Secreted growth factors and their receptors play eminent roles in development and differentiation, and it is increasingly recognized that their activity is regulated by proteins functioning in secretory protein traffic. Thus, despite the presumed housekeeping role of genes involved in the 

characteristic pattern encompassing organs with high secretory activity. The ER synexpression group also contains genes without known function, and their synexpression predicts that they are involved in protein secretion as well (10–13). For example the ER synexpressed 18F9 gene (10) was subsequently shown to encode the guanine nucleotide exchange factor, msec12, involved in ER export (14).

One of the growth factor cascades, which is regulated at the level of secretory protein traffic is the Wnt/β-catenin cascade, where the ER chaperones Mesd and Shisa regulate folding and transport to the cell surface of the Wnt receptors LRPs 5 and 6 and Frizzled 7 and 8, respectively (5, 8). Likewise, we have shown that LRPs 6 is negatively controlled by regulated endocytosis via its antagonist Dickkopf1 (Dkk1) and the Dkk1 coreceptor Kremen2 (Krm2), a single transmembrane-spanning protein. Dkk1, LRPs 6, and Krm2 form a ternary complex which is rapidly endocytosed, leading to inhibition of Wnt/β-catenin signaling (15). This regulatory process plays an important role during early embryonic head induction and antero-posterior (a-p) patterning in the Xenopus embryo (15–17). To gain a deeper insight into the regulation of Dkk1 mediated Wnt inhibition we aimed to identify binding partners of Krm2. In this study we describe a novel protein, Erlectin, which contains mannose 6-phosphate receptor homology (MRH) domains and interacts with Krm2. The on-line version of this article (available at http://www.jbc.org) contains supple-

mental Table 1 and Fig. 1.

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4 The abbreviations used are: ER, endoplasmic reticulum; a-p, antero-posterior; MRH, mannose 6-phosphate receptor homology; GNPTAG, γ-subunit of GlcNAc-1-phos-

photransferase; HA, hemagglutinin; LC-MS/MS, liquid chromatography tandem mass spectrometry; IP, immunoprecipitation; RT-PCR, reverse transcription PCR; HEK, human embryonic kidney; MO, morpholino.

The MRH Protein Erlectin Is a Member of the Endoplasmic Reticulum Synexpression Group and Functions in N-Glycan Recognition

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Krm1 and 2 (Krm1/2) are coreceptors for Dickkopf1 (Dkk1), an antagonist of Wnt/β-catenin signaling, and play a role in head induction during early Xenopus development. In a proteomic approach we identified Erlectin, a novel protein that interacts with Krm2. Erlectin (XTP3-B) is member of a protein family containing mannose 6-phosphate receptor homology (MRH-, or PRKCSH-) domains implicated in N-glycan binding. Like other members of the MRH family, Erlectin is a luminal resident protein of the endoplasmic reticulum. It contains two MRH domains, of which one is essential for Krm2 binding, and this interaction is abolished by Krm2 deglycosylation. The overexpression of Erlectin inhibits transport of Krm2 to the cell surface. Analysis of its embryonic expression pattern in Xenopus reveals that Erlectin is member of the endoplasmic reticulum synexpression group. Erlectin morpholino antisense injection leads to head and axial defects during organogenesis stages in Xenopus embryos. The results indicate that Erlectin functions in N-glycan recognition in the endoplasmic reticulum, suggesting that it may regulate glycoprotein traffic.

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regulates glycoprotein traffic and is required for late head and axial development.

**EXPERIMENTAL PROCEDURES**

**Erlectin Sequences and Homologies**—The following sequences of Erlectin were obtained from databases: human (NM_015701, GeneID: 27248), mouse (NM_025745), chicken (XM_419295), Danio rerio (BC044498), Xenopus tropicalis (AAH67973), Ciona intestinalis (AK114497), Strongylocentrotus purpuratus (XP_784270), and Drosophila melanogaster (NM_135693). The two Xenopus laevis alleles are BC074469 (allele1) and CB558691 (allele2). CB558691 is a partial sequence of contig XGI TC228200.

**Expression Constructs**—Human erlectin cDNA was obtained from RZPD (IMAGE ID 3447840). Tagged erlectin constructs were generated by inserting erlectin into a pCS-based vectors containing N-terminal FLAG and V5 epitopes after the signal peptide of mouse krm2 or by using the endogenous signal peptide in a pCS vector providing a C-terminal HA tag. Erlectin point mutation (G379S) and PKRCK domain deletions were prepared by PCR directed mutagenesis: (ΔD1, ΔS111-N180; ΔD2, ΔS342-G418; ΔD1/D2, ΔS111-N180, ΔS342-G418). pCS-V5-mdkk3 was constructed like pCS-V5-erlectin. pCS-myc-mkr2 was generated by insertion of mkr2m into a vector containing an N-terminal myc-tag. pCS-flag-mkr2aTMC was generated by PCR-mediated mutagenesis. Other expression constructs were as described (15).

**Large Scale Purification of the myc-Krm2 Protein Complex**—HEK293 cells stably expressing myc-mKr2m and flag-LRP6 were generated by transfection with pCI-Neo (Promega), pCS-myc-mkr2m, and pCS-flag-LRP6. Cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 0.5 mg/ml neomycin, and selected clones were transfection with pCI-Neo (Promega), pCS-myc-mkr2m or pCS-myc-mkr2mD1/D2, with myc peptide (0.5 mg/ml) in 150 mM ammonium carbonate, 0.1% buffer) for 1 h on ice. Following a clarifying spin (30 min, 226,000 × g) detergent extracts were subjected to IP with anti-V5 antibody beads (Sigma), washed four times with Nonidet P-40 buffer, and incubated with recombinant Flag-Erlectin for 5 h. CoIPs were analyzed by SDS-PAGE and Western blotting.

For deglycosylation of Krm2, cell lysates from Krm2-transfected cells were subjected to Endo H (Roche Diagnostics) treatment (0.3 units/ml) in 100 mM NaAc, pH 5.5, or N-glycosidase F (Roche Diagnostics) treatment (62 units/ml) for 40 min at 37 °C, and analyzed by SDS-PAGE and Western blotting. For the deglycosylation experiment followed by in vitro CoIP, recombinant Krm2ΔTMC-V5 was treated with 62 units/ml N-glycosidase F for 1 h at 37 °C, resuspended in Nonidet P-40 buffer containing 0.2% (w/v) Nonidet P-40, and subjected to IP with anti-V5 antibody beads overnight at 4 °C. IPs were washed four times with Nonidet P-40 buffer, incubated with recombinant Flag-Erlectin for 5 h, and analyzed by SDS-PAGE and Western blotting.

Cell surface biotinylation was carried out on HEK293T cells transfected in a 12-well plate with 5 ng of pCS2-mkr2m-V5, 2.5 ng of pCS2-GFP and 200 ng of pCS2-flag-erlectin using 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce) according to the manufacturer. Cells were lysed with Nonidet P-40 buffer, containing 0.8% (w/v) Nonidet P-40, and Krm2-V5 was immunoprecipitated with anti-V5 antibody beads and analyzed by SDS-PAGE and Western blotting. As control for cell surface biotinylation, cells were transfected with 50 ng of pCS2-V5-nme1 (nucleoside diphosphate kinase A, (36)). Antibodies used for Western blotting were anti-V5 (Invitrogen), anti-FLAG M2 (Sigma), anti-HA (Roche), anti-GFP (Chemikon), and anti-myc 9E10 (M. Eilers).

**Immunofluorescence Staining**—HeLa cells were transfected with 200 ng of pCS2-erlectin-HA and 100 ng of EYFP-ER (Clontech). 24 h after transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.1% Triton X-100, phosphate-buffered saline, and subjected to immunostaining. Erlectin-HA was detected with rat anti-HA antibody and cy3 or fluorescein isothiocyanate-conjugated anti-rat secondary antibody (Dianova). The trans-Golgi network and endosomes were stained with mouse anti-TGN38- and anti-EEA1-antibody, respectively (BD Biosciences), followed by cy3-conjugated anti-mouse antibody (Dianova).

**Fractination of Cultured Cells and Protease Protection Assay**—The microsomal fraction was isolated from HEK293T cells after transfection of erlectin-HA using an ER isolation kit protocol (Sigma, E01010). Aliquots were treated ± 1% Triton X-100 for 30 min on ice, then incubated ± 250 μg/ml Proteinase K (Gerbu), for 1 h on ice. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM, and samples were analyzed by SDS-PAGE and Western blotting. Antibodies used were mouse anti-calnexin antibody (BD Biosciences) and mouse anti-tubulin antibody (Sigma, B512).

Embryos, in situ Hybridization, and RT-PCR—In vitro fertilization, embryo culture, staging, whole-mount in situ hybridization, and micro-injection were carried out as described (37). Primers used for RT-PCR of erlectin were 5’-TGAAGGAGAAGTTCTCCAGT-3’ (forward) and 5’-GGTTGGAGCTTTAGTTGACTC-3’ (reverse) for allele1 and...
**Erlectin Functions in N-Glycan Recognition**

5′-TGAAAGAGAAGTTCTCCACC-3′ (forward) and 5′-GTTTGCACTTTAATTTCACAACG-3′ (reverse) for allele2.

Morpholino Injections and Histology—Morpholino (MO) antisense oligonucleotides with the following sequences were obtained from Gene Tools, LLC: MO1, 5′-GAGAATGTGCAGGAGTTACCGGTTA-3′, MO2, 5′-AGAGAATGCGCAGGAGCGACAGGTT-3′, and a standard control oligonucleotide designed by Gene Tools. Unless indicated otherwise, embryos were injected at 4 cell stage four times into the marginal zone. For histological analysis 10-μm sections of paraffin-embedded embryos were cut and stained with Mayer's hemalaun/eosin following standard procedures.

**RESULTS**

Erlectin Is a Novel MRH Domain Protein and Specifically Interacts with Krm2—To identify binding partners of Krm2 we employed a proteomic approach. We generated a HEK293 cell line stably expressing mouse Krm2 and LRP6 and performed a large scale affinity purification of the Krm2 protein complex from cellular membrane lysates. The purified protein complex was separated by SDS-PAGE, and individual bands were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). As control for background binding we performed a similar purification from HEK293 cells expressing only LRP6.

One of the proteins found in two independent Krm2 purifications but not in the LRP6 control purification was a novel protein we termed Erlectin (for ERlectin, see below; other GenBank designations are XTP3-B, C2orf30, CL25084). We identified seven independent peptides that all matched this protein (Fig. 1A).

The open reading frame of erlectin consists of 483 amino acids with a calculated molecular mass of 53 kDa. Data base searches revealed homologues of erlectin in deuterostomes (chordates, echinoderms) and protostomes including Drosophila and Caenorhabditis elegans (Fig. 1B).

Erlectin is a member of the MRH domain protein family. It contains a signal peptide and two MRH domains (Fig. 1C).

To confirm the physical association of Erlectin with Krm2 we carried out in vitro binding studies of recombinant Erlectin and Krm2ΔTMC, a soluble form of Krm2 lacking the transmembrane domain and the C-terminal tail (15). As control protein we used recombinant Dkk3, which unlike Dkk1 does not bind to Krm2 (15). Recombinant proteins were produced as conditioned medium from cells individually overexpressing secreted forms of the proteins. Under the conditions tested Erlectin specifically binds to Krm2ΔTMC (Fig. 2A, lane 1) but not to Dkk3 (lane 2). Also in the reverse CoIP, Krm2ΔTMC is precipitated by Erlectin (Fig. 2B, lane 1).

Krm2 forms a ternary complex with Dkk1 and LRP6 that triggers internalization of LRP6 (15). Therefore, we tested whether Erlectin also interacts with LRP6. LRP6ΔTMC, a soluble form of LRP6 consisting of the extracellular domain of the protein, does not bind to Erlectin (Fig. 2C, lane 1). As shown previously (38), LRP6ΔTMC also does not interact with Dkk3 (Fig. 2C, lane 2). In addition, Erlectin neither binds full-length LRP6 nor Dkk1 (supplemental Fig. 1).

**FIGURE 1.** Erlectin is a novel protein related to oligosaccharide-processing proteins. A, amino acid sequence of Erlectin. Highlighted in red are peptides identified by LC-MS/MS. B, Erlectin homology tree and matrix showing amino acid identity between indicated species. C, structure of Erlectin and comparison with domain relatives (adapted from SMART). Erlectin contains a signal peptide and two MRH domains (PRKCSH by SMART/InterPro, indicated as D1, D2). The scheme shows a comparison of all four human proteins containing an MRH domain. Also shown is an alignment of a conserved region of the MRH domains, with conserved residues labeled in black and gray. A point mutation in a conserved residue (G106S, arrow) of GNPTAG leads to a lysosomal storage disease (28). hu, human; mo, mouse; ch, chicken; X.t., X. tropicalis; D.r., D. rerio; C.i., C. intestinalis; S.p., S. purpuratus; D.m., D. melanogaster; PRKCSH, β-subunit of glucosidase II.
In summary, Erlectin specifically interacts with the Dkk1 coreceptor Krm2. The transmembrane and the cytoplasmic domain of Krm2 are not required for this interaction.

The MRH Domain 2 of Erlectin Mediates Binding to Krm2—We performed in vitro binding assays using recombinant proteins to test the requirement of the two Erlectin MRH domains for Krm2 binding. Deletion within the MRH domain 2 but not 1 impairs the binding of Erlectin to Krm2 (Fig. 3). Because a point mutation (G106S) in the MRH domain of GNPTAG (see Fig. 1C, arrow) leads to the lysosomal storage disease mucolipidosis type III in humans (28), we also tested the homologous mutation of Erlectin in the MRH domain 2. Interestingly, this mutation abolishes binding of Erlectin to Krm2 (Fig. 3, lane 5).

The Krm2 Kringle Domain and N-Glycans Are Required for Interaction with Erlectin—Krm2 contains an extracellular kringle-, WSC-, and CUB-domain, followed by a transmembrane domain and a cytoplasmic tail (Fig. 4A). To determine which domain interacts with Erlectin, we analyzed Krm2 deletion constructs for their ability to bind Erlectin in CoIP. While deletion of the WSC- as well as the CUB-domain does not influence binding to Erlectin (Fig. 4B, lanes 4 and 5), deletion of the kringle domain strongly impairs this interaction (Fig. 4B, lane 3). Thus, the interaction of Krm2 with Erlectin is mediated by the kringle domain.

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Erlectin Functions in N-Glycan Recognition

Because MRH domains are implicated in N-glycan recognition, we analyzed whether binding of Erlectin to Krm2\Delta TMC is glycan-dependent. When Krm2\Delta TMC is subjected to deglycosylation with N-glycosidase F, a shift in apparent molecular weight is observed, indicating that Krm2\Delta TMC is indeed N-glycosylated. Furthermore, deglycosylated Krm2\Delta TMC fails to bind Erlectin in vitro (Fig. 4C, lane 2). These results suggest that Erlectin recognizes and binds to oligosaccharides linked to the kringle domain of Krm2. Consistent with this, the Krm2 kringle domain contains one potential N-glycosylation site (N48 in mouse, N55 in X. laevis).

Erlectin Is a Member of the Endoplasmic Reticulum Synexpression Group in Xenopus—We studied the expression pattern of erlectin during Xenopus development by in situ hybridization and RT-PCR. Two alleles are present in X. laevis showing 82% identity in their open reading frames. We therefore designed PCR primer pairs as well as in situ probes specific for each allele. RT-PCR analysis shows no difference in the expression pattern of both alleles. Likewise, in situ hybridization indicates a comparable expression pattern of both alleles.

RT-PCR analysis shows that erlectin is expressed maternally. Levels of mRNA decrease during gastrulation (stages 10.5–13) and are then increasingly up-regulated during neurulation (stages 17–21) and tailbud stages (Fig. 5A).

By whole mount in situ hybridization, erlectin expression is observed in the animal hemisphere of blastula stage embryos (Fig. 5C). During neurula stages expression is seen in the notochord (Fig. 5E). At late neurula stage strong expression is also detected in the anlagen for cement gland and hatching gland and is very similar to the expression of XAG, a marker for these tissues (39) (Fig. 5, F and G). In tailbud embryos erlectin expression occurs in otic vesicle and pronephros and continues in cement and hatching gland (Fig. 5I). A weak ubiquitous expression of erlectin is observed at all stages.

This expression is highly reminiscent of the expression pattern of the ER synexpression group (11), of which the KDEL receptor is one example (Fig. 5H). We conclude that erlectin is a member of the ER synexpression group, which strongly suggests that the protein plays a role in secretory protein traffic.

Erlectin Is a Luminal Protein of the Endoplasmic Reticulum—To further corroborate an involvement of Erlectin in ER function, we studied its subcellular distribution in cultured cells. When expressed in HEK293T cells, trans-Golgi network marker TGN38 (40) (Fig. 6, B–B'), and with the luminal ER protein calnexin (data not shown). In contrast, the localization of Erlectin-HA does not overlap with that of the trans-Golgi network marker TGN38 (40) (Fig. 6, B–B'), nor with the endosome marker EEA1 (41) (Fig. 6, C–C').

To further clarify the membrane topology of Erlectin, we performed a protease protection assay with membranes prepared from transfected...
Erlectin Functions in N-Glycan Recognition

To investigate this further, we monitored plasma membrane levels of Krm2 by cell surface biotinylation. Following cotransfection with erlectin, cell surface levels of Krm2 are strongly reduced, whereas the total cellular Krm2 is mostly unaffected (Fig. 7D, upper two panels). The cytoplasmic protein nucleoside diphosphate kinase A (NME1) serves as control and is not biotinylated (Fig. 7D, lower two panels). In addition, expression of erlectin does not influence the expression of LRP6 (Fig. 7E), indicating that reduced cell surface localization of Krm2 does not result from a general impairment of protein traffic. The results demonstrate that coexpression of Erlectin specifically inhibits transport of Krm2 to the cell surface and induces intracellular accumulation of Krm2, most likely in the ER.

Erlectin Is Essential for Head and Axial Development in Xenopus—To study the function of Erlectin in vivo we first overexpressed mRNA in Xenopus embryos. Embryos injected with up to 4 ng of mRNA develop normally (data not shown).

Because Krm2 overexpression anteriorizes embryos (17), we tested whether overexpression of Erlectin can modify this Krm2 gain-of-function phenotype by coinjection of Erlectin and Krm2 mRNA. Neither an enhancement nor a rescue of the embryonic phenotype was observed (data not shown). To further investigate the role of Erlectin during development we made use of MO antisense oligonucleotides, which are in widespread use in developmental biology because of their high specificity and low toxicity (43, 44). We designed two MOs targeted against each of the two alleles found in X. laevis. Both MOs also target the X. tropicalis erlectin allele (two mismatches each) (Fig. 8A).

Injection of both MOs at 4-cell stage leads to a very similar, characteristic phenotype in X. laevis and X. tropicalis embryos, although at different doses (Fig. 8B). Embryos develop morphologically normally until late tailbud stage. Tadpole embryos show axial defects including anterior head defects and shorter, bent tails, as well as retarded development and consequently reduced size. Histological analysis of MO-injected embryos shows reduced size of axial organs including notochord and somites. Embryos exhibit microcephaly, with reduced brain tissue that lacks a ventricle. The cement gland is present but abnormally shaped, and the heart is absent (Fig. 8C). The specificity of this phenotype is supported by two different MOs that give the same characteristic phenotype in both X. laevis and X. tropicalis.

Krm1+2 MO-injected embryos show microcephaly and reduced expression of the forebrain marker b61 because of a role of the proteins in early a-p patterning (17). We therefore asked whether the observed axial defects in Erlectin MO-injected embryos are due to a similar influence of Erlectin on early a-p patterning. We injected Erlectin MO into Xenopus embryos and analyzed b61 (forebrain) (45) and krox20 (hindbrain) (46) (Fig. 8E). Neither of these markers is affected by Erlectin MO injection. Likewise, other a-p markers such as XAG (cement/hatching gland), otx2 (fore/midbrain) (47), and Xnot2 (notochord) (48) are unaffected (not shown). This indicates that head defects induced by Erlectin depletion occur after initial a-p patterning is established and are thus most likely unrelated to the role of Krm2 during this early phase of development.

DISCUSSION

In eukaryotic cells, the ER is the entry site for proteins destined for the secretory pathway, and the site where folding, disulfide bond formation, N- and O-glycosylation, oligomerization, and quality control of newly synthesized proteins occurs (49–53). MRH domain proteins are a small family of N-glycan-recognition proteins, two of which have been shown to reside in the ER. Glucosidase II β-subunit functions in glycan proc-
Erlectin Functions in N-Glycan Recognition

Erlectin is a novel member of the MRH domain family, and our results indicate that it also functions in the ER. It is a member of the ER synexpression group, which strongly predicts an ER function, and it localizes to the ER lumen in transfected cells. The fact that it lacks a canonical KDEL or HDEL retention signal raises the possibility that Erlectin is part of a protein complex retained in the ER. Because the binding of Erlectin is abolished by N-glycosidase F treatment of Krm2 this strongly suggests that like other MRH domain proteins, Erlectin recognizes N-glycans.

Erlectin contains two MRH domains that do not appear equivalent in substrate binding abilities. MRH domain 1 is dispensable for binding to Krm2, and it remains an open question whether it is inactive in N-glycan recognition or whether it has other specificities. Interestingly, a point mutation in the MRH domain 2, which mimics a homologous mutation in GNPTAG linked to mucolipidosis type III (28), abolishes binding of Erlectin to Krm2, indicating that this is a functionally conserved amino acid.

Cotransfection with Erlectin specifically inhibits transport to the cell surface and induces intracellular accumulation of Krm2, most likely in the ER. Because Krm2 is a negative regulator of Wnt signaling, one would predict that Erlectin derepresses Wnt signaling. However, in Wnt reporter assays Erlectin cotransfection with Krm2 is mildly inhibitory on Wnt signaling (not shown), suggesting that Erlectin overexpression may have other effects as well. On the other hand, knock down of Erlectin in Xenopus embryos results in disturbed axial development and head defects, which are generally associated with enhanced Wnt signaling. Phenotypically these embryos do resemble embryos depleted of Krm1/2. However, early a-p markers are unaffected, in contrast to Krm1/2-depleted embryos, in which reduction of the forebrain marker bfl1 is observed (17). Moreover, we could not rescue the Erlectin MO-injected embryos with krm2 mRNA or other Wnt inhibitors like dkk1, dnWnt8 mRNA, or β-catenin MO (not shown), providing further evidence that this phenotype is not because of excessive Wnt signaling. Thus, Erlectin is not required for Wnt-mediated early a-p patterning. Yet, the severe phenotypic defects observed after depletion of Erlectin indicate an essential, pleiotropic function of this gene, because multiple tissues, including brain, notochord, and heart are affected. Maternal Erlectin, which would be unaffected by MO injection, may account for normal early development. In conclusion, although its interaction with Krm2 is useful for studying the effects of Erlectin, the physiological relevance of this interaction is unclear.

What then may be the physiological role of Erlectin? Its ability to retain Krm2 intracellularly suggests that Erlectin may act as an ER chaperone, similar to Mesd or Shisa, which regulate receptor folding or transport to the cell surface (5, 8). However, because Erlectin is a member of the ER synexpression group, a specific role in any one given developmental pathway seems unlikely, and this rather points to a more general involvement of the protein in ER-mediated processes. Similar to the glucosidase II β-subunit, Erlectin may be part of an enzyme complex involved in recognizing and/or processing N-glycans. This would be supported by its lack of an ER retention signal as well as of any other functional protein domain. Erlectin was suggested as a possible functional protein domain. Erlectin was suggested as a possible functional protein domain. Erlectin was suggested as a possible functional protein domain. Erlectin was suggested as a possible functional protein domain.

In summary, Erlectin is a novel member of the MRH domain family, whose function in N-glycan recognition makes it a potential component of an enzyme complex involved in processing N-glycans. This would require further investigation to confirm its physiological role in N-glycan processing.
tional homolog to Yos9p (31), an MRH protein playing a role in ER-associated degradation (31–34). It is therefore an interesting possibility that the phenotype of Erlectin depletion in Xenopus embryos reflects disturbed protein degradation.

Of note, two other MRH domain proteins, glucosidase II β-subunit and GNPTAG cause human disease when mutated (25, 54), and our loss of function analysis suggests that Erlectin too may be essential in humans as it is in frogs.

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14.転写調節因子Erlectinは、N-糖の認識に重要である。