *Mybl2*, *Myc*, *Mrg15*, and *MrgX* mRNAs were detected by Northern blotting. EtBr staining of RNAs (28S and 18S) served as a control for equal amounts of loading of samples. WT, wild type.

compared with control cells (33) and that $p21^{Sdi1/Cip1/Waf1}$ is upregulated in these MEFs at earlier passages. To determine whether MRGX had a similar effect on cell growth, we bred $MrgX^{+/-}$ females with $MrgX^{-}$ males and isolated MEFs from E13.5 embryos. Daily cell counts revealed no significant differences in the growth rates of wild-type (WT), $MrgX^{+/-}$ (+/−), and *MrgX*-deficient (knockout [KO]) MEFs (Fig. 3A).

Since both MRGX and MRG15 interact with Rb, we then determined whether *MrgX*-deficient MEFs responded to serum stimulation with expected changes in expression of the cell cycle-related genes encoding cyclin E1 (*Ccne1*), cyclin D1 (*Ccnd1*), B-myb (*Mybl2*), and c-*myc* (*Myc*), which are known to be expressed at G_1/S. The expression of all four genes in wild-type (WT), $MrgX^{+/-}$ (+/−), and *MrgX*-deficient (KO)

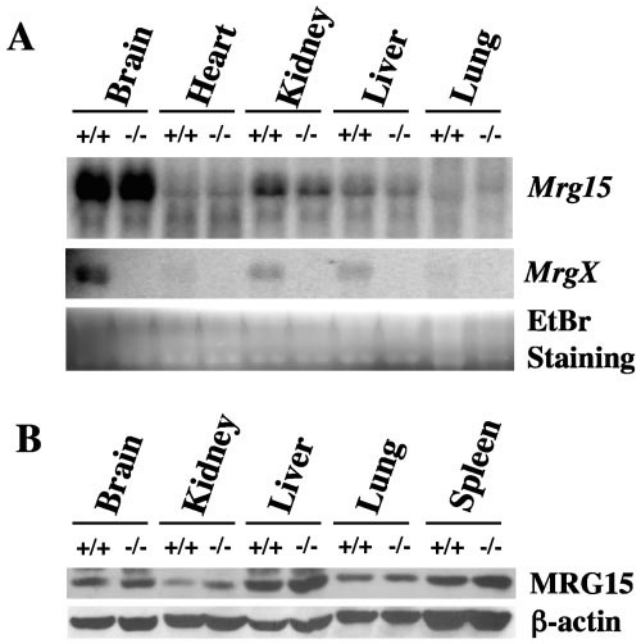

FIG. 4. *Mrg15* expression in *MrgX*-deficient mice. (A) *Mrg15* and *MrgX* mRNA expression in various tissues from wild-type (+/+) and $MrgX^{-/-}$ (−/−) mice. Expression of mRNA was determined by Northern blotting. No *MrgX* transcript was present in $MrgX^{-/-}$ tissues. EtBr staining of RNAs (28S and 18S) was the control for equal amounts of loading of samples. (B) MRG15 protein in various tissues from wild-type (+/+) and $MrgX^{-/-}$ (−/−) mice was determined by Western blot analysis. Equal amounts of loading of wild-type and $MrgX^{-/-}$ samples was confirmed using an anti-β-actin antibody. These results are representative of analyses of multiple male or female mice.

MEFs was low in the quiescent state (0 h) and increased in a time-dependent manner after serum stimulation (Fig. 3B). Again, no difference was observed in the expression patterns of the three genotypes. Cyclin E1 expression is upregulated at G_0 in Rb-deficient MEFs because Rb repression of cyclin E1 expression is abrogated (13, 14). However, cyclin E1 expression remained repressed in *MrgX*-deficient MEFs at G_0 (0 h). The expression levels of *Mrg15* remained constant during this time and, importantly, there was no difference in *Mrg15* expression levels among the three genotypes. We confirmed that half the amount of *MrgX* transcript and no *MrgX* transcript was expressed in $MrgX^{+/-}$ and *MrgX*-deficient MEFs, respectively, using the same membrane. Ethidium bromide (EtBr) staining of 28S and 18S rRNAs was used as a loading control (Fig. 3B).

Collectively, these data indicate that MRGX is not essential for cell growth control, and this is most likely due to a compensatory effect of MRG15.

***Mrg15* expression in *MrgX*-deficient mice.** The lack of phenotype of *MrgX* deficiency indicates that MRG15 or some other protein(s) must compensate for the function(s) of *MrgX* in deficient mice. We therefore determined *Mrg15* expression in tissues from adult *MrgX*-deficient mice. *Mrg15* mRNA expression in brain, heart, kidney, liver, and lung tissues from $MrgX^{-/-}$ mice was the same as that of the wild type (Fig. 4A) despite the confirmation that *MrgX* mRNA was not detected in tissues from *MrgX* null mice. MRG15 protein levels in brain, kidney, liver, lung, and spleen tissues were also not different in wild-type versus *MrgX* null mice (Fig. 4B). β-Actin demonstrated equal loading in the various samples. These results indicate that *Mrg15* mRNA, as well as protein, was not upregulated in the absence of *MrgX*.

TABLE 1. Genotype analysis of $Mrg15^{+/-}$ $MrgX^{-}$ male × $Mrg15^{+/-}$ $MrgX^{-/-}$ female intercrosses

Age	No. with genotype of:			No. of litters
	$Mrg15^{+/+}$ $MrgX^{-}$ or $MrgX^{-/-}$	$Mrg15^{+/-}$ $MrgX^{-}$ or $MrgX^{-/-}$	$Mrg15^{-/-}$ $MrgX^{-}$ or $MrgX^{-/-}$	
E10.5	2	2	2[a]	1
E11.5	5	10	0	2
E14.5	4	13	0	3

[a] Note that these embryos were smaller than other littermates.

Overlapping functions of *MrgX* and *Mrg15* in early embryogenesis. To investigate the possibility of functional redundancy of *MrgX* and *Mrg15* during embryogenesis, we crossed $Mrg15^{+/-}$ $MrgX^{-}$ males and $Mrg15^{+/-}$ $MrgX^{-/-}$ females, collected embryos at the indicated embryonic stages, and determined the genotype of the embryos (Table 1). We did not obtain $Mrg15^{-/-}$ $MrgX^{-}$ or $MrgX^{-/-}$ (DKO) embryos at E14.5, the stage at which we were able to identify *Mrg15* single-KO embryos occurring at the expected Mendelian frequency (33). DKO embryos were present at E10.5 and were smaller than their littermates. The results indicate that *Mrg15* and *MrgX* have overlapping functions during early mouse embryogenesis.

MRGX protein shares common motifs in the C-terminal area with MRG15 such as the HLH and LZ (2, 3), although both proteins have specific and different sequences in the N-terminal region. The common motifs between MRGX and MRG15 are important for protein-protein interactions, such as Rb and HDAC1 (20, 34, 37), and transcriptional regulation of the *B-myb* promoter. Thus, it is very likely that the *MrgX* deficiency is compensated for by MRG15, although *Mrg15* expression (both mRNA and protein levels) in *MrgX*-deficient mice and MEFs is not upregulated.

Mrg15 null embryos are lethal at E14.5, and MEFs exhibit growth defects. In contrast, *MrgX* deficiency has no obvious phenotype in development, adult mice, and MEF cultures. MRG15 is highly conserved evolutionarily from yeast to humans, although it is not known whether it has a fundamental function(s) in cellular processes in all of these organisms. MRGX is present only in mammals, and therefore the possibility exists that it evolved to perform some very specific and subtle function(s) in mammals that we have not yet discovered. For example, because MRGX is highly expressed in the brain, it may be important for more sophisticated brain functions such as learning and memory. Thus, the *MrgX*-deficient mouse model has the potential to determine additional MORF4/MRG family gene functions in vivo in the future.

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