Mex3c regulates insulin-like growth factor 1 (IGF1) expression and promotes postnatal growth

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ABSTRACT Insulin-like growth factor 1 (IGF1) mediates the growth-promoting activities of growth hormone. How IGF1 expression is regulated posttranscriptionally is unclear. Caenorhabditis elegans muscle excess 3 (MEX-3) is involved in cell fate specification during early embryonic development through regulating mRNAs involved in specifying cell fate. The function of its mammalian homologue, MEX3C, is unknown. Here we show that MEX3C deficiency in Mex3c homozygous mutant mice causes postnatal growth retardation and background-dependent perinatal lethality. Hypertrophy of chondrocytes in growth plates is significantly impaired. Circulating and bone local production of IGF1 are both decreased in mutant mice. Mex3c mRNA is strongly expressed in the testis and the brain, and highly expressed in resting and proliferating chondrocytes of the growth plates. MEX3C is able to enrich multiple mRNA species from tissue lysates, including Igf1. Igf1 expression in bone is decreased at the protein level but not at the mRNA level, indicating translational/posttranslational regulation. We propose that MEX3C protein plays an important role in enhancing the translation of Igf1 mRNA, which explains the perinatal lethality and growth retardation observed in MEX3C-deficient mice.

INTRODUCTION
Insulin-like growth factor 1 (IGF1) is a prime mediator of the growth-promoting effects of growth hormone (GH; Rodriguez et al., 2007), which promotes postnatal growth through stimulating endocellular and local production of IGF1 (Ohlsson et al., 2009). The liver produces 75% of circulating IGF1 in the bloodstream. Eliminating hepatic production of IGF1 in mice does not affect postnatal growth, indicating that 25% of normal circulating IGF1 is sufficient for normal postnatal growth (Sjogren et al., 1999; Yakar et al., 1999; Stratakopoulos et al., 2008). Local production of IGF1 in developing bone is also essential for normal postnatal growth. Chondrocyte- or osteoblast-specific knockout of Igf1 in mouse causes postnatal growth retardation, although the manipulation does not affect circulating IGF1 levels (Govoni et al., 2007a, 2007b).

Humans and mice have multiple forms of Igf1 transcripts because of the usage of different polyadenylation sites; some have a short 3′ untranslated region (3′ UTR) of 150–400 nucleotides (nt) (e.g., mouse NM_184052 and human NM_001111285), but some have a long 3′ UTR of more than 6000 nt (e.g., mouse NM_010512 and human NM_001111283). Polysome association analysis found that, of the liver Igf1 mRNAs with different lengths of 3′ UTR, only the short species were found on polysomes, suggesting that some aspect of the long 3′ UTR may prevent translation (Foyt et al., 1991). Whereas the liver expresses more Igf1 transcripts with a short 3′ UTR (Bell et al., 1986), bone-forming cells predominantly express Igf1 transcripts with a long 3′ UTR (Delany and Canalis, 1995). Recently it was found that nocturnin regulates Igf1 mRNA stability through binding to its 3′ UTR (Kawai et al., 2010). It remains unclear how the translation of Igf1 mRNA, especially in transcripts with a long 3′ UTR, is regulated.

Caenorhabditis elegans muscle excess 3 (MEX-3) is a heterogenous nuclear ribonucleoprotein (hnRNP) K homology (KH) domain-containing RNA binding protein. It is involved in cell fate specification in the early embryonic stage of C. elegans and in the maintenance of the totipotency of the germ line in adult worms (Draper et al., 1996; Hunter and Kenyon, 1996; Ciosk et al., 2006). MEX-3 is thought to prevent the translation of pal-1 and nanos mRNA in the anterior blastomeres or germ cells.
RESULTS

Background-dependent postnatal lethality of Mex3c mutant mice

Mex3c mutant mice were generated from a gene trap ES cell line, where the Mex3c gene was mutated by gene trapping. Based on the National Center for Biotechnology Information (NCBI) EST sequence C.J072395.1 and the mRNA sequence BC125427, the mouse Mex3c gene contains three exons, with C.J072395.1 spanning exons 1 and 2, and BC125427 spanning exons 2 and 3. This Mex3c mRNA transcript is predicted to encode a protein of 464 AA, containing two hnrNp KH RNA-binding domains (Grishin, 2001) and one ZNF domain (Figure 1A). The NCBI mouse Mex3c reference sequence NM_001039214.4 regards intron 1 in Figure 1A as exon sequences, resulting in a protein with an extra 189 AA at the N terminal. In our gene trap mouse, the trapping vector pGT1xrT2 was integrated into the second intron of Mex3c (Figure 1B). Reverse transcription PCR (RT-PCR) and DNA sequencing analyses revealed that most transcripts from the trapped allele (written as “tr” hereafter) contained the LacZ cDNA from the trapping vector, but lacked the third exon of Mex3c, producing a peptide containing the N-terminal 56 AA of the 464 AA Mex3c and the full-length β-galactosidase (β-gal) peptide. Because this fusion peptide lacks the hnrNp KH RNA-binding and the ZNF domains, it is expected to be nonfunctional. The β-gal moiety in the fusion protein permits tracing of the expression of Mex3c mRNA through detecting β-gal activity.

Transcripts from the trapped allele, however, also included a low percentage of intact full-length Mex3c mRNA due to alternative splicing. Quantitative RT-PCR (qRT-PCR) revealed that authentic Mex3c mRNA in +/+, +/tr, and tr/tr mice comprised ~55% and 2.2% of that of +/+ mice, respectively (Figure 1C). Thus tr/tr mice were not Mex3c null but expressed <5% of normal levels of Mex3c mRNA. Because neither customized nor commercially available anti-Mex3c antibodies were specific enough to detect endogenous MEX3C protein, the expression level of MEX3C protein in mutant mice is unclear.

Trapping both alleles of the Mex3c gene did not affect embryonic development, because the tr/tr pups from +/tr × +/tr matings were born normal with Mendelian ratios. Postnatal survival, however, was affected to variant degrees depending on background. Under a C57BL/6 background, more than 85% of mutants died within several hours after birth. Only seven homozygous mutant mice from more than 160 pups of heterozygous parents survived to weaning time. Mutant pups most likely died from poor breathing, and their bodies were cyanotic. On histological analysis, alveolar spaces in lungs from mutant mice were poorly expanded compared to wild-type lungs and the ZNF domains, it is expected to be nonfunctional. The β-gal moiety in the fusion protein permits tracing of the expression of Mex3c mRNA through detecting β-gal activity.

Mex3c, as assessed by tracing β-gal activity in organs of 1-d-old pups, was highly expressed in muscle (Figure 2A) and multiple internal organs such as the lung and the spleen (Figure 2B). Mex3c is expressed at low levels in the liver, and β-gal activity was seen in blood vessel cells but not hepatic cells on liver sections (unpublished data). Because of the important roles of the lung
and bone in normal respiration, Mex3c expression was further examined by tracing β-gal activity on cryosections from 1-d-old pups. β-Gal activity was observed in all cell types in the lung (Figure 2C). In the developing tibia bone, resting and proliferating chondrocytes showed high β-gal activity, whereas hypertrophic chondrocytes showed low activity, and cells in the ossification center showed intermediate β-gal activity (Figure 2D). High Mex3c expression in the skeleton and lung may underlie perinatal lethality in Mex3c mutant mice, but the mechanism warrants further study. Adult Mex3c mutant mice were deficient in IGF1 (see later in the text), and IGF1 deficiency causes perinatal lethality because of poor respiration (Liu et al., 1993; Govoni et al., 2007b). Thus it is likely that perinatal lethality in Mex3c mutant mice is also related to IGF1 deficiency.

Postnatal growth retardation in Mex3c mutant mice

In a mixed B6/129 or a mainly FVB/N background, more than 80% of tr/tr mice lived to adulthood. Mutant mice from these backgrounds were growth retarded, however, and had an average of 87% normal body length at various time points (Table 1). The few tr/tr mice of mainly C57/BL6 background which survived to adulthood were also growth retarded (unpublished data). Heterozygous mice showed normal growth. Consistent with reduced body length in tr/tr mice, tibias from mutant mice were also, on average, 15% shorter (unpublished data).

Histological analysis of tibial proximal growth plates from 20-d-old mutant mice, when the epiphyseal growth plate is most actively proliferating (Walker and Kember, 1972), revealed that the length of the growth plate was significantly decreased in mutant mice (Table 2). Although the lengths of the resting and the proliferating zones were not different between control and tr/tr mutant mice, that of the hypertrophic zone was significantly decreased in mutant mice (Table 2; Figure 3, A and B). Consistent with the attenuated hypertrophic zone, the mean height of terminal hypertrophic chondrocytes in mutant mice was significantly decreased (Table 2). In addition, a trend

![Image of Figure 2: Examination of Mex3c expression in newborn mice by tracing β-gal activity. (A) High β-gal activity was detected in skeletal muscle. Bodies of 1-d-old pups were eviscerated followed by whole-mount X-Gal staining. The muscle tissues of +/tr and tr/tr pups were strongly positive for β-gal activity. The residual skin around the mouth and paws was negative, demonstrating the specificity of the staining. (B) Detecting β-gal activity in internal organs of newborn mice. β-Gal activity was high in most internal organs except for the liver. (C) All cells in the lung of newborn pups were β-gal positive. Scale bar: 50 μm. (D) Detecting β-gal activity in tibia of newborn mice. Cryosections of the proximal ends of tibia from +/tr mice were stained by X-Gal, followed by eosin counterstaining. Scale bar: 100 μm.](image)
mice. Circulating GH concentrations of adult mutant mice were significantly higher than those of control mice (Table 3). Circulating IGF1 concentrations in mutant mice, however, were 40% lower than in normal mice (Table 3). IGF1 deficiency causes growth retardation in mice (Liu et al., 1993; Powell-Braxton et al., 1993) and impairs chondrocyte hypertrophy (Wang et al., 1999). Sixty percent of normal serum IGF1, however, would be sufficient to maintain normal postnatal growth in Mex3c mutant mice if their local IGF1 production in the bone were unaffected (Sjogren et al., 1999; Yakar et al., 1999; Govoni et al., 2007a, 2007b). IGF1 concentrations in bone (tibia and femur) extracts were compared by enzyme-linked immunosorbent assay (ELISA). IGF1 levels in extracts from mutant mice were found to be 42% lower than that of normal mice (Table 3), confirming IGF1 deficiency in bone.

Immunohistochemical analysis of developing bone revealed that in 20-d-old mutant mice, IGF1 protein was significantly decreased in resting and proliferating chondrocytes (Figure 4B, middle and right panels). The specificity of the signal was confirmed by the blocking peptide to significantly reduce the staining in these cells (Figure 4, left panel). The addition of blocking peptide only slightly decreased the signals in hypertrophic chondrocytes (Figure 4C), which could be the result of high IGF1 expression in hypertrophic chondrocytes (Gil-Pena et al., 2009). IGF1 expression in cells of primary spongiosa of the mutant mice was also significantly reduced (Figure 4D). These observations were reproducible in tibia growth plates of multiple pairs of control and mutant mice and corroborated with our ELISA data that bone-forming cells from mutant mice produced reduced IGF1. These data confirmed IGF1 deficiency in bones of mutant mice. Thus we observed bone IGF1 deficiency and postnatal growth retardation in Mex3c mutant mice.

**Mex3c is highly expressed in the testis, ovary, brain, and developing bone**

The expression of Mex3c in tissues of 4-wk-old mice was examined by tracing the activities of β-gal. Mex3c was highly expressed in the testis and the brain, but low in other internal organs, including the liver (Figure 5A). This finding was also confirmed by qRT-PCR, with the liver showing the lowest Mex3c expression among all the tissues examined (Figure 5B). Although Mex3c is highly expressed in the testis, Mex3c mutant males had normal spermatogenesis and were fertile with normal litter size. Mex3c was also readily detected in the female reproductive system including the ovary, the uterus, and the oviducts (Figure 5A). Mutant females, however, were also fertile, although they tended to have their first litters 2–3 wk later than do normal mice, which might be the result of growth retardation. Because there is still a low level (2%) of authentic Mex3c mRNA in

![Figure 3: Analysis of growth plates from control and Mex3c mutant mice.](image)

| Control | Mutant | p Value |
|---------|--------|---------|
| HZ (μm) | 249.0 ± 23.2 | 166.4 ± 18.0 | <0.05 |
| HZ cell height (μm) | 21.7 ± 0.5 | 17.5 ± 0.4 | <0.0001 |

Data are means ± SEM for five 20-d-old mice per group. Control mice included three +/+ mice and two +/tr mice. Values represent the longitudinal dimension of the structure parallel to the long axis of the bone. Twenty terminal chondrocytes from each of the five mice were measured to obtain the mean of HZ cell height.

**TABLE 2: Growth plate parameters in control and tr/tr mice.**
homozygous mutant mice, the residual MEX3C protein might have been enough to maintain normal gametogenesis in both sexes. A null Mex3c mutant is thus needed to test whether Mex3c is necessary for normal reproduction. Consistent with high Mex3c expression in the brain, we found that mutant mice had reduced fat deposition related to abnormal energy expenditure (unpublished observation).

Chondrocyte hypertrophy is apparently affected in Mex3c mutant mice; thus we examined Mex3c gene expression in the growth plates of developing tibias. Mex3c was highly expressed in chondrocytes of the resting and proliferating zones (Figure 5C). In the hypertrophic zone, Mex3c showed decreased expression, and β-gal activity was barely detectable in fully hypertrophic chondrocytes. Mex3c expression was high in stromal cells in the matrix of spongy bone (Figure 5D) and compact bone (Figure 5E). Strong β-gal activity was also observed in periosteum, a membrane structure generating osteoblasts. Comparing the pattern of IGF1 deficiency and Mex3c expression in the growth plate, IGF1 deficiency was pronounced in cells of mutant mice that normally had high Mex3c expression. MEX3C is an intracellular RNA-binding protein; high expression of Mex3c in multiple types of bone cells suggests that IGF1 deficiency in these cells of mutant mice could be a direct effect of MEX3C deficiency. The data also suggest that Mex3c mutation has varied effects on organs with high Mex3c expression.

IGF1 deficiency in Mex3c mutant mice is not caused by decreased Igf1 mRNA level

qRT-PCR revealed that total Igf1 transcripts (including those with short and long 3' UTRs) in tissues (such as the liver, kidney, spleen, and testis) and muscle from mutant mice were not significantly decreased (Figure 6A). Although the liver and kidney of mutant mice showed 23% less expression of Igf1 mRNA, this marginal decrease did not account for the 40% reduction in serum IGF1 levels, because serum IGF1 levels that were 71% of normal levels were observed in animals with 28% of normal levels of the 1-kb short-form Igf1 transcripts and 52% of normal levels of 7-kb long-form Igf1 transcripts (Liu et al., 1998). Cells in tibia from mutant mice were clearly deficient in IGF1 protein.
Mex3c regulates IGF1 and growth

MEX3C regulates IGF1 and growth. It enriches IGF1 mRNA from tissue lysates. GST-MEX3C and GST-MEX3C/N pulled down significantly more IGF1 mRNA than did GST and GST-MEX3C/C, which lack RNA-binding domains. Although mRNA of Gapdh and all five other mRNA species tested were also enriched by GST-MEX3C and GST-MEX3C/N (unpublished data), their enrichment was all KH.

qRT-PCR analysis found that Igf1 mRNA levels in tibias of mutant mice were not different from those of controls (Figure 6B), demonstrating that IGF1 deficiency in Mex3c mutant mice was not caused by decreased Igf1 mRNA levels. Thus our data suggest that MEX3C deficiency most likely affected Igf1 mRNA translation, but not Igf1 mRNA transcription or turnover.

MEX3C enriched Igf1 mRNA from tissue lysates

MEX3C has two KH RNA-binding domains; we examined whether MEX3C interacted with Igf1 mRNA. Glutathione S-transferase (GST), GST-MEX3C, GST-MEX3C/C (GST fused to the C-terminal 263 AA of MEX3C which contains the ZNF domain but not the two KH RNA-binding domains; Figure 6C), and GST-MEX3C/N (GST fused to the N-terminal 296 AA of MEX3C, containing the two KH RNA-binding domains but not the ZNF domain) were tested for their capability to enrich Igf1 mRNA from brain lysate. We chose the brain because it expresses Igf1 and has high Mex3c expression. qRT-PCR revealed that GST-MEX3C and GST-MEX3C/N pulled down significantly more Igf1 mRNA than did GST and GST-MEX3C/C, which lack RNA-binding domains (Figure 6D). Although mRNA of Gapdh and all five other mRNA species tested were also enriched by GST-MEX3C and GST-MEX3C/N (unpublished data), their enrichment was all KH.

FIGURE 5: Analysis of Mex3c expression in mouse tissues. (A) Expression of Mex3c in tissues of developing mice. Tissues from 4-wk-old heterozygous and normal mice were stained for β-gal; positive tissues were stained blue. The epididymis were positive even in normal mice (+/+) due to endogenous β-gal activity, which indicates the reliability of the staining condition. Lu: lung; Spl: spleen; Kid: kidney; Liv: liver; Musc: skeletal muscle; Fat/epi: gonadal fat/epididymis; Ov/Ut: ovary/uterus; Tes: testis; He: heart; Bla/Ure: bladder/ureter. (B) qRT-PCR analysis of Mex3c mRNA in various tissues from normal mice. Means ± SEM of data from four mice are presented. (C) Mex3c expression in chondrocytes of proximal tibia growth plates of 20-d-old mice indicated by tracing β-gal activity. β-Gal–positive cells were stained blue. No β-gal–positive cells were observed in control (+/+) mice. The sections were counterstained with eosin to show the matrix in red. RZ, resting zone; PZ, proliferating zone; HZ, hypertrophic zone. (D) Expression of Mex3c in stromal cells of the spongy bone. (E) Expression of Mex3c in stromal cells of the compact bone. Scale bars: 50 μm.

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mouse MEX3C are nearly 90% identical to *C. elegans* MEX-3 KH domains. This high identity suggests that mouse MEX3C might also interact with multiple mRNA molecules. Indeed, human MEX3C is able to associate with poly(A), poly(U), and multiple mRNA species (Buchet-Poyau et al., 2007). Whether Igf1 mRNA interacts directly with MEX3C is still an open question, however.

**DISCUSSION**

Here we report that Mex3c mutant mice showed growth retardation, background-dependent perinatal lethality, and Igf1 deficiency. Although MEX3C deficiency also affects energy balance and decreases fat deposition, rescuing the slim phenotype by introducing an ob/ob mutation did not rescue growth retardation (our unpublished data), excluding the possible effects of negative energy balance on postnatal growth. We postulate that Igf1 deficiency might be a major mechanism underlying the phenotypes in the mutants described in this work.

Eighty-five percent of Mex3c mutant mice in the C57BL/6 background died from poor respiration soon after birth, similar to knockout mouse models deficient in Igf1 signaling. Constitutive Igf1 knockout causes background-dependent perinatal lethality (Liu et al., 1993), and osteoblast-specific Igf1 knockout causes similar postnatal death (Govoni et al., 2007b). In both cases, poor breathing was the cause of death. We did not examine whether Igf1 was deficient in newborn mutant pups. Because Mex3c is highly expressed in multiple tissues of newborn pups, and Igf1 deficiency is observed in adult mutant mice, Igf1 deficiency could also be present in newborn pups, and this could underlie their perinatal lethality.

Although serum Igf1 levels in Mex3c mutant mice are 60% of normal levels and by themselves do not account for postnatal growth retardation, we observed local Igf1 deficiency in the bone. Chondrocyte- and osteoblast-specific Igf1 knockout affects postnatal growth even though circulating Igf1 levels are normal (Govoni et al., 2007a, 2007b). Thus local deficiency of Igf1 in the bone of Mex3c mutant mice might underlie the observed attenuated growth. Consistent with local deficiency of Igf1 in Mex3c mutant mice, Mex3c is highly expressed in resting and proliferating chondrocytes, as well as in bone stromal cells. Our analysis of growth plate from Mex3c mutant mice showed that chondrocyte hypertrophy was affected, the same phenomenon noted in Igf1 mutant mice (Wang et al., 1999). Thus our data suggest that Igf1 deficiency most likely underlies the growth retardation observed in Mex3c mutant mice.

Although a specific MEX3C antibody is unavailable to confirm MEX3C deficiency at the protein level, the finding that tr/tr mice have only 2% normal Mex3c mRNA expression suggests that MEX3C protein deficiency is highly likely. Although the possibility of toxic gain of function of MEX3C-β-gal fusion protein cannot be excluded at the moment, its likelihood is low. So far, nearly 1000 articles have been published describing various phenotypes of gene trap mice, and many of these mice express a fusion protein from the trapped host gene and the reporter LacZ. To the best of our knowledge, toxic gain of function of the host peptide-β-gal fusion protein has not been reported to account for the observed phenotypes. In addition, Mex3c is highly expressed in the testis, ovary, and brain; the toxic effects would also be evident in these organs if MEX3C-β-gal is indeed a toxic protein. Mutant mice, however, have normal gametogenesis and are fertile. Although tr/tr mice showed abnormal energy expenditure and reduced fat deposition, tr/tr;ob/ob double mutant mice were obese but still growth retarded (our unpublished observations), excluding a role of energy expenditure on growth.

**FIGURE 6:** qRT-PCR analysis of Igf1 mRNA in mutant mouse and in GST pull-down assays. (A) Comparison of Igf1 mRNA levels in liver, kidney, spleen, testis, and muscle tissues from +/+ and tr/tr mice. Four 4–5-wk-old male mice for each genotype were analyzed. Expression in tissues of control mice was set as 1. Means ± SEM are presented. (B) Igf1 mRNA expression in +/+ and tr/tr tibia. (C) Diagram showing domains in MEX3C, MEX3C/N, and MEX3C/C. KH: KH RNA-binding domain. (D) GST pull-down analysis of Igf1 mRNA enrichment by partial and full-length MEX3C proteins. Means ± SEM of three independent assays are presented. mRNA levels in GST-only assays were set as 1. Numbers above the columns are means ± SEM of Ct numbers.

domain-dependent. The sequence of *C. elegans* MEX-3 recognition element (MRE) was defined as (A/G/U)(G/U)AGN(0–8)U(U/A/C)UA) and was found in the 3’-UTR of ~26.2% of all genes in *C. elegans* (Pagano et al., 2009). Considering genes with this MRE in the 5’-UTR and coding region, a higher proportion of *C. elegans* mRNA is expected to contain this MRE and bind MEX-3. Thus MEX-3 is able to bind multiple mRNA species. The KH RNA binding domains of
The tissue- and pathway-specific effects of Mex3c mutation also argue against the possibility of a toxic gain-of-function mechanism in the growth retardation of tr/tr mice.

Igf1 mRNA was decreased by 23% in the liver and the kidney of Mex3c mutant mice but not in the other organs, including the bone. Although it is unclear why Igf1 transcription is not increased in the mutants, which had higher levels of GH than did control mice, the marginal decrease of Igf1 mRNA in the liver and the kidney could be indirect effects of MEX3C deficiency, because Mex3c has relatively low expression in these two organs. Neither the 40% decrease of circulating IGFl nor the decrease of local IGFl production in the bone of mutant mice can be explained by decreased Igf1 mRNA levels. Although deficiency of acid-labile subunit and IGF binding proteins leads to decreased circulating IGFl (Ueki et al., 2000; Ning et al., 2006), this deficiency does not cause perinatal lethality or dramatic growth retardation. In addition, deficiency of these proteins does not decrease local expression of IGFl in bone. IGFl production must have been affected in Mex3c mutant mice. Our data suggest that MEX3C deficiency may not affect Igf1 mRNA transcription or stability, it most likely affects Igf1 expression at the protein level.

MEX3C protein is an RNA-binding protein and contains two KH-type RNA-binding domains and one ZNF domain mediating protein–protein interaction. GST pull-down assays showed that GST-MEX3C and GST-MEX3C/N (the N-terminal MEX3C containing two RNA-binding domains) pulled down 200- and 400-fold more Igf1 mRNA from mouse brain lysate than did GST, demonstrating that the MEX3C N terminus containing the RNA-binding domains is necessary and sufficient to enrich Igf1 mRNA. We noted that in this GST pull-down assay, GST-MEX3C and GST-MEX3C/N were able to enrich all the mRNA species we had tested to various degrees, reminiscent of human MEX3C, which associates with poly(A), poly(U), and multiple mRNA species including Gapdh (Buchet-Poyau et al., 2007). C. elegans MEX-3 binds to a consensus MRE which is present in a large proportion of C. elegans genes (Pagano et al., 2009). Thus our observation that MEX3C, a MEX-3 homologue, can pull down multiple mRNAs including Igf1 mRNA from mouse tissue lysates is not surprising. We noted that the C terminus of MEX3C showed inhibitory effects on RNA pull-down. It is likely that the C terminus of MEX3C provides a mechanism for mRNA selectivity. Although it remains to be determined whether Igf1 mRNA is a direct or an indirect target of MEX3C and the region of Igf1 mRNA necessary for the interaction, our data suggest that Igf1 mRNA could be an mRNA target for MEX3C.

MEX3C’s C. elegans homologue MEX-3 repressed the translation of pal-1 and nanos mRNA through unknown mechanisms (Draper et al., 1996; Hunter and Kenyon, 1996; Jadhav et al., 2008). Our data suggest that MEX3C deficiency inhibits the translation of Igf1 mRNA. Bone-forming cells express predominantly Igf1 transcripts of ~7000 nt with a long 3’ UTR (Delany and Canalis, 1995), and, in the liver, Igf1 transcripts with a long 3’-UTR have low translatability (Foyt et al., 1991). Local production of IGFl in the bone is essential for normal postnatal growth (Govoni et al., 2007a, 2007b). We propose that Igf1 mRNA with a long 3’-UTR may need trans-acting factors to improve their translatability in bone-forming cells, and MEX3C or its interacting partner could be one of these trans-acting factors. This proposal would explain why Mex3c mutation impairs bone IGFl production and postnatal growth. Our data and those of others suggest that MEX3C seems able to bind multiple mRNA molecules. How MEX3c mutation affects the translation of certain mRNA molecules, such as Igf1 mRNA, but not others needs further study. In summary, our data suggest that MEX3C plays an important role in enhancing the translation of Igf1 mRNA directly or indirectly and promoting postnatal growth.

**MATERIALS AND METHODS**

**Generation of Mex3c gene trap mice**

The Mex3c gene trap ES cell line DD0642 was obtained from the Sanger Institute Gene Trap Resource (SIGTR, Cambridge, UK). The ES cells were microinjected into mouse blastocysts, and the resulting chimeric males were mated with C57/BL6 females to obtain heterozygous Mex3c gene trap mice. Heterozygous mice were intercrossed to obtain mutant mice of 129sv/C57 mixed background. Heterozygous mice were backcrossed to C57/BL6 or FVB/N for six generations to obtain mutant mice of mainly C57/BL6 or FVB/N background. Mice were housed in the animal facility of Wake Forest University Health Sciences (Winston-Salem, NC). Experiments were conducted in accordance with the National Research Council publication Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Wake Forest University. Mice were kept in microisolator cages with 12-h light/dark cycles and were fed ad libitum. A chow diet (Prolab, RMH3000; PMI Nutrition International, Henderson, CO) was used for colony maintenance.

**Genotyping**

Ear biopsies were lysed as previously described for genotyping (Agoulinik et al., 2002). The gene trap allele was detected by PCR amplification of a 419–base pair product with primer TrapF (ttacacatcagccgtacag) and TrapR (ctctgcctgacgttctca). Homozygous gene trap mice were distinguished from heterozygous mice by PCR with primer pair D18Mit210F (gggccaagtaacttaactaata) and D18Mit210R (ttacacgtagctgccctcc). The thermal cycle parameters were: 94°C 4 min, followed by 35 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s. A 122–base pair PCR product was obtained from the C57BL/6 and FVB alleles, and a 146–base pair PCR product was obtained from the gene trap 129/Sv allele. The size difference was resolved on a 3% agarose gel stained with ethidium bromide.

**Expression and purification of GST fusion proteins**

*Escherichia coli* BL21(DE3) transformed with plasmid DNA (pGEX4T1, pGEX4T1/MEX3C, pGEX4T1/MEX3C/C, and pGEX4T1/MEX3C/N) was used for GST fusion expression as described (Zhou et al., 2005). Glutathione sepharose 4B beads (GE Life Sciences, Pittsburgh, PA) were used to purify the GST fusion proteins according to the manufacturer’s instructions.

**GST pull-down assay**

GST pull down and immunoprecipitation were performed as described (Lu and Bishop, 2003; Zhang et al., 2005). The final precipitates were either lysed in 50 μl of 1X SDS loading buffer for SDS–PAGE and Western blotting analysis or used for RNA extraction using the RNeasy Protect kit (QIAGEN, Valencia, CA) for qRT-PCR analysis.

**qRT-PCR**

Mice were killed by CO2 overdose, and tissues were snap frozen in liquid nitrogen and then stored at –80°C before RNA extraction. To extract RNA from developing tibia, all muscle tissue was removed from the bone, and the bone marrow cells were removed by several flushes with ice-cold phosphate-buffered saline (PBS) solution. Then bones were ground in liquid nitrogen, and RNA was extracted from bone powders with TRIzol (Invitrogen, Life Technologies, Grand Island, NY). Total RNA from soft tissues was extracted with an RNeasy Mini Kit (QIAGEN) as instructed by the manufacturer. Reverse
transcription was performed with the SuperScript First-Strand Synthesis System from Invitrogen.

Real-time PCR was performed on a 7300 real-time PCR system (Applied Biosystems, Foster City, CA). For mouse Gapdh, Hprt1, and IGF1, TaqMan probes (Applied Biosystems) were used. The IGF1 Taqman probe recognizes both the short form and long forms of IGF1 transcripts. For Mex3c (Mex3cF: atctgtctccacgcctac, and Mex3cR: agtgcttaatatttacacctg) and Ppib (PpibF: tcgctttgagacttttggaa and PpibR: aggcgtccatatagtc) real-time PCR, SYBR Green PCR Master Mix (Applied Biosystems) was used. After the PCR amplification, a dissociation program was run, and the amplified product was analyzed by electrophoresis to verify the specificity of the amplification. Relative gene expression levels were calculated using the CT method (Livak and Schmittgen, 2001). In cases where mRNA was not detected after 40 cycles, the cycle threshold (Ct) number was set as 40 for analysis. Three to five animals were assayed for each group. Three independent experiments were performed with each experiment performed in triplicate. Results were presented as mean ± SEM.

Growth plate analysis
Tibia from newborn, 20-d-old, and 8-wk-old mice were fixed in 4% paraformaldehyde (PFA)/PBS at 4°C for 4 h. Bones from 20-d-old and 8-wk-old mice were decalcified in Immunocal (Decal Chemical, Tallman, NY) at 4°C for 30 min to 1 h. Bones were dehydrated and embedded in paraffin to obtain paraffin sections for hematoxylin and eosin staining or Masson’s trichrome staining.

The longitudinal dimensions of the proximal tibial growth plate and the heights of terminal chondrocytes were measured on photomicrographs of anatomically matched midsagittal sections, taken under a 20× objective with an Axio M1 microscope and an AxioCam MRc digital camera (Carl Zeiss, Thornwood, NY). Measurements were performed with ImageJ software.

Immunostaining of tibia sections
For histochemical analysis, decalcified bones were immersed in 30% sucrose/PBS overnight at 4°C before they were embedded in optimal cutting temperature (OCT) compound for cryosectioning. To examine the expression of Mex3c through detecting β-gal activity, bone cryosections were stained with X-GAL as described (Beddington et al., 1989). To examine the expression of IGF1 in the bone, bone cryosections were fixed with 4% PFA, and treated with 3% H2O2/PBS for 30 min to inactivate endogenous peroxidase activity. Then the sections were blocked with Protein Block (Dako, Carpinteria, CA) for 1 h, and incubated with anti-IGF1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100) at 4°C overnight. To confirm specificity, anti-IGF antibody was preincubated with a fivefold concentration of IGF1 blocking peptide (Santa Cruz Biotechnology) for 2 h before the antibody was applied to the sections. After three 5-min washes in Tris-buffered saline and Tween 20 (TBS/T), the sections were incubated with biotin-conjugated anti-goat secondary antibody (1:300; Vector Labs). After three 5-min washes, the signals were visualized by ImmPACT DAB (Vector Labs). The sections were counterstained with hematoxylin before mounting. Images were taken with an Axio M1 microscope equipped with an AxioCam MRc digital camera (Carl Zeiss).

Hormone and growth factor assays
Serum was obtained from the saphenous vein of mice at ages listed in Table 1. GH was assayed with the mouse/rat GH ELISA kit purchased from Millipore (cat. E2R228G45K; Billerica, MA) as instructed by the manufacturer. Serum IGF1 was assayed with a mouse/rat IGF1 immunoassay kit from R&D Systems (Minneapolis, MN). To assay IGF1 in bone extracts, tibia and femur bones were rinsed of muscle tissues manually. Bones were homogenized with a motor homogenizer in RIPA buffer (1 g of bone in 5 ml of buffer), and lysates were incubated with shaking at 4°C for 1 h. The lysates were centrifuged at 12,000 g at 4°C for 10 min. The cleared supernatants were diluted 20 times for the ELISA.

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REFERENCES
Agoulnik AI, Lu B, Zhu Q, Truong C, Ty MT, Arango N, Chada KK, Bishop CE (2002). A novel gene, Pog, is necessary for primordial germ cell proliferation in the mouse and underlies the germ cell deficient mutation, gcl. Hum Mol Genet 11, 3047–3053.
Beddington RS, Morgernstern J, Land H, Hogan B (1988). An in situ transgenic enzyme marker for the midgestation mouse embryo and the visualization of inner cell mass clones during early organogenesis. Development 106, 37–46.
Bell GI, Stempken MM, Fong NM, Rall LB (1986). Sequences of liver cDNAs encoding two different mouse insulin-like growth factor I precursors. Nucl Acids Res 14, 7873–7882.
Buchet-Poyau L, Courchet J, Hr HL, Seraphin B, Scaozeac J-Y, Duret L, Domon-Dell C, Freund J-N, Billaud M (2007). Identification and characterization of human Mex-3 proteins, a novel family of evolutionarily conserved RNA-binding proteins differentially localized to processing bodies. Nucl Acids Res 35, 1289–1300.
Ciosk R, DePalma M, Priess JR (2006). Translational regulators maintain totipotency in the Caenorhabditis elegans germline. Science 311, 851–853.
Delany AM, Canalis E (1995). Transcriptional repression of insulin-like growth factor I by glucocorticoids in rat bone cells. Endocrinology 136, 4776–4781.
Donnini M, Lapucci A, Papucci L, Witort E, Jacquier A, Brewer G, Nicolin A, Capaccioli S, Schiavone N (2004). Identification of TINO: a new evolutionarily conserved BCL-2 Au-rich element RNA-binding protein. J Biol Chem 279, 20154–20166.
Draper BW, Mello CC, Bowerman B, Hardin J, Priess JR (1996). MEX-3 is a KH domain protein that regulates blastomere identity in early C. elegans embryos. Cell 87, 205–216.
Foyt HL, LeRoith D, Roberts CT, Jr (1991). Differential association of insulin-like growth factor I mRNA variants with polysomes in vivo. J Biol Chem 266, 7300–7305.
Gil-Pena H, Garcia-Lopez E, Alvarez-Garcia O, Loredo V, Carbajo-Perez E, Ordoniez FA, Rodriguez-Suarez J, Santos F (2009). Alterations of growth plate and abnormal insulin-like growth factor I metabolism in growth-retarded hypokalemic rats: effect of growth hormone treatment. Am J Physiol Renal Physiol 297, F639–F645.
Govoni KE, Lee SK, Chung YS, Behringer RR, Wegedal JE, Baylink DJ, Mohan S (2007a). Disruption of insulin-like growth factor-I expression in type Ilalphal collagen-expressing cells reduces bone length and width in mice. Physiol Genomics 30, 354–362.
Govoni KE, Wegedal JE, Florin L, Angel P, Baylink DJ, Mohan S (2007b). Conditional deletion of insulin-like growth factor-I in collagen type IIalphaI collagen-expressing cells reduces bone length and width in mice. J Biol Chem 282, 2646–2663.
Grishin NV (2001). KH domain: one motif, two folds. Nucl Acids Res 29, 638–643.
Guzman B, Cormand B, Ribases M, Gonzalez-Nunez D, Botey A, Poch E (2006). Implication of chromosome 18 in hypertension by sibling pair and association analyses: putative involvement of the RKHD2 gene. Hypertension 48, 883–891.
Hunter CP, Kenyon C (1996). Spatial and temporal controls target pal-1 blastomere-specification activity to a single blastomere lineage in C. elegans embryos. Cell 87, 217–226.
Jadhav S, Rana M, Subramaniam K (2008). Multiple maternal proteins coordinate to restrict the translation of C. elegans nanos-2 to primordial germ cells. Development 135, 1803–1812.
Kawai M, Delany AM, Green CB, Adamo ML, Rosen CJ (2010). Nocturnin suppresses igf1 expression in bone by targeting the 3' untranslated region of igf1 mRNA. Endocrinology 151, 4861–4870.

Liu JL, Grinberg A, Westphal H, Sauer B, Accili D, Karas M, LeRoith D (1998). Insulin-like growth factor-I affects perinatal lethality and postnatal development in a gene dosage-dependent manner: manipulation using the Cre/loxP system in transgenic mice. Mol Endocrinol 12, 1452–1462.

Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75, 59–72.

Livak KJ, Schmitthenner TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-\(\Delta \Delta CT\) method. Methods 25, 402–408.

Lu B, Bishop CE (2003). Mouse GGN1 and GGN3, two germ cell specific proteins from the single gene Ggn, interact with mouse POG and play a role in spermatogenesis. J Biol Chem 278, 16289–16296.

Lu B, Geurts AM, Poirier C, Petit DC, Harrison W, Overbeek PA, Bishop CE (2007). Generation of rat mutants using a coat color-tagged Sleeping Beauty transposon system. Mamm Genome 18, 338–346.

Ning Y, Schuller AG, Bradshaw S, Rotwein P, Ludwig T, Frystyk J, Pintar JE (2006). Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. Mol Endocrinol 20, 2173–2186.

Ohlsson C, Mohan S, Sjogren K, Tivesten A, Isgaard J, Isaksson O, Jansson JO, Svensson J (2009). The role of liver-derived insulin-like growth factor-I. Endocr Rev 30, 494–535.

Pagano JM, Farley BM, Essien KI, Ryder SP (2009). RNA recognition by the embryonic cell fate determinant and germline totipotency factor MEX-3. Proc Natl Acad Sci USA 106, 20252–20257.

Powell-Braxton L, Hollingshead P, Warburton C, Dowd M, Pitts-Meek S, Dalton D, Gillett N, Stewart TA (1993). IGFI-I is required for normal embryonic growth in mice. Genes Dev 7, 2609–2617.

Rodriguez S, Gaunt TR, Day IN (2007). Molecular genetics of human growth hormone, insulin-like growth factors and their pathways in common disease. Hum Genet 122, 1–21.

Sjogren K et al. (1999). Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proc Natl Acad Sci USA 96, 7088–7092.

Skarnes WC et al. (2004). A public gene trap resource for mouse functional genomics. Nat Genet 36, 543–544.

Stratikopoulos E, Szabolcs M, Dragatis I, Klinakis A, Efstratiadis A (2008). The hormonal action of IGF1 in postnatal mouse growth. Proc Natl Acad Sci USA 105, 19378–19383.

Ueki I, Ooi GT, Tremblay ML, Hurst KR, Bach LA, Boisclair YR (2000). Inactivation of the acid labile subunit gene in mice results in mild retardation of postnatal growth despite profound disruptions in the circulating insulin-like growth factor system. Proc Natl Acad Sci USA 97, 6868–6873.

Walker KV, Kember NF (1972). Cell kinetics of growth cartilage in the rat tibia. I. Measurements in young male rats. Cell Tissue Kinet 5, 401–408.

Wang J, Zhou J, Bondy CA (1999). Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. FASEB J 13, 1985–1990.

Yakar S, Liu J, Stannard B, Butler A, Accili D, Sauer B, LeRoith D (1999). Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci USA 96, 7324–7329.

Zhang J, Wang Y, Cao Z, Huang P, Lu B (2005). Yeast two-hybrid screens imply that GGNBP1, GGNBP2 and OAZ3 are potential interaction partners of testicular germ cell-specific protein GGN1. FEBS Lett 579, 559–566.

Zhou Y, Zhao Q, Bishop CE, Huang P, Lu B (2005). Identification and characterization of a novel testicular germ cell-specific gene Ggnbp1. Mol Reprod Dev 70, 301–307.