N-Myristoyltransferase 1 Is Essential in Early Mouse Development*

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N-Myristoyltransferase (NMT) transfers myristate to an amino-terminal glycine of many eukaryotic proteins. In yeast, worms, and flies, this enzyme is essential for viability of the organism. Humans and mice possess two distinct but structurally similar enzymes, NMT1 and NMT2. These two enzymes have similar peptide specificities, but no one has examined the functional importance of the enzymes in vivo. To address this issue, we performed both genetic and biochemical studies. Northern blots with RNA from adult mice and in situ hybridization studies of day 13.5 embryos revealed widespread expression of both Nmt1 and Nmt2. To determine whether the two enzymes are functionally redundant, we generated Nmt1-deficient mice carrying a β-galactosidase marker gene. β-Galactosidase staining of tissues from heterozygous Nmt1-deficient (Nmt1+/−) mice and embryos confirmed widespread expression of Nmt1. Intercrosses of Nmt1+/− mice yielded no viable homozygotes (Nmt1−/−), and heterozygotes were born at a less than predicted frequency. Nmt1−/− embryos died between embryonic days 3.5 and 7.5. Northern blots revealed lower levels of Nmt2 expression in early development than at later time points, a potential explanation for the demise of Nmt1−/− embryos. To explore this concept, we generated Nmt1−/− embryonic stem (ES) cells. The Nmt2 mRNA could be detected in Nmt1−/− ES cells, but the total NMT activity levels were reduced by ~95%, suggesting that Nmt2 contributes little to total enzyme activity levels in these early embryo cells. The Nmt1−/− ES cells were functionally abnormal; they yielded small embryoid bodies in vitro differentiation experiments and did not contribute normally to organogenesis in chimeric mice. We conclude that Nmt1 is not essential for the viability of mammalian cells but is required for development, likely because it is the principal N-myristoyltransferase in early embryogenesis.

N-Myristoylation is a lipid modification of proteins that facilitates the targeting of proteins to membrane surfaces. The process is catalyzed by N-myristoyltransferase (NMT),1 an enzyme that transfers myristate from myristoyl-Coenzyme A to the amino group of an amino-terminal glycine (1). There is little doubt that this posttranslational modification is important for eukaryotic cells. NMT is essential for cell viability in Saccharomyces cerevisiae (2), Candida albicans (3), and Cryptococcus neoformans (4). NMT is also required for the viability of flies (5), worms (6, 7), trypanosomes (8), and Leishmania (9). Because its activity is essential, NMT has sparked interest as a target for antifungal, antiparasitic, and even anticancer therapy (9). N-Myristoylation is also required to produce infectious human immunodeficiency virus type 1, suggesting that NMT could be a potential anti-human immunodeficiency virus type 1 target (10, 11).

Mammals (e.g. human, mouse, rat, and cow) and other vertebrates (e.g. chicken, frog, and zebrafish) possess two NMTs, NMT1 and NMT2, which are products of distinct genes (www.ensembl.org/). Human NMT1 and NMT2 are 77% identical at the amino acid level and have similar selectivities for peptide substrates (12). The two mouse genes, Nmt1 and Nmt2, encode enzymes that are >95% identical to the human enzymes at the amino acid level (12).

Why mammals and other higher organisms have two NMTs is not clear. Northern blots comparing the patterns of Nmt1 and Nmt2 expression have not yet been published for either mouse or human, and it is not known whether the two enzymes are functionally redundant in vivo. In this study, we sought to address this issue by creating and characterizing Nmt1 knockout mice and by analyzing the biochemical and functional properties of an Nmt1-deficient cell line.

EXPERIMENTAL PROCEDURES

Nmt1-deficient Mice—A mouse embryonic stem (ES) cell line (XE400, strain 129/Ola) with an insertional mutation in Nmt1 was created in a gene-trapping program, BayGenomics (baygenomics.ucsf.edu/). The gene-trapping vector, pGTL1fx, was designed to create an in-frame fusion between the 5′ exons of the trapped gene and a reporter, βgeo (a fusion of β-galactosidase and neomycin phosphotransferase II). Nmt1 spans 13 exons on mouse chromosome 11. The insertional mutation in XE400 occurred in intron 5. Thus, the gene-trapped locus is predicted to yield a fusion transcript containing exons 1−5 of Nmt1 and βgeo. The ES cells were injected into C57BL/6 blastocysts to create chimeric mice, which were bred with C57BL/6 mice to generate heterozygous (+/−).

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‡‡ The abbreviations used are: NMT, N-myristoyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ES, embryonic stem; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; E, embryonic day.

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Nmt1-deficient mice. All mice had a mixed genetic background (~50% C57BL/6 and ~50% 129/Ola). The mice were weaned at 21 days of age, housed in a barrier facility with a 12-h light/dark cycle, and fed chow containing 4.5% fat (Ralston Purina, St. Louis, MO).

Genotyping by Southern Blot and PCR—Genomic DNA (10–20 μg) from tail biopsies, yolk sacs, or embryos was digested with BglII and analyzed by Southern blot with an Nmt1 probe located 3’ of the insertion site. The probe was amplified from mouse genomic DNA with primers 5’-GACCTGTAGTGGCCACATTG-3’ and 5’-ATGCCAGGGGGGACAGCTGATC-3’. The wild-type allele yielded a 17.5-kb band, and the mutant allele yielded a 6.2-kb band.

Blastocysts or cellular outgrowths from blastocysts were genotyped by PCR. The cells were lysed in 20 μl of PCR buffer (10 μM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 0.1 μg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 200 μg/ml proteinase K) at 56 °C for 1 h and then at 95 °C for 15 min (13). The PCR contained primer A (5’-CCGAGAGTTCCAGATTTAAA-3’) and primer B (5’-ATCCCTTGGTTGCTACACC-3’), which correspond to sequences upstream and downstream, respectively, of the insertion point, and primer C (5’-CCACACGCGGTTCTTCCTGTT-3’), which is specific to gptII1x sequences. Genomic DNA (100 ng) and primers A, B, and C (50 ng each) were placed in 10X Titanium PCR buffer (Clontech) containing 0.2 mM deoxyxylulose triphosphates and 1.25 unit of Taq polymerase (Clontech) for 30 s at 95 °C. An initial denaturing amplification for 35 cycles (1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C), the PCR products were size-fractionated on a 1.5% agarose gel in 1X Tris-acetate-EDTA buffer. Primers A and B amplify a 560-bp band from a wild-type allele; primers A and C amplify a 960-bp fragment from a mutant allele.

RNA Isolation and Northern Blot Analysis—Total RNA (25 μg) isolated from 50–150 mg of mouse tissue with TRIzol reagent (Invitrogen) (OriGene Technologies, Rockville, MD) and mouse embryo poly(A)+ riboprobes were performed as described from wild-type blastocysts with the RNase Mini Kit (Qiagen, Valencia, CA). The quality and quantity of the RNA were assessed with spectrometry and by examining the RNA on an ethidium bromide-stained 1% agarose gel. To generate first-strand cDNA, total RNA (1.0 μg) was reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) at 25 °C for 10 min and then at 37 °C for 2 h. Ten ng of cDNA was used for each real-time PCR. Primer and probe sets from Applied Biosystems (Nmt1, Mm00500829_m1; Nmt2, Mm00476437_g1) were used to quantify Nmt1 and Nmt2 expression. A real-time PCR assay for GAPDH was used for normalization (Applied Biosystems). Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems). The thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15-s denaturation at 95 °C, 10 min at 60 °C, and a 1.5-min melt at 95 °C. All assays were performed in triplicate and normalized against GAPDH. The results are expressed as relative expression with the 2−ΔΔCT method (17).

Analysis of Nmt1−/− ES Cells—The number of chromosomes in Nmt1−/− and the parental Nmt1+/+ ES cells was counted (18, 19). To determine whether the Nmt1−/− ES cells could differentiate into beating heart cells, Nmt1−/− ES cells were diluted in ES cell medium (25,000 cells/ml) and then pipetted in 20-μl droplets into a 96-well V-bottomed polypolyene plate (EK-21281; lids, EK26161; E&K Scientific Plates). The plates were flipped gently with one smooth motion and placed upside down in a 37 °C incubator with 7% CO2 for 2–3 days. The plates were then flipped right-side up, and differentiation medium (1X Glasgow minimal essential medium/BHK21 medium, 2 mM glutamine, 1X nonessential amino acids, 1X nonessential fatty acid supplement, 50 μg/ml G418 (baygenomics.ucsf.edu/) and grown for 24 h. A high concentration of G418 (5.0 mg/ml) was added, and the concentration was gradually increased to 12.0 mg/ml. After 7–10 days, single colonies were picked and grown in individual wells of a 96-well plate for 5–7 days. Cell lines were genotyped by both Southern blot and PCR. In parallel control studies, homozygous mutant ES cell lines were created from an ES cell line with a mutation in Linna (the gene encoding prelin and a lamin C).

RESULTS

Nmt1 and Nmt2 are expressed in a wide variety of adult mouse tissues, as judged by Northern blots (Fig. 1). In situ hybridization studies of mouse embryos at embryonic day (E) 13.5 revealed widespread expression of Nmt1 in multiple tissues, for example in the liver, lung, and neural tube (Fig. 2). Nmt2 was also expressed in these same tissues, although the level of expression was consistently lower (Fig. 2).

To determine whether both enzymes are essential in mammals, we produced Nmt1−/+ mice with a mutant ES cell line containing an insertional mutation in intron 3 of Nmt1 (Fig. 3A). A PCR genotyping approach was developed (Fig. 3B), which was particularly useful for genotyping blastocysts. The mutation introduced a new BglII site, facilitating Southern blot

Safe Liquid Scintillation mixture in a Beckman liquid scintillation counter. One unit of NMT activity was defined as 1 pmol of myristoyl-peptide formed per minute.

Real-time PCR—Total RNA isolated from wild-type blastocysts with the RNase Mini Kit (Qiagen, Valencia, CA). The quality and quantity of the RNA were assessed with spectrometry and by examining the RNA on an ethidium bromide-stained 1% agarose gel. To generate first-strand cDNA, total RNA (1.0 μg) was reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) at 25 °C for 10 min and then at 37 °C for 2 h. Ten ng of cDNA was used for each real-time PCR. Primer and probe sets from Applied Biosystems (Nmt1, Mm00500829_m1; Nmt2, Mm00476437_g1) were used to quantify Nmt1 and Nmt2 expression. A real-time PCR assay for GAPDH was used for normalization (Applied Biosystems). Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems). The thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15-s denaturation at 95 °C, 10 min at 60 °C, and a 1.5-min melt at 95 °C. All assays were performed in triplicate and normalized against GAPDH. The results are expressed as relative expression with the 2−ΔΔCT method (17).

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Northern blots. in situ embryos, as judged by genotyping (Fig. 3). As predicted, the insertional mutation resulted in the production of an Nmt1-βgeo fusion transcript (Fig. 3D). Nmt1 mRNA levels in Nmt1−/− mice were approximately one-half of those in wild-type controls. Full-length Nmt1 transcripts were undetectable in Nmt1−/− ES cells (Fig. 3E). Because NMT is an essential enzymatic activity in yeast (2), we predicted that Nmt2 would be expressed in the Nmt1−/− ES cells. Indeed, Nmt2 expression was detectable by Northern blot after a long exposure (36 h) (Fig. 3E). Nmt1+/+ mice were normal in terms of weight, growth rate, and appearance; we observed no postnatal deaths in these mice. X-gal staining of tissues from adult Nmt1+/+ mice revealed widespread expression of Nmt1 (Fig. 4). In the brain, β-galactosidase expression was largely confined to neurons and was prominent in the Purkinje cells of the cerebellum and the pyramidal neurons of the CA1 region of the hippocampus. The facial nucleus in the medulla and the gray matter of the spinal cord also stained intensely. β-Galactosidase expression was identified in hepatocytes, renal tubular cells, and cardiac myocytes.

Because the specificities of NMT1 and NMT2 for peptide substrates are similar (12) and because the expression patterns of Nmt1 and Nmt2 are similar in adult mice and E13.5 embryos (Figs. 1 and 2), we predicted that the two genes would play entirely redundant roles and that Nmt1−/− mice would be viable and phenotypically normal. This prediction was not upheld. In genotyping 247 offspring of Nmt1+/− intercrosses by Southern blot, we identified 121 Nmt1+/+ mice, 126 Nmt1−/− mice, and no Nmt1+/+ mice. Interestingly, Nmt1−/− mice were born less frequently than predicted by Mendelian genetics (p < 0.01, χ² test). Thus, heterozygous Nmt1 deficiency adversely affected survival.

Additional genotyping revealed a complete absence of Nmt1−/− embryos at E7.5, E8.5, E11.5, E15.5, and E17.5 (data not shown), indicating that Nmt1 is essential for development. Not surprisingly, β-galactosidase was expressed at high levels in multiple tissues of Nmt1+/− embryos at E13.5, including the central nervous system, heart, lung, and liver (Fig. 5A). Strong β-galactosidase expression was also identified in embryos at E6.5 (data not shown). Although Nmt1−/− embryos were absent at E7.5, we identified 5 Nmt1−/− blastocysts (E3.5) among 31 that were genotyped. When placed in culture, both Nmt1−/− and Nmt1+/+ blastocysts yielded cellular outgrowths (Fig. 5B).

One potential explanation for the demise of Nmt1−/− embryos is that Nmt1 is the “main NMT” early in mouse development. In line with this concept, Nmt1 expression was 6.76 ± 0.37-fold higher in wild-type 3.5-day mouse embryos (blastocysts) than Nmt2 expression, relative to GAPDH, as judged by TaqMan real-time PCR. In addition, Nmt1 expression was higher at E7 than at E11 and E15, as judged by Northern blot analysis (Fig. 6). Nmt2 expression was relatively low at E7 and then increased gradually (Fig. 6).

To further explore the issue of Nmt1 expression in early embryonic cells, we isolated Nmt1−/− ES cells from Nmt1+/− ES cells by selection in high concentrations of G418 (20). The Nmt1−/− cells, like the parental Nmt1+/− ES cells, had a normal chromosome count (n = 40). Based on the Northern blot studies, we predicted that the majority of the NMT activity in these early embryonic cells might result from the expression of Nmt1 rather than Nmt2. If so, one would expect to observe much lower levels of enzymatic activity in Nmt1−/− cells than in Nmt1+/+ ES cells. Indeed, this was the case (Fig. 7). NMT activity levels were intermediate in Nmt1+/− cells (Fig. 7).

To assess the functional capabilities of Nmt1−/− ES cells, we assessed their ability to differentiate into beating cardiac myocytes in embryoid bodies in vitro. Nmt1−/− ES cells yielded embryoid bodies (Fig. 8A) that stained brightly for β-galactosidase (Fig. 8B). However, the embryoid bodies and the area of beating heart cells within them were smaller with Nmt1−/− ES cells than with Nmt1+/+ ES cells (Fig. 8, C and D).

Because Nmt1 is obviously required for mouse development, we suspected that the Nmt1−/− ES cells, when injected into blastocysts, might be defective in populating the tissues of the chimeric mice. The mice (n > 40) generated from blastocysts that had been injected with Nmt1−/− ES cells exhibited minimal evidence of chimerism, as judged by coat color. Moreover, β-galactosidase staining of 1-day-old pups from the injected blastocysts revealed a complete absence of β-galactosidase expression in the brain, lung, and liver (tissues that normally exhibit strong β-galactosidase staining in Nmt1+/+ mice). However, the injected blastocysts did indeed yield chimeras because we identified β-galactosidase staining in enterocytes in
every pup that was examined (n = 8) (Fig. 9). In parallel control experiments, high G418 selection was used to produce euploid ES cells that were homozygous for a mutant *Lmna* allele. *Lmna* is not essential for embryonic development (21). Unlike the *Nmt1*+/− ES cells, the homozygous *Lmna* ES cells consistently yielded 80–90% chimeric mice.

**DISCUSSION**

The fact that *N*-myristoylation of proteins is important in eukaryotes is beyond dispute. Yeast, fungi, flies, worms, trypanosomes, and *Leishmania* cannot survive without the enzyme that carries out this protein modification (2–5, 8). However, in mammals as well as several other vertebrates, two distinct *N*-myristoyltransferases exist (12). Although the two human enzymes have similar peptide specificities and kinetic properties (12), it has remained mysterious whether the enzymes are entirely redundant or whether one enzyme might play a unique, vital role *in vivo*. In the current study, we provide new insights into that issue, demonstrating that *Nmt1* is essential for mouse development. Homozygous *Nmt1*-deficient mice died between 3.5 and 7.5 days of embryonic development. This result was unexpected, not just because of the similar properties of the two enzymes (12), but also because the tissue-specific expression patterns of the enzymes are similar, as judged by Northern blots and *in situ* hybridization studies of mouse embryos (Figs. 1 and 2). Interestingly, only about one-half of the heterozygous knock-out mice survived. The demise of significant numbers of heterozygous knock-out mice has been observed previously with mutations in other essential genes, for example *Apob* (22) and *Cul-4A* (13).

The *Nmt1* knock-out mice were generated with an ES cell line containing an insertional mutation in intron 3 of *Nmt1*. A theoretical concern with gene trap mutations is that the splicing
machinery might “splice around” the entire insertion, leading to the production of low levels of a wild-type transcript. In the case of the mutant Nmt1 ES cell line, this did not occur, because we were unable to find any full-length Nmt1 transcripts in Nmt1−/− ES cells by Northern blot, even on long exposures. The insertion of the gene trap vector led to the production of a fusion transcript (consisting of a small segment of Nmt1 and βgeo), which could be detected by Northern blot. The production of the Nmt1-βgeo fusion protein was fortunate because it made it possible for us to assess Nmt1 expression in tissue sections with X-gal stains. The fusion protein would not be predicted to retain any NMT enzymatic activity, inasmuch as it would lack important functional domains of NMT, for example regions that bind the protein substrate and myristoyl-CoA (23, 24).

Why would Nmt1 be essential for development, given that a similar gene (Nmt2) exists within the genome? It seems unlikely that Nmt1 is simply required for cell viability, as it is in yeast, given that we had no difficulty in isolating Nmt1−/− ES cells. Those ES cells that expressed Nmt2 retained the capacity to differentiate into enterocytes in chimeric mice. A more attractive explanation is that Nmt1 is simply “the main Nmt enzyme” early in embryonic development. By Northern blot, Nmt2 expression was lower at E7 than at later time points, whereas Nmt1 expression was strong at E7. In addition, Nmt2 was expressed at far lower levels than Nmt1 in wild-type blastocysts (E3.5), as judged by real-time PCR. Also, total enzymatic activity levels were quite low in the Nmt1−/− ES cells, suggesting that Nmt2 contributes minimally to enzyme activity levels in those cells. The Nmt1−/− ES cells created within this study were functionally defective, lacking the capacity to contribute to the formation of organs in which Nmt1 is normally expressed at high levels (e.g., brain, liver, and lung).

The ES cell clone used to create the Nmt1 knock-out mice was produced by BayGenomics, one of the gene-trapping programs that form the International Gene Trap Consortium (www.igtc.org.uk/). Thus far, the International Gene Trap Consortium lists ~27,000 mutant ES cell lines covering ~32% of the ~30,000 mouse genes (25). Within BayGenomics, we have documented that the likelihood of trapping a particular mouse gene is directly correlated to the size of the gene and its level of expression in ES cells.7 Thus, large genes and genes expressed

\[a\] B. Conklin, A. Nord, W. Skarnes, and S. G. Young, unpublished observations.
at high levels in ES cells are more likely to be trapped than small genes and genes that are expressed at low levels in ES cells. The preference for larger genes makes perfect sense, inasmuch as they provide a greater opportunity for the random gene-trapping insertional event. Trapping of highly expressed genes also makes sense because the generation of a drug-resistant ES cell colony depends on the expression of \( \beta \text{geo} \), which is driven by the promoter of the trapped gene. Of note, Nmt2 is approximately 5 kb longer than Nmt1, so by that criterion, one would expect Nmt2 to be trapped by the International Gene Trap Consortium more often than Nmt1. However, as we showed, NMT activity levels are quite low in Nmt1 cells, suggesting that Nmt1 might normally be expressed more highly in ES cells than Nmt2. If so, one would predict that Nmt1 would have been trapped by the IGTC much more frequently than Nmt2. Indeed, this has been the case: Nmt1 has been trapped 12 times, whereas Nmt2 has never been trapped.

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REFERENCES

1. Johnson, D. R., Bhatnagar, R. S., Knoll, L. J., and Gordon, J. I. (1994) *Annu. Rev. Biochem.* 63, 869–914
2. Duronio, R. J., Towler, D. A., Heuckeroth, R. O., and Gordon, J. I. (1989) *Science* 245, 796–800
3. Weinderg, R. A., MacWherter, C. A., Freeman, S. K., Wood, D. C., Gordon, J. L., and Lee, S. C. (1995) *Mol. Microbiol.* 16, 241–250
4. Lodge, J. K., Jackson-Machelski, R., Toffaletti, D. L., Perfect, J. R., and Gordon, J. I. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 12006–12012
5. Ntwasa, M., Aapies, S., Schiffmann, D. A., and Gay, N. J. (2001) *Exp. Cell Res.* 262, 134–144
6. Gonczy, P., Echeverri, C., Oegema, K., Coulson, A., Jones, S., Copley, R., Duperion, J., Oegema, J., Brehm, M., Cassin, E., Hannak, E., Kirkham, M., Pichler, S., Flohrs, K., Goessen, A., Leidel, S., Allame, A., Martin, C., Ozu, N., Bork, P., and Hyman, A. (2000) *Nature* 408, 331–336
7. Kamath, R., Fraser, A., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D., Zipperlen, P., and Ahringer, J. (2003) *Nature* 421, 231–237
8. Price, H., Menon, M., Pasetthymilaki, C., Goulding, D., McKeon, P., and Smith, D. (2003) *J. Biol. Chem.* 278, 7206–7214
9. Selvakumar, P., Pasha, M., Ashakumar, L., Dimmock, J., and Sharma, R. (2002) *Int. J. Mol. Med.* 10, 493–500
10. Takamune, N., Hamada, H., Misumi, S., and Shoji, S. (2002) *FEBS Lett.* 527, 138–142
11. Shiraishi, T., Misumi, S., Takama, M., Takahashi, I., and Shoji, S. (2001) *Biochem. Biophys. Res. Commun.* 282, 1201–1205
12. Giang, D. K., and Cravatt, B. F. (1998) *J. Biol. Chem.* 273, 6595–6598
13. Li, B., Ruiz, J., and Chun, K. (2002) *Mol. Biol. Cell* 12, 4997–5005
14. Wilkinson, D. G., and Nieto, M. A. (1993) *Methods Enzymol.* 255, 361–373
15. Raju, R. S., Datla, R. S., and Sharma, R. K. (1999) *Biochem. Biophys. Res. Commun.* 257, 284–288
16. King, M., and Sharma, R. (1991) *Anal. Biochem.* 199, 149–153
17. Livak, K. J., and Schmittgen, T. D. (2001) *Methods* 25, 402–408
18. Cronmille, C., and Mintz, B. (1978) *Dev. Biol.* 67, 465–477
19. Mcburney, M., and Rogers, B. (1982) *Dev. Biol.* 89, 503–508
20. Mortensen, R., Conner, D., Chao, S., Geisterfer-Lowrance, A. A., and Seidman, J. G. (1999) *Mol. Cell. Biol.* 12, 2391–2395
21. Sullivan, T., Escalante-Accende, D., Bhat, H., Anver, M., Bhat, N., Nakashima, K., Stewart, C., and Burke, B. (1999) *J. Cell Biol.* 147, 913–920
22. Huang, L., Voytiainas, E., Markenson, D., Sokol, K., Hayek, T., and Breslow, J. (1995) *J. Clin. Invest.* 95, 5152–5161
23. Raju, R., Anderson, J., Datla, R., and Sharma, R. (1997) *Arch. Biochem. Biophys.* 348, 134–142
24. Raju, R., Datla, R., Warrington, R., and Sharma, R. (1996) *Biochemistry* 37, 14928–14936
25. Skarnes, W., von Melchner, H., Wurst, W., Hicks, G., Nord, A., Cox, T., Young, S., Ruiz, P., Soriano, P., and Tessier-Lavigne, M. (2004) *Nat. Genet.* 36, 921–924