

Meltrin β mini, a new ADAM19 isoform lacking metalloprotease and disintegrin domains, induces morphological changes in neuronal cells¹

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Abstract Meltrin β (ADAM19) is a metalloprotease-disintegrin expressed in the peripheral nervous system and other organs during embryogenesis. We report here an alternatively spliced isoform, meltrin β mini, that lacks the prodomain, metalloprotease and disintegrin domains. A comparison of the cDNA and genomic sequences suggested the existence of a new exon. This isoform was detected in murine dorsal root ganglion and neuronal cell lines by RT-PCR. Overexpression of meltrin β mini but not meltrin β induced neurite outgrowth in neuronal cells. These studies suggest that the novel meltrin β isoform has a distinct function related to neurogenesis.

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Key words: Dorsal root ganglion; N1E-115; Neurite outgrowth; Neurogenesis

1. Introduction

The metalloprotease-disintegrin meltrin β (ADAM19) belongs to the ADAM (a disintegrin and metalloprotease) family. Its specific patterns of expression during embryogenesis suggest that it is involved in the development of the peripheral nervous system, skeletal muscle, bone and heart tissue [1–3]. To date, more than 30 ADAMs have been identified in worms, flies, rodents, primates and humans [4,5]. ADAMs are thought to be involved in numerous biological functions, including fertilization, myogenesis, neurogenesis, and epithelial tissue development [4,6,7]. Some ADAMs are catalytically active metalloproteases and participate in the proteolytic processing of the extracellular domains of membrane-anchored proteins [8,9]. For example, TACE (ADAM17) is involved in the processing of tumor necrosis factor- α in this manner [10,11]. Another example is meltrin β (ADAM19), which has been implicated in the processing of neuregulin β , another membrane-anchored growth factor [12]. However, the metalloprotease domains of about half of the known ADAMs lack a catalytic site, although the domain is otherwise highly con-

served. Protease-defective ADAMs also have an essential role in murine development (ADAM2, 3, 23), indicating that ADAMs function other than as proteases. Other studies suggest that ADAMs are also involved in cell–cell or cell–extracellular matrix interactions through their interaction with integrins [13–16] or proteoglycans [17]. Here, we report the properties of meltrin β mini, a new splice variant of meltrin β (ADAM19) that lacks the prodomain, metalloprotease and disintegrin domains.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Sigma (St. Louis, MO, USA) and nacalai tesque (Kyoto, Japan). Restriction enzymes and molecular biology reagents were purchased from TaKaRa (Kyoto, Japan) and Toyobo (Osaka, Japan), unless otherwise indicated.

2.2. Isolation of β mini cDNA

An initial cDNA clone encompassing the disintegrin and cysteine-rich domains of meltrin β was identified among RT-PCR products amplified from murine myogenic cellular RNA [1]. This clone was used as a probe to isolate cDNA clones encoding meltrin β and β mini from a cDNA library prepared from a murine myoblast cell line, C2, in λ Zap II (Stratagene, La Jolla, CA, USA). Sequencing was carried out by the dideoxy chain termination method using a dye primer cycle sequencing kit (Applied Biosystems/Perkin Elmer, Foster City, CA, USA) and an ABI 373A DNA sequencer (Applied Biosystems/Perkin Elmer).

2.3. RT-PCR analysis

mRNA was extracted from murine embryos with the QuickPrep *micro* mRNA Purification Kit (Amersham Pharmacia Biotech) and used as a template for cDNA synthesis with the SuperScript First-Strand Synthesis System (Invitrogen). PCR was carried out with AmpliTaq Gold DNA polymerase (PE Applied Biosystems) for 40 cycles at 96°C for 15 s, 62°C for 30 s and 72°C for 30 s. The primers used to identify meltrin β mRNAs were 5'-CGCAGAATGAAACGGGAAGATCT-3' and 5'-TACACCAATGGCATTCTCGGAGTG-3' (for meltrin β ; 421 bp) and 5'-CTTCAGAGCGAGTGTGGAGAG-3' and 5'-AGAGTGCAGTTGGAGGGCATTG-3' (for β mini; 211 bp). The G3PDH 0.45 kb Control Amplimer Set (Clontech) was used as a positive control. To determine the 3'-terminus of the alternatively spliced form of meltrin β , RT-PCR was carried out with primer 1, specific to the end of the meltrin β coding region (5'-TTCTCGACACTTCTAGATCTTGG-3'), and primer 2, specific to sequences in the alternatively spliced form (5'-CTTCAGAGCGAGTGTGGAGAG-3').

2.4. Plasmids

A cDNA sequence encoding the presumed β mini protein was constructed by ligating a β mini partial fragment to a meltrin β fragment encoding the region from the cysteine-rich domain through the cytoplasmic tail. The E347Q protease-deficient meltrin β construct has been previously described [12]. The plasmid pBIE was generated by replacement of the human cytomegalovirus promoter region of

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¹ Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank data bases under the accession number AB096093.

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Abbreviations: DRG, dorsal root ganglion

pIRES2-EGFP (Clontech) with the promoter region of pEF-BOS [12]. All meltrin β constructs were inserted into pEF-BOS, which bears the human EF-1 α promoter [18].

2.5. Cell culture and neurite outgrowth

COS-7 cells or the murine neuroblastoma cell line N1E-115 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The cells were transfected with Lipofect-

AMINE 2000 (Invitrogen) according to the manufacturer's instructions. All constructs were transfected together with pBIE. For neuronal differentiation, N1E-115 cells were washed twice with phosphate-buffered saline (PBS) at 4–6 h after transfection and then allowed to differentiate in DMEM. For quantification of neurite outgrowth, cells were allowed to differentiate for 24 h after transfection, and three to five random photographs were taken per well. Cells bearing processes five times longer than the cell diameter were

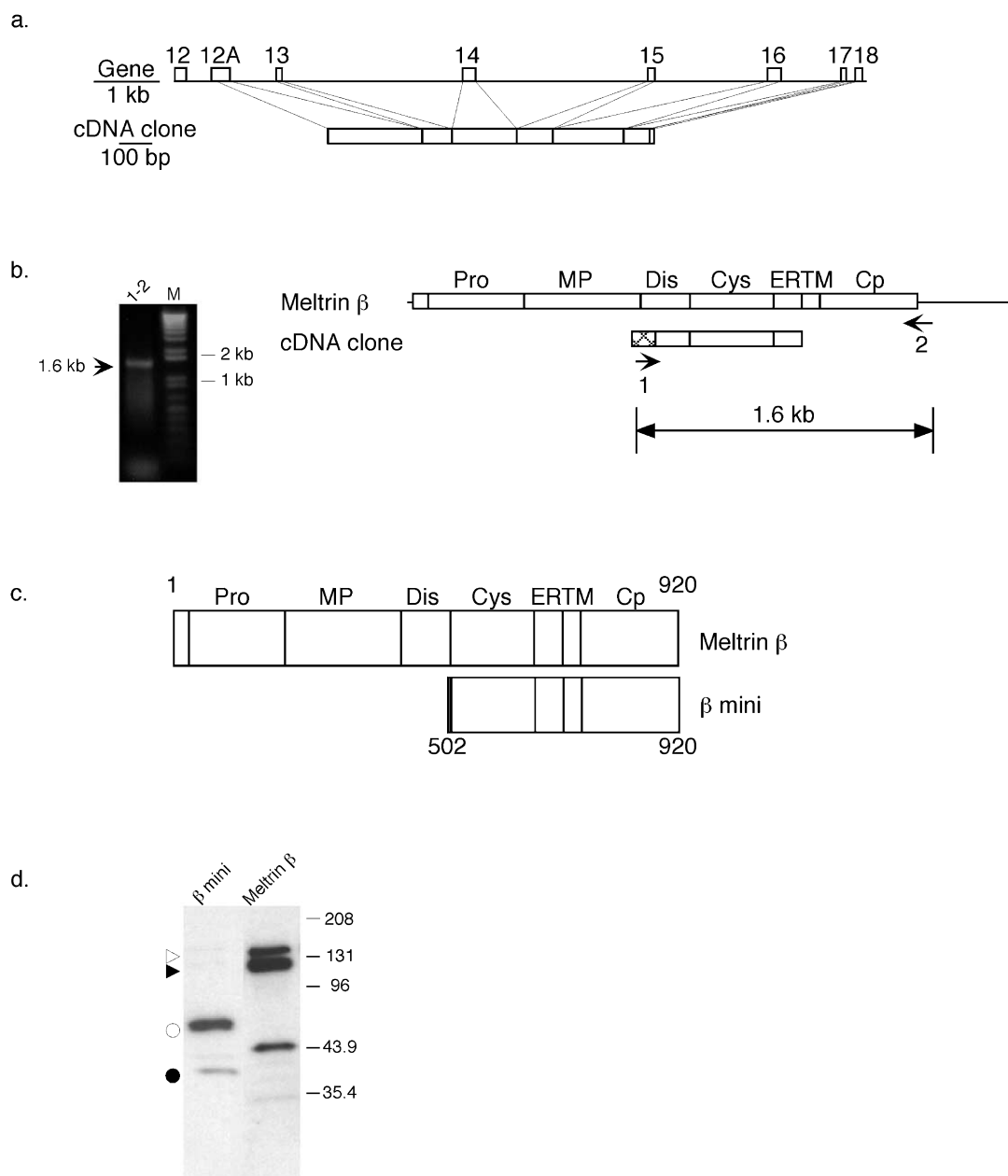


Fig. 1. Alternatively spliced transcripts generated from the murine meltrin β gene. a: Schematic representation of a cDNA clone showing an alternatively spliced transcript generated from the murine meltrin β gene. The white box represents an exon. The number above the box indicates the exon number according to NCBI LocusLink (ID: 11492). 12A indicates a putative β mini-specific exon. b: Predicted β mini cDNA, as determined by RT-PCR. (Left) A 1.6 kb RT-PCR product generated by the primer set (1,2), as described in Section 2. (Right) Pro, prodomain; MP, metalloprotease domain; Dis, disintegrin domain; Cys, cysteine-rich domain; ER, EGF-like repeat domain; TM, transmembrane domain; Cp, cytoplasmic domain. The hatched box represents the β mini-specific sequence derived from exon 12A. c: Domain organization of murine meltrin β (upper) and β mini (lower) proteins. Numbers indicate meltrin β amino acid residues. d: Immunoblot analysis of meltrin β and β mini proteins expressed in COS-7 cells. The cell lysates were prepared as described in Section 2 and subjected to immunoblot analysis with an antibody specific for the carboxy-terminus of meltrin β , which recognizes both the meltrin β and β mini proteins. Open and filled triangles indicate the ~120 kDa prodomain-containing inactive form and the ~100 kDa prodomain-excised active form of meltrin β , respectively. Open and filled circles also indicate the ~60 kDa full-length form and the ~40 kDa processed form of β mini, respectively. Prestained molecular mass standards are shown on the right.

counted as positive. The cells were analyzed under a immunofluorescence microscope (Axiovert 200M; Zeiss) equipped with a Plan-Achro-mat 20× lens. The images were acquired with Metamorph software.

2.6. Immunoblotting

Four to six hours after transfection, N1E-115 cells were differentiated in DMEM for 24 h. The cells were scraped from 60 mm dishes with a rubber policeman and homogenized in 300 μ l 500 mM sodium carbonate containing a protease inhibitor cocktail (25955-11, Nacalai Tesque), pH 11.0, with a loosely fitting homogenizer. The homogenate was sonicated with three 20 s pulses and the lysates were subjected to immunoblot analysis. Primary antibodies were applied for 1 h in PBS containing 5% skim milk at the following dilutions: anti-meltrin β antiserum, 1:300; polyclonal anti-GAP-43 antibody (AB5220, Chemicon), 1:5000; polyclonal anti-GFP antibody (598, MBL, Japan), 1:300. Immunoblots were developed using horseradish peroxidase-conjugated secondary antibody (PI-1000, Vector) followed by enhanced chemiluminescence. The intensity of each band was determined by laser densitometry of immunoblots using Molecular Dynamics Densitometer and ImageQuant software (Sunnyvale, CA, USA). Scans of multiple exposures were made to ensure that the results fell into the linear range of the instrument. For GAP-43 immunoblot analysis, the amount of protein loaded was normalized to the level of GFP as a control.

3. Results

We used a meltrin β probe to screen a cDNA library derived from the C2 myoblast cell line. Thirteen positive clones were identified among 2.5×10^5 primary plaques. Analyses of the inserts by restriction digestion and sequence analysis allowed two independently isolated clones to be classified as alternatively spliced forms of meltrin β . Both clones consisted of novel ~ 200 bp sequences followed by a truncated disintegrin domain and entire cysteine-rich domains of meltrin β (amino acid residues 438–707) (Fig. 1b; supplemental data, <http://www.elsevier.com/PII/S0014579302037328>). A search of the mouse genome database revealed that a new exon encoding the novel isoform-specific sequence was located between exons 12 and 13 of meltrin β .

To determine the 3'-terminus of the alternatively spliced form of meltrin β , we designed PCR primers specific to the end of the meltrin β coding region and to sequences unique to the alternatively spliced form. RT-PCR performed with these primers yielded a 1.6 kb PCR product (Fig. 1b). Sequence analysis of this PCR product revealed that it encoded amino acid residues 438–920 of meltrin β (DDBJ accession number AB096093). The first methionine (502) of this translated sequence lies just upstream of the cysteine-rich domain coding region (Fig. 1c) and is preceded by a termination codon downstream of the β mini-specific exon; the deduced amino acid sequence thus lacks the prodomain, metalloprotease and disintegrin domains. When this clone was transiently expressed in COS-7 cells, ~ 60 kDa and ~ 40 kDa bands were detected by immunoblot analysis with an antibody spe-

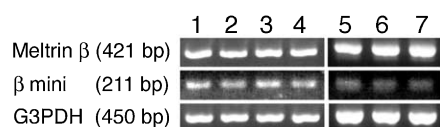


Fig. 2. Expression of β mini in the DRG and in N1E-115 cells. β mini expression was detected in the DRG and in N1E-115 cells by RT-PCR analysis. Lanes 1–4, DRG of E11.5, E13.5, E16.5 and P1 embryos, respectively; lanes 5–7, N1E-115 cells analyzed 0, 1, 3 days after the induction of differentiation, respectively.

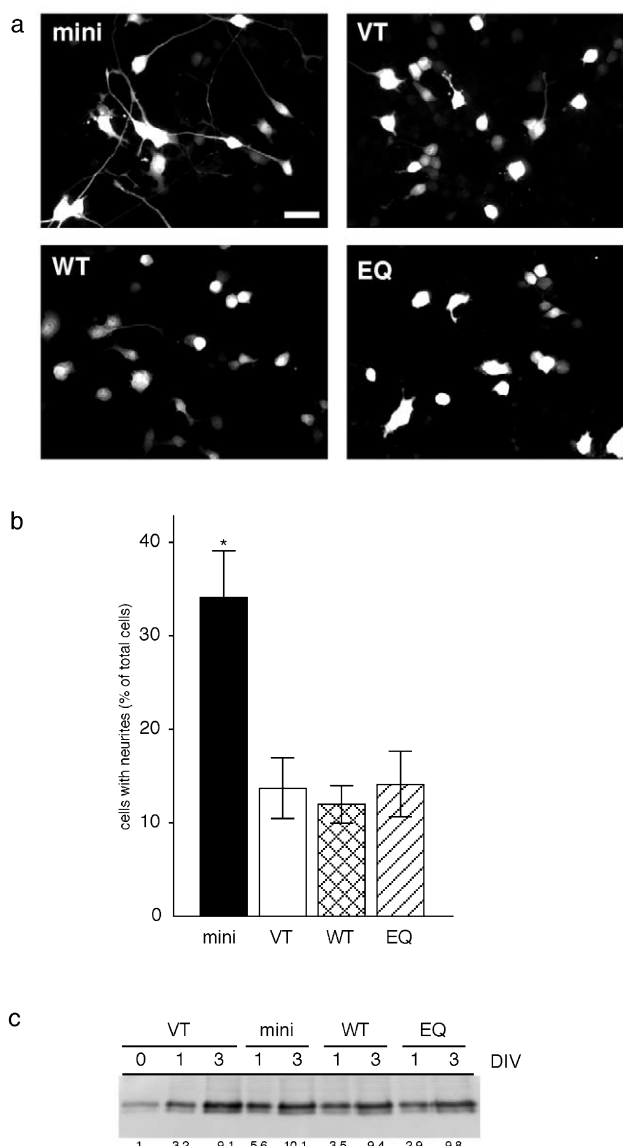


Fig. 3. Neuritogenesis of N1E-115 cells is accelerated by the overexpression of β mini protein. **a**: Morphology of N1E-115 cells transfected with cDNAs encoding the indicated constructs. All constructs were transfected together with the EGFP expression vector. mini, β mini; VT, EGFP expression vector alone; WT, wild type meltrin β ; EQ, protease-deficient meltrin β . Scale bar, 50 μ m. **b**: Neuronal differentiation of N1E-115 cells. Cells with neurites five times longer than the cell diameter were counted as positive. The percentage of the total cells is shown. * $P < 0.01$ between the experimental groups. **c**: Western blot analysis of GAP-43 protein levels in cells transfected with the indicated cDNA constructs following differentiation for the indicated periods of time. Numbers under each lane show the relative intensity of the bands against the control (0 DIV). DIV, days in vitro.

cific to the meltrin β carboxy-terminus (Fig. 1d). These bands were not detected in COS-7 cells transfected with meltrin β . Together, these results indicate that this cDNA clone can generate a novel alternatively spliced form of meltrin β as a translation product, which we have named meltrin β mini (β mini).

It has been reported that meltrin β is expressed in the dorsal root ganglia (DRG) of murine embryos. β mini was also ex-

pressed in the DRG (from embryonic day (E) 11.5 through postnatal day (P) 1) (Fig. 2). We also detected meltrin β and β mini mRNAs in murine neuroblastoma N1E-115 cells, which differentiate into neuronal cells after serum depletion. No major changes in the levels of meltrin β and β mini mRNAs could be seen upon the induction of differentiation. The endogenous β mini protein could not be detected by available anti-meltrin β antibodies, probably due to the low level of β mini expression (data not shown).

We investigated the effects of overexpression of β mini in N1E-115 cells. After 24 h of differentiation, N1E-115 cells expressing β mini exhibited elaborate neurite-like structures (Fig. 3a,b). Such morphological changes were never seen in N1E-115 cells expressing either meltrin β or a protease-deficient meltrin β mutant (EQ). To determine whether β mini augments neurite outgrowth as a result of differentiation, we examined the expression levels of GAP-43, a protein marker for neuronal differentiation [19], in transfected N1E-115 cells. As shown in Fig. 3c, N1E-115 cells transfected with β mini express more GAP-43 than control cells. Three days after induction, however, the level of expression of GAP-43 and the morphology of β mini-expressing cells were comparable to those seen in cells transfected with other constructs. These data suggest that β mini accelerates neurite outgrowth.

4. Discussion

Alternatively spliced forms of ADAM have previously been reported. ADAM12-S, a secreted form of human ADAM12 (meltrin α), lacks the transmembrane domain and has a unique 34 amino acid carboxy-terminus [20]. To our knowledge, ours is the first report of an alternatively spliced form of ADAM that encodes a protein lacking the prodomain, metalloprotease and disintegrin domains. An inspection of the presumed amino acid sequence indicates that β mini does not have an obvious signal sequence, and we did not detect β mini on the surface of transiently expressed COS-7 cells (data not shown). Whether β mini is integrated as a membrane protein or whether β mini protein is stably expressed in vivo remains elusive. Fertilin β (ADAM2) is proteolytically processed to a mature form that lacks the metalloprotease and disintegrin domains and covalently associates with fertilin α [21,22]. Although β mini is the product of alternative splicing and not of post-translational processing, it has a domain structure similar to that of the mature form of fertilin β . We could not detect interaction of β mini with either meltrin β or meltrin α by immunoprecipitation (data not shown), but β mini may still be associated with other ADAMs. Although the function of β mini is unknown, in vitro overexpression studies suggested that this novel meltrin β isoform has roles distinct from that of meltrin β , especially in neuritogenesis. β mini-specific knock-down experiments such as gene targeting or RNA interference assays are likely to reveal its in vivo roles.

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References

- [1] Yagami-Hiromasa, T., Sato, T., Kurisaki, T., Kamijo, K., Nabeshima, Y. and Fujisawa-Sehara, A. (1995) *Nature* 377, 652–656.
- [2] Kurisaki, T., Masuda, A., Osumi, N., Nabeshima, Y. and Fujisawa-Sehara, A. (1998) *Mech. Dev.* 73, 211–215.
- [3] Inoue, D., Reid, M., Lum, L., Kratzschmar, J., Weskamp, G., Myung, Y.M., Baron, R. and Blobel, C.P. (1998) *J. Biol. Chem.* 273, 4180–4187.
- [4] Wolfsberg, T.G., Primakoff, P., Myles, D.G. and White, J.M. (1995) *J. Cell Biol.* 131, 275–278.
- [5] Blobel, C.P. (1997) *Cell* 90, 589–592.
- [6] Primakoff, P. and Myles, D.G. (2000) *Trends Genet.* 16, 83–87.
- [7] Schlondorff, J. and Blobel, C.P. (1999) *J. Cell Sci.* 112, 3603–3617.
- [8] Black, R.A. and White, J.M. (1998) *Curr. Opin. Cell Biol.* 10, 654–659.
- [9] Turner, A.J. and Hooper, N.M. (1999) *Biochem. Soc. Trans.* 27, 255–259.
- [10] Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J. and Cerretti, D.P. (1997) *Nature* 385, 729–733.
- [11] Moss, M.L., Jin, S.L., Milla, M.E., Bickett, D.M., Burkhardt, W., Carter, H.L., Chen, W.J., Clay, W.C., Didsbury, J.R., Hassler, D., Hoffman, C.R., Kost, T.A., Lambert, M.H., Leesnitzer, M.A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L.K., Schoenen, F., Seaton, T., Su, J.L. and Becherer, J.D. et al. (1997) *Nature* 385, 733–736.
- [12] Shirakabe, K., Wakatsuki, S., Kurisaki, T. and Fujisawa-Sehara, A. (2001) *J. Biol. Chem.* 276, 9352–9358.
- [13] Zhou, M., Graham, R., Russell, G. and Crouche, P.I. (2001) *Biochem. Biophys. Res. Commun.* 280, 574–580.
- [14] Bridges, L.C., Tani, P.H., Hanson, K.R., Roberts, C.M., Judkins, M.B. and Bowditch, R.D. (2002) *J. Biol. Chem.* 277, 3784–3792.
- [15] Eto, K., Puzon-McLaughlin, W., Sheppard, D., Sehara-Fujisawa, A., Zhang, X.P. and Takada, Y. (2000) *J. Biol. Chem.* 275, 34922–34930.
- [16] Nath, D., Slocumbe, P.M., Webster, A., Stephens, P.E., Doherty, A.J. and Murphy, G. (2000) *J. Cell Sci.* 113, 2319–2328.
- [17] Iba, K., Albrechtsen, R., Gilpin, B., Frohlich, C., Loechel, F., Zolkiewska, A., Ishiguro, K., Kojima, T., Liu, W., Langford, J.K., Snaderson, R.D., Brakebusch, C., Fassler, R. and Wewer, U.M. (2000) *J. Cell Biol.* 149, 1143–1156.
- [18] Mizushima, S. and Nagata, S. (1990) *Nucleic Acids Res.* 18, 5322.
- [19] Basi, G.S., Jacobson, R.D., Virag, I., Schilling, J. and Skene, J.H. (1987) *Cell* 49, 785–791.
- [20] Gilpin, B.J., Loechel, F., Mattei, M.G., Engvall, E., Albrechtsen, R. and Wewer, U.M. (1998) *J. Biol. Chem.* 273, 157–166.
- [21] Carroll, D.J., Dikegoros, E., Koppel, D.E. and Cowan, A.E. (1995) *Dev. Biol.* 168, 429–437.
- [22] Blobel, C.P., Wolfsberg, T.G., Turck, C.W., Myles, D.G., Primakoff, P. and White, J.M. (1992) *Nature* 356, 248–252.