The intracellular domain of teneurin-2 has a nuclear function and represses zic-1-mediated transcription

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Summary

Teneurin-2, a vertebrate homologue of the *Drosophila* pair-rule gene *ten-m/odz*, is revealed to be a membrane-bound transcription regulator. In the nucleus, the intracellular domain of teneurin-2 colocalizes with promyelocytic leukemia (PML) protein in nuclear bodies implicated in transcription control. Since *Drosophila ten-m* acts epistatically to another pair-rule gene *opa*, we investigated whether gene regulation by the mammalian opa homologue zic-1 was influenced by the intracellular domain of teneurin-2. We found that zic-mediated transcription from the apolipoprotein E promoter was inhibited. Release of the intracellular domain of teneurin-2 could be stimulated by homophilic interaction of the extracellular domain, and the intracellular domain was stabilized by proteasome inhibitors. We have previously shown that teneurin-2 is expressed by neurons belonging to the same functional circuit. Therefore, we hypothesize that homophilic interaction enables neurons to identify their targets and that the release of the intracellular domain of teneurin-2 provides them with a signal to switch their gene expression program from growth towards differentiation once the proper contact has been made.

Key words: Pair-rule, PML, RIP, Ten-m, Transcription

Introduction

Studies in *Drosophila* have revealed the existence of two members of a new protein family, namely, Ten-a and Ten-m (Baumgartner and Chiquet-Ehrismann, 1993; Baumgartner et al., 1994; Minet and Chiquet-Ehrismann, 2000; Fascetti and Baumgartner, 2002). *Ten-m*, also known as *odz* (Levine et al., 1994), is a pair-rule gene (Baumgartner et al., 1994). This is surprising, as Ten-m is a cell-surface or secreted protein, and all other pair-rule genes are transcription factors (Lawrence and Struhl, 1996). Baumgartner et al. proposed that Ten-m acts as a pair-rule gene by binding to a receptor (Baumgartner et al., 1994), which in turn transmits the extracellular signal into the nucleus. Mutational analysis by Baumgartner et al., indicated that Ten-m initiates a signal transduction cascade via or in concert with *opa* receptor (Baumgartner et al., 1994), another pair-rule gene that encodes a zinc finger transcription factor.

During the later stages of development, Ten-a and Ten-m/Odz are predominantly expressed in the nervous system (Levine et al., 1997; Minet et al., 1999; Fascetti and Baumgartner, 2002). The predominant neuronal expression is conserved in the vertebrate homologues ten-m1, 2, 3 and 4 in the mouse (Oohashi et al., 1999; Ben-Zur et al., 2000), neurestin in the rat (Otaki and Firestein, 1999) and ten-m3 and ten-m4 in zebrafish (Mieda et al., 1999).

Most of the functional studies have been performed on the avian ten-m family members. Three family members have been described in the chicken so far and have been termed teneurin-1 (Minet et al., 1999), teneurin-2 (Rubin et al., 1999) and teneurin-4 (Tucker et al., 2000). Teneurin-2 is a type II transmembrane protein containing a furin cleavage site in the extracellular domain (Rubin et al., 1999). Both teneurin-1 and -2 promote neurite outgrowth in vitro (Minet et al., 1999; Rubin et al., 1999). Teneurin-2 also acts as a homophilic adhesion protein and may play a role in the specification of neuronal circuits in the developing visual system (Rubin et al., 2002). In addition to being found in the nervous system, teneurin-2 and -4 are expressed in two important organizing centers of limb development: the apical ectodermal ridge and the zone of polarizing activity, respectively (Tucker et al., 2001; Tucker et al., 2000).

As all members of the teneurin family are type II transmembrane proteins (Rubin et al., 1999; Feng et al., 2002), one potential scenario by which such membrane-spanning proteins can fulfill their role as signaling molecules is by a mechanism recently described as regulated intramembrane proteolysis (RIP) (reviewed in Brown et al., 2000). RIP is a two-step mechanism that leads to the cleavage of transmembrane proteins at and in the lipid bilayer. The cleavage and release of the extracellular or intraluminal parts of the protein is a prerequisite for a second cleavage, which leads to the separation of the intracellular part from the membrane. The latter takes place within the transmembrane domain. The resulting soluble intracellular part translocates to the nucleus, where it participates in transcription. RIP was first proposed as a signaling model by which the sterol regulatory element binding protein (SREBP) regulates lipid metabolism (Brown and Goldstein, 1997). It is now known to control diverse cellular and developmental processes (Brown et al., 2000). The study of Notch, another protein exerting function by this mechanism, was crucial to discover important features of RIP (Chan and Jan, 1998). Also Ire1 (Niwa et al., 1999) and ATF6 (Haze et al., 1999), both of which are involved in the unfolded secretory protein pathway (endoplasmatic reticulum

stress response), signal through RIP. Amyloid precursor protein (APP), which is thought to be involved in the Alzheimer's disease, is a prominent example of this mechanism (Haass and De Strooper, 1999; Ebinu and Yankner, 2002). Not only does proteolysis of APP lead to the accumulation of the toxic APP peptide underlying Alzheimer disease, but RIP may be part of normal APP signaling (Gao and Pimplikar, 2001). The most recently recognized and least described examples of RIP include CD44 (Okamoto et al., 2001), ErbB-4 (Ni et al., 2001; Lee et al., 2002), luman (Raggo et al., 2002) and E-cadherin (Marambaud et al., 2002). These diverse examples of RIP could well be just the tip of the iceberg of a large group of transmembrane proteins undergoing proteolytic cleavage to initiate a signal transduction cascade.

It was the aim of the present work to determine whether a similar proteolytic mechanism is responsible for the signaling by teneurins, thus reconciling the enigma of *Drosophila ten-m* being a pair-rule gene and a bona fide transcription regulator despite its cell-surface location. We found that indeed the intracellular domain of teneurin-2 can be released from the cell membrane and that it translocates to the nucleus where it is able to influence the transcription activity of zic, a vertebrate homologue of the *Drosophila* Opa (Yokota et al., 1996).

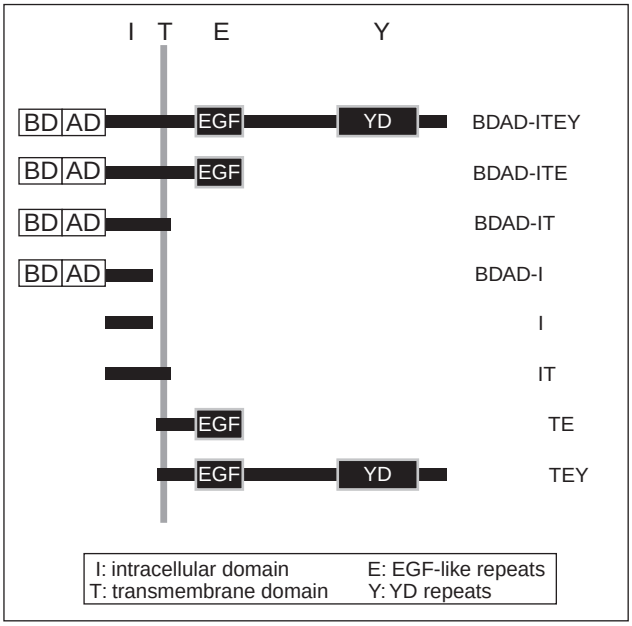

Fig. 1. Schematic models of all teneurin-2 proteins used in this study. BD stands for Gal4 DNA-binding domain and AD for NFκB activation domain.

Materials and Methods

Antibodies and DNA constructs

The following primary antibodies were used anti-teneurin-2 (Rubin et al., 1999), anti-VSV (affinity-purified peptide antibody, from Andrew Matus, Friedrich Miescher-Institute), anti-FLAG (M2, Stratagene), anti-Gal4 (DGB RK5C1, Santa Cruz), anti-PML (PG-M3, Santa Cruz), anti-myc (c-Myc 9E10, Santa Cruz) and anti-HA (12CA5, Roche). Secondary antibodies used were Alexa594- and Alexa488-conjugated goat anti-mouse and goat anti-rabbit IgG (all from Molecular Probes), horseradish-peroxidase-coupled anti-mouse and anti-rabbit IgG (Soccochim).

The following DNA constructs were used: pFR-luc (luciferase reporter plasmid; Stratagene), pSV-β-Galactosidase (Promega), pBD-NFκB (encodes BDAD), pCMV-AD and pCMV-BD (Stratagene), p-CMX-PML and p-CMX-PML-RAR (expression plasmids encoding PML or PML-RAR fusion protein, respectively, kindly provided by Ronald M. Evans, San Diego) (Kakizuka et al., 1991; Lin and Evans, 2000), pEF-zic1 (expression plasmid encoding myc-tagged zic-1, a generous gift from Jun Aruga, Saitama, Japan) (Aruga et al., 1996), pXP2-*APOE*189 (luciferase reporter plasmid under the control of an apolipoprotein E promoter; kindly provided by Francisco Zafra, Madrid) (Salero et al., 2001).

Teneurin-2 constructs

Eight different teneurin-2 constructs were used in this study (Fig. 1). They are named according to the teneurin-2 protein domains contained within their coding regions. Two of them (constructs TE and TEY) were described before (Rubin et al., 2002). Construct I represents the soluble intracellular domain of teneurin-2. It encodes the first 372 amino acids of the teneurin-2 sequence as described previously (Rubin et al., 1999), followed by a VSV tag for detection. Construct IT contains, in addition to the intracellular domain, amino acids 373-406, including the membrane-spanning domain and 10 extracellular amino acids followed by an HA tag for detection. In four constructs teneurin-2 was coupled to the Gal4-binding domain (BD) and the NFκB activation domain (AD), generating BDAD-teneurin-2 fusion proteins (see Fig. 1). These constructs were cloned by multiple PCR. The product of the PCRs comprised bases 675-1118 of pCMV-BD, coding for BD, bases 703-1267 of pCMV-AD coding for AD and bases 1-630 of teneurin-2 coding for the first 210 amino acids of the intracellular domain of teneurin-2 until the *Blp*I site. These fragments were connected by the method of SOE (Horton, 1995), and the resulting construct was cloned into the *Bam*HI/*Blp*I site of the pre-existing pcDNA3 vectors containing teneurin-2 constructs of different lengths (Rubin et al., 2002).

Transient transfections

HT1080 fibrosarcoma and COS-7 green monkey kidney cells were routinely maintained in DMEM medium supplemented with 10% FCS. For transient transfections, the cells were seeded in six-well plates or 35 mm dishes containing four internal wells (Greiner). 12 hours later they were transfected with the indicated expression vectors (1 μg of each vector) by using FUGENE-6 (3 μl, 6 μl or 9 μl for one, two to three or four different plasmids, respectively; Roche). 24 hours after transfection the cells were rinsed in PBS and processed for either measuring luciferase and β-galactosidase activities, western blotting or immunofluorescence.

Where indicated the cells were treated with the following substances at least 5 hours after transfection: ALLN (25 μg/ml; N-acetyl-leu-leu-norleu-AL; Sigma); tunicamycin (2 μg/ml; Sigma) or lactacystin (10 μM; Sigma) for 4 or 8 hours prior to harvesting.

Stable cell lines

Construct I was subcloned into the ecdyson-inducible expression vector pIND (Invitrogen) and transfected into EcR-293 cells (Invitrogen) according to the supplier's manual, resulting in the cell line EcR-293-I. Clones were tested for the inducible expression of construct I after the addition of increasing concentrations of ponasterone (1-10 μg/ml; Invitrogen) by immunoblotting using anti-VSV antibodies.

Clones of HT1080 cells stably expressing TEY (TEY cells) or TE (TE cells) on their surfaces, respectively, have been described previously (Rubin et al., 2002).

Luciferase and β-galactosidase assays

The cells were lysed by adding reporter lysis buffer (Promega). Appropriate dilutions of the lysed cell suspension were then pipetted into Microlite™ luciferase plates (Dynex Technologies), and the luciferase activity was measured in a Microlumat (LB96P, EG+G Berthold) by automatic injection of luciferin substrate solution (2 mM luciferin, 100 mM ATP in 250 mM glycin pH 7.8, 150 mM $MgSO_4$). All luciferase activities were normalized with respect to the transfection efficiency by co-transfecting a β-galactosidase vector. To determine β-galactosidase activity the diluted cell suspensions were incubated with the substrate solution (4.5 mM 2-nitrophenyl-b-D-galactopyranoside in 0.2 M Na-phosphate, 2 mM $MgCl_2$, 0.1 mM β-mercaptoethanol) for 30 minutes at 37°C. To stop the enzymatic reaction, 1 M Na_2CO_3 was added and the OD was measured at 405 nm in a microplate reader (BioRad).

Western blotting

Teneurin-2 constructs I, IT and ITE were extracted by adding SDS sample buffer containing 20% β-mercaptoethanol directly to the cells. Extraction of the nuclear constructs BDAD and BDAD-I was achieved by performing nuclear fractionation. The transfected cells were harvested by scraping off the cell layer in lysis buffer [10 mM HEPES pH 7.5, 0.5% triton X-100, 300 mM sucrose, 100 mM NaCl, 2 mM $MgCl_2$, protease inhibitors (Complete™, Roche Diagnostics)] on ice and subsequent centrifugation for 10 minutes at 420 ***g*** in an Eppendorf centrifuge. The resulting pellet was resuspended in lysis buffer and centrifuged again. The final pellet was then dissolved in SDS sample buffer containing 20% β-mercaptoethanol, 6 M urea and protease inhibitors (Complete™). Before loading on an 8% SDS-PAGE gel, DTT was added to a final concentration of 10 mM.

The transmembrane constructs BDAD-ITE and BDAD-ITEY were extracted from the cells by the following procedure. The cells were extracted on ice by a hypotonic buffer (2 mM Na-phosphate buffer pH 7.5, 20 mM KCl, 1 mM β-mercaptoethanol), scraped off and centrifuged for 10 minutes at 6800 ***g*** at 4°C in an Eppendorf centrifuge. The resulting pellet was reconstituted in detergent buffer [50 mM Tris pH 8, 1% NP40, 150 mM NaCl, 5 mM EDTA, 6 M urea, protease inhibitors (Complete™)], incubated for 20 minutes at 37°C and centrifuged for 10 minutes at 17,900 ***g***. SDS sample buffer containing 20% β-mercaptoethanol, 6 M urea and protease inhibitors (Complete™) was added to the supernatant and incubated for 1 hour at 52°C. After DTT was added (10 mM), the samples were loaded on a 6% SDS-PAGE gel.

The gels were transferred to PVDF membranes. The proteins were detected by anti-Gal4 antibody (BDAD and BDAD-I) or by anti-teneurin-2 serum (BDAD-ITE and BDAD-ITEY), horseradish-peroxidase-coupled secondary antibodies and ECL SuperSignal® (Pierce).

Immunofluorescence

The cells grown on 35 mm four-well staining dishes (Greiner) were fixed with 4% PFA for 30 minutes at room temperature and, where indicated, permeabilized with 0.1% triton X-100 for 5 minutes. Incubation with primary antibodies was performed for 60 minutes and that with secondary antibodies for 30 minutes, both at room temperature, and the cells were washed in PBS after each incubation. Finally, the specimens were mounted in Moviol and examined and photographed using a Zeiss Axiophot microscope (Carl Zeiss Ltd.) connected to a 3CCD camera (Sony).

Results

Colocalisation of the teneurin-2 intracellular domain with PML

Pilot results with a yeast two-hybrid assay indicated transcriptional activity for the intracellular part of teneurin-2 (data not shown). Consistent with this we found the intracellular domain of teneurin-2 (referred to as construct I, see schematic representation of constructs in Fig. 1) to be translocated to the nucleus if transfected into HT1080 cells. To our surprise, transfection of construct I did not lead to a uniform nuclear accumulation but its expression instead was confined to discrete spots within the nucleus (Fig. 2b,d). This staining pattern contrasted with that of the transmembrane teneurin-2 construct IT, which accumulated on the cell surface (Fig. 2a). The nuclear localization coincided with a very similar punctate pattern obtained by staining for PML protein (promyelocytic leukemia protein) (reviewed in Seeler and Dejean, 1999) and may represent nuclear bodies, termed promyelocytic oncogenic domains (PODs) or PML bodies (Fig. 2e). Double immunofluorescence staining of construct I transfected cells did show substantial, but not complete, overlap of PML with the intracellular domain of teneurin-2 (Fig. 2d-f). Since PML bodies are involved in a number of functions associated with transcriptional control (Zhong et al., 2000), it was of interest to determine whether there was a genuine colocalisation of teneurin-2 and PML in nuclear bodies. We therefore cotransfected construct I with an expression plasmid encoding PML on the one hand and with PML-RAR (PML-retinoic acid receptor fusion protein) on the other. Transfection of PML into cells containing endogenous PML protein leads to a massive enlargement of the nuclear bodies (Doucas et al., 1996). In contrast, transfection of PML-RAR should result in a destruction of the PML body architecture (Dyck et al., 1994; Mu et al., 1994). We did indeed observe these effects, as illustrated in Fig. 2h,k,l. Interestingly, for teneurin-2 I an equivalent staining pattern was detected after cotransfection with PML, and I was pulled into the enlarged PML bodies (Fig. 2g-i). Furthermore, destruction of the PML body architecture after transfection with PML-RAR also changed the expression pattern of cotransfected I, which was no longer accumulated in discrete spots in the nucleus but seemed to be expressed homogeneously throughout the cells (Fig. 2j). These results indicate that the intracellular domain of teneurin-2 accumulates within nuclear bodies, thus supporting our previous hypothesis that the intracellular domain of teneurin-2 is involved in transcriptional regulation.

Functional interaction of teneurin-2 with zic

In *Drosophila*, ten-m was postulated to modulate the activity of Opa protein (Baumgartner et al., 1994). It was therefore of interest to investigate whether the zinc finger transcription factor zic, a vertebrate homologue of Opa, would influence or would be influenced by the intracellular domain of teneurin-2.

When both proteins were expressed in COS-7 cells by transient transfections we observed a marked downregulation of the intracellular domain I of teneurin-2 compared with its usual expression level (Fig. 3A). On the other hand, co-transfection of the two constructs did not have an effect on the level of zic (Fig. 3C). In contrast to its effect on teneurin-2, zic did not downregulate another co-transfected transcription factor BDAD (Gal4 DNA-binding domain fused to the NFκB activation domain) in an analogous analysis (Fig. 3B,D). The zic-induced downregulation of the intracellular domain I of teneurin-2 was counteracted by the addition of the proteasome

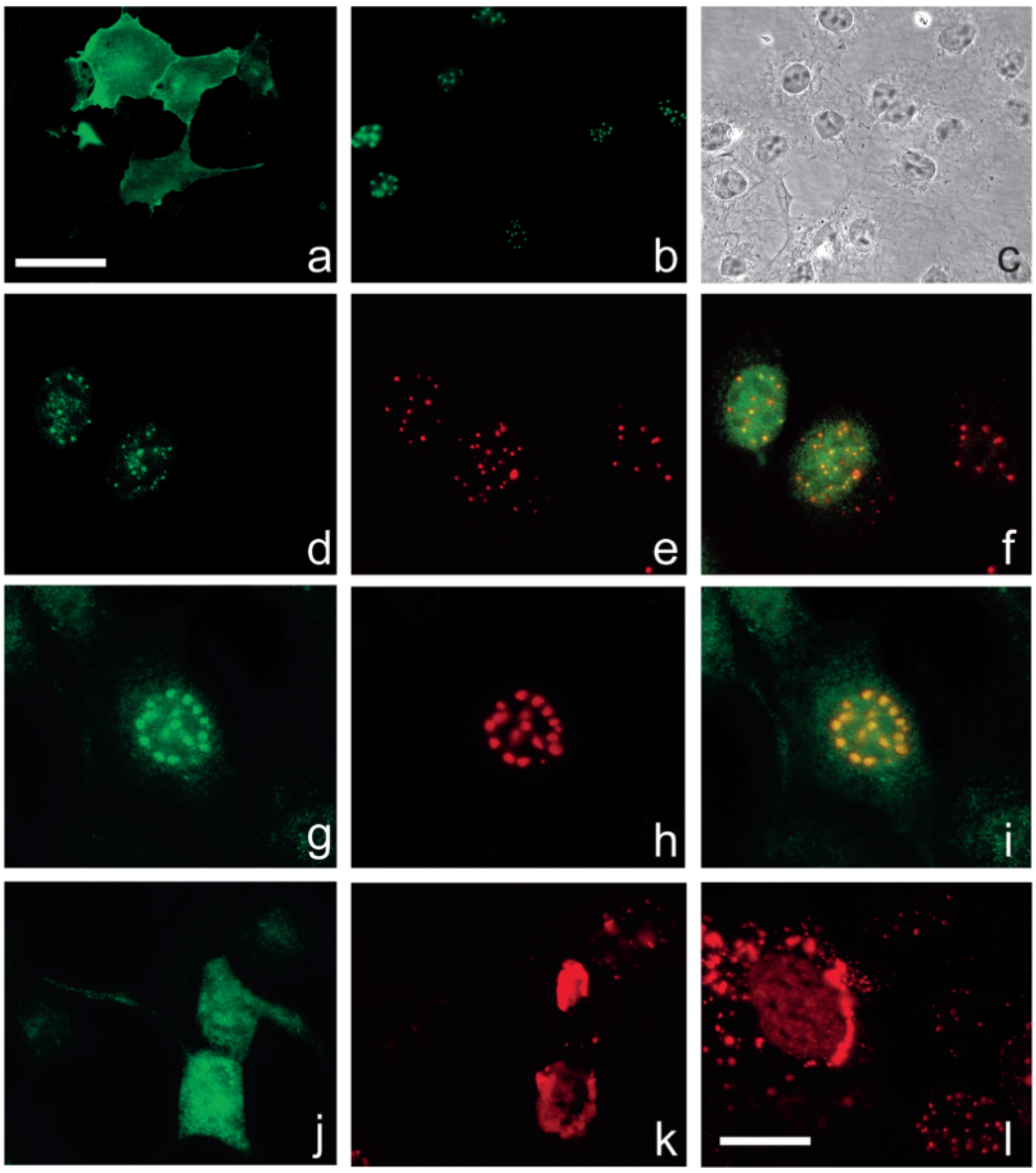

Fig. 2. The teneurin-2 intracellular domain colocalizes with PML. Microscopic analysis of HT1080 cells transfected with teneurin-2 variants IT (a), I (b-k), PML (g-i) and PML-RAR (j-l). Staining of non-permeabilized cells with an anti-HA antibody detecting the C-terminal tag of construct IT. (a) Staining of permeabilised cells with anti-VSV detecting the C-terminal tag of construct I (b) and same field analysed in phase contrast (c). Double-immunofluorescence of I transfected cells for I (anti-VSV; d) and endogenous PML (anti-PML; e) with an overlap shown in f. Double immunofluorescence staining of cells co-transfected with I and PML for I (anti-VSV; g) and PML (anti-PML; h) with the overlap shown in i. Double-immunofluorescence staining of cells co-transfected with I and PML-RAR for I (anti-VSV; j) and PML (anti-PML; k). For comparison, l shows PML-RAR single transfected cells (anti-PML). Bar, 50 μm (a-c) or 10 μm (d-l).

inhibitor lactacystin (Fig. 3A). Thus the nuclear intracellular domain of teneurin-2 seems to be subject to degradation by the proteasome pathway. By immunofluorescence staining of the transfected cells we observed that zic-transfected cells revealed a relatively diffuse nuclear staining (Fig. 3E,F) and in nuclei containing high amounts of zic protein, the punctate staining of teneurin-2 I disappeared and became diffuse (Fig. 3F). Thus, the presence of zic prevented the association of the teneurin-2 intracellular domain with PML bodies and made it amenable to proteasome-mediated degradation.

To examine the potential effect of the teneurin-2 I on the transcriptional activity of zic, stably transfected EcR-293 cell lines were produced. In these EcR-I cells teneurin-2 I was only expressed upon addition of ponasterone (Fig. 4A). EcR-I cells were transiently transfected with zic and a luciferase reporter construct under the control of the apolipoprotein E (ApoE) promotor known to be activated by zic (Salero et al., 2001). Whereas the ApoE-luciferase reporter construct alone did not show any activity, the presence of zic led to a dramatic increase in luciferase acitivity (Fig. 4B). After the induction of teneurin-2 I by ponasterone we observed a marked reduction in the expression level of the reporter gene only in EcR-I cells (Fig. 4B) and not in EcR control cells (Fig. 4C). This result suggested that the intracellular domain of teneurin-2 did have an inhibiting effect on the transcriptional activity of zic, and this effect was more pronounced in the presence of the proteasome inhibitor ALLN, which stabilizes teneurin-2 I (Fig. 4A).

Release of the intracellular domain from the plasma membrane

To be a functional regulator of transcription, wild-type transmembane teneurin-2 would have to be specifically cleaved in or at the plasma membrane, possibly upon a signal by ligand binding. In turn its intracellular part must be released and translocated to the nucleus in a manner similar to that established for proteins regulated by RIP (reviewed in Brown

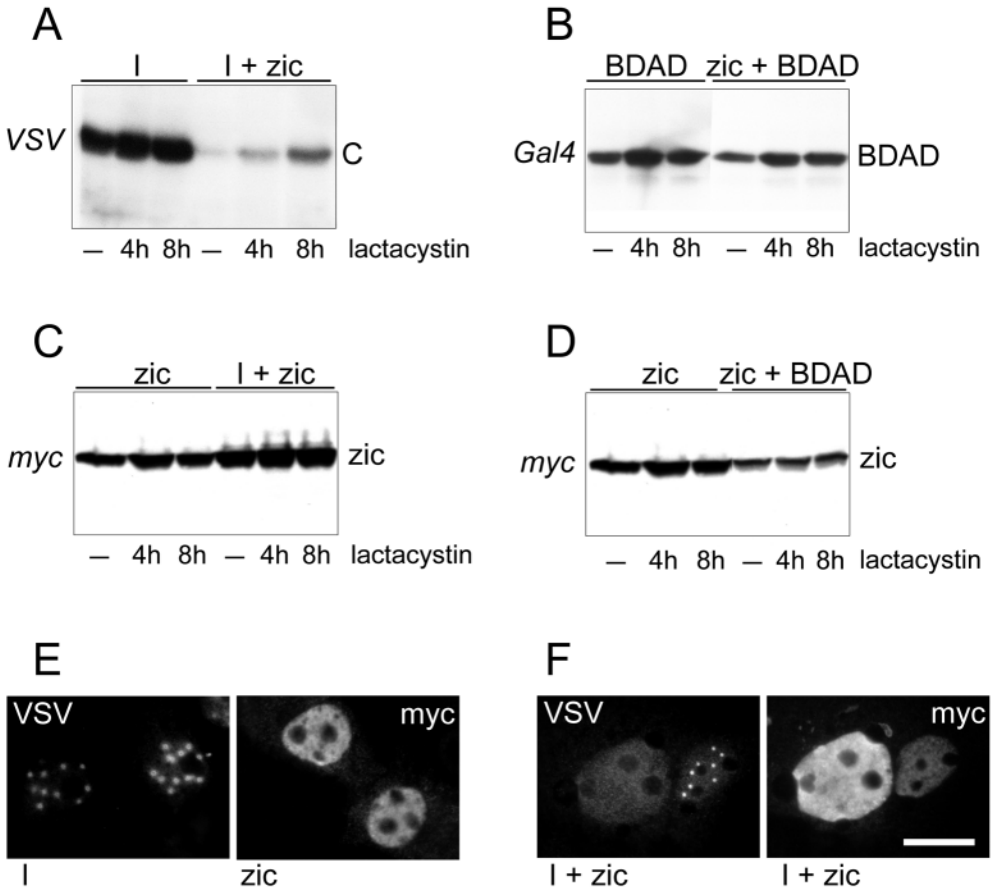

Fig. 3. Zic promotes degradation of the teneurin-2 intracellular domain. Western blot analysis of cell extracts of COS-7 cells transfected with teneurin-2 I (A, left side), the recombinant transcription factor BDAD (pBD-NFκB) (B, left side) and zic (C and D, left side) alone and in combination, as indicated above the lanes (A-D right sides). Extracts of cells without lactacystin treatment were compared to extracts made from cells grown with the addition of lactacystin for the time periods indicated before harvesting. In A, the teneurin-2 intracellular domain was detected by anti-VSV, in B, BDAD was detected by anti-Gal4, and in C and D, zic was detected by anti-myc. Zic caused downregulation of the cotransfected teneurin-2 I but not of BDAD. Immunofluorescence staining of cells transfected with teneurin-2 I (anti-VSV antibody) and zic (anti-myc antibody) alone (E) and after co-transfection (F) revealed that the anti-VSV signal in PML bodies is lost in nuclei showing high expression levels of zic. Bar, 10 μm.

et al., 2000; Ebinu and Yankner, 2002). To test this hypothesis we developed a sensitive method to detect the released intracellular domain of teneurin-2 in the nucleus. We expressed fusion proteins of full-length teneurin-2 (or of smaller transmembrane versions truncated in their extracellular domain) fused to a Gal4 DNA-binding domain (BD) and a NFκB activation domain (AD; see Fig. 1). If cleavage and translocation to the nucleus occurred, BDAD-I could be detected by binding to specific Gal4 recognition sequences in the promotor of the cotransfected luciferase reporter plasmid, and subsequent initiation of luciferase gene expression activated by AD could be monitored.

Fig. 5 illustrates the correct expression of the transfected fusion proteins. BDAD and BDAD-I, serving as positive controls in this experiment, were detectable on a western blot of nuclear extracts by anti-Gal4 antibodies (Fig. 5A), and their accumulation in the nucleus was confirmed by immunofluorescence staining of permeabilised cells (Fig. 5B). At the same time BDAD-ITE and BDAD-ITEY could be identified as part of the plasma membrane by western blots of membrane fractions and by immunofluorescence of non-permeabilized cells (Fig. 5A,B).

For analysis of the luciferase activity induced by the teneurin-2 fusion constructs, HT1080 cells were cotransfected with the respective BDAD-teneurin-2 constructs, the luciferase reporter plasmid, as well as a β-galactosidase construct for normalization of transfection efficiencies. As displayed in Fig. 6A, BDAD-ITE, BDAD-IT and BDAD-I did indeed lead to an induction of luciferase activity above the negative control (BD construct). However, BDAD-ITEY, being the largest fusion protein, did not lead to a significantly enhanced activity. This might partly be explained by the fact that the larger the transmembrane construct the lower the expression level. Alternatively, cleavage of the shorter constructs might be constitutive, whereas cleavage of the full-length construct might have to be specifically induced. This is the case for Notch: processing of Notch expressed on the cell surface is specifically activated by binding to its ligand Delta (Kidd et al., 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). Teneurin-2 has recently been shown to bind homophilically by its extracellular domain (Rubin et al., 2002). We therefore speculated that this interaction could induce cleavage and translocation of the intracellular domain of the BDAD-teneurin-2 fusion proteins, which in turn would be represented by enhanced luciferase activities.

To test whether homophilic interaction of teneurin-2 represents a signal for cleavage of its intracellular domain we transfected BDAD-ITEY and BDAD-IT into HT-1080 cells (HT control) or clones that constitutively express the entire extracellular domain TEY on their surface (TEY cells). The luciferase activity produced from the co-transfected reporter plasmid was then compared (Fig. 6B). As can be seen in Fig. 6B, luciferase activity obtained after transfection of BDAD-ITEY into the TEY cells was 2.6-fold higher than after transfection into HT control cells, whereas the opposite was the case for the BDAD-IT construct, which gave lower luciferase activities in the TEY cells than in the HT control cells, with a ratio of 0.4 fold (Fig. 6B). Thus, we see a 6.5-fold

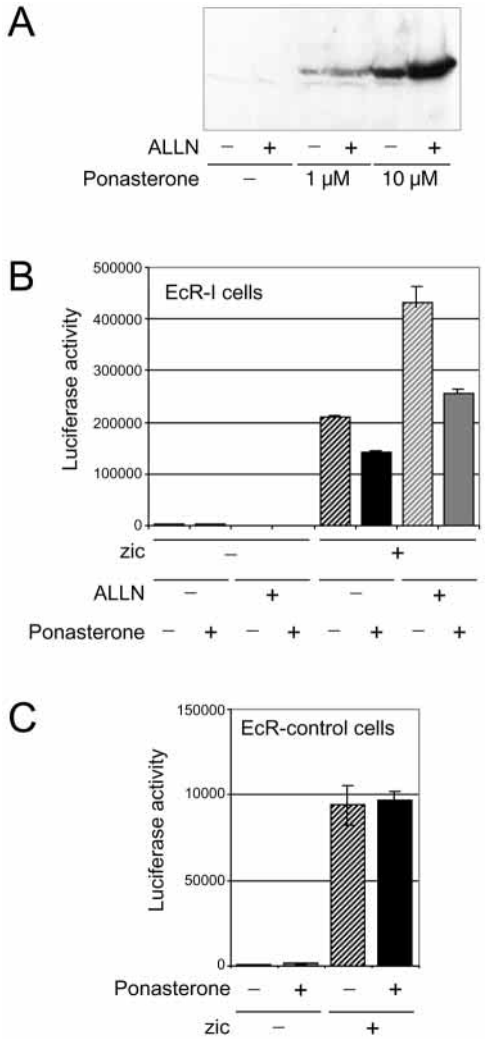

Fig. 4. Teneurin-2 attenuates the transcriptional activity of zic. (A) Expression of teneurin-2 I could be induced by addition of increasing concentrations of ponasterone in EcR-293 cells stably transfected with the teneurin-2 construct I, and the addition of ALLN resulted in the stabilization of the teneurin-2 I protein. (B) Transcriptional activity of zic was studied by transfecting pXP2-*APOE*189 alone (– zic) or together with a zic-expressing construct (+ zic) into the teneurin-2 I stable transfectant EcR-293-I cells in the absence or presence of proteasome inhibitor ALLN (–/+ ALLN). Luciferase activity was dependent on the presence of zic, and induction of teneurin-2 I by ponasterone resulted in decreased luciferase expression, which was even more pronounced in the presence of ALLN, which is known to stabilize teneurin-2 I. (C) Transcriptional activity of zic after transfecting pXP2-*APOE*189 alone (– zic) or together with a zic expressing construct (+ zic) into EcR-control cells. Addition of ponasterone had no effect on the resulting luciferase activity measured.

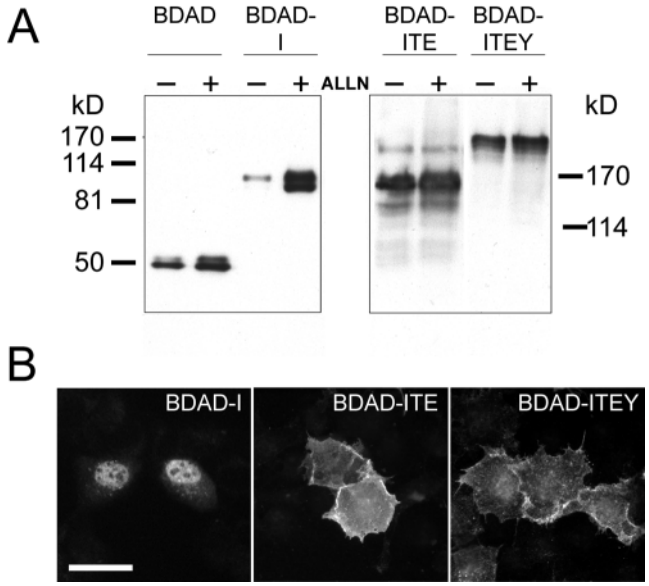

Fig. 5. Expression of fusion proteins between teneurin-2 and the recombinant transcription factor BDAD. Western blot analysis (A) and immunofluorescence staining (B) of BDAD and three different BDAD-teneurin-2 fusion constructs in HT1080 cells using anti-Gal4 antibodies. (A) Transient transfection with constructs pBD-NFκB expressing BDAD, BDAD-I, BDAD-ITE and BDAD-ITEY in the presence (+) or absence (–) of ALLN, as indicated above the lanes. (B) Immunofluorescence analysis of permeabilized cells transfected with BDAD-I and of non-permeabilized cells with either BDAD-ITE or BDAD-ITEY using anti-Gal4 to detect BDAD-I and anti-teneurin-2 for BDAD-ITE and BDAD-ITEY. Staining of BDAD-ITE- or BDAD-ITEY-transfected cells with anti-Gal4 after permeabilization resulted in addition to the cell surface staining in ER and Golgi staining, but no nuclear staining could be detected (data not shown). BDAD-I did not accumulate in PML bodies but was diffusely distributed throughout the nucleus and was strongly protected from degradation in the presence of the proteasome inhibitor ALLN. Bar, 20 μm.

induction of luciferase activity of BDAD-ITEY in TEY cells compared with BDAD-IT-transfected cells. When we did the same experiment comparing transfection of BDAD-ITEY with BDAD-IT into TE cells versus HT control cells we did not see any difference between the two transfected constructs and the fold induction remained 1.0 (Fig. 6C; Table 1). Each of these experiments was repeated five times with consistent results (Table 1). We, therefore, conclude that interactions through the C-terminal half of the extracellular domain are able to stimulate the release of the intracellular domain of teneurin-2, as indicated in the model presented in Fig. 6C.

The induction of luciferase activity following transfection of BDAD-ITEY could again be markedly upregulated by the addition of protease inhibitors, such as ALLN and lactacystin (Fig. 7A). Thus, also the cleaved intracellular domain is subject to rapid degradation by the proteasome pathway. This was confirmed when the ITE protein of transfected cells was analysed on a western blot. The addition of ALLN led to the stabilization of two particular cleavage products, of which one matched the size of the entire intracellular domain and one was a fragment thereof (Fig. 7B, arrow and arrowhead).

Taken together, we conclude that the activity of the luciferase reporter gene originated from cleavage of the BDAD-teneurin-2 fusion proteins at (or in the vicinity of) the membrane. However, cleavage of full-length teneurin-2 led to a significant induction of the luciferase gene only when processing was upregulated by homophilic binding of the extracellular C-terminal part of teneurin-2. Furthermore, the cleaved intracellular domain is subject to rapid degradation by the proteasome pathway.

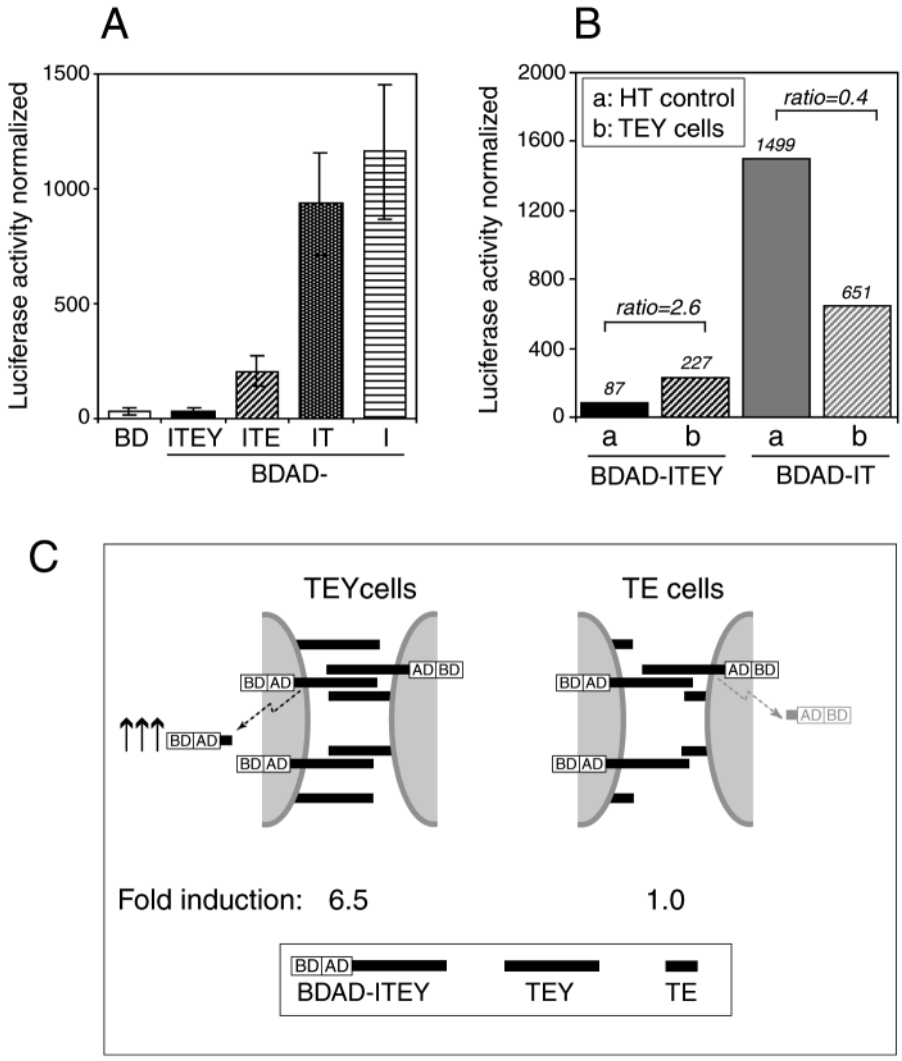

Fig. 6. The intracellular domain of teneurin-2 is released from the cell membrane. (A) Detection of nuclear activity of transmembrane BDAD-teneurin-2 fusion proteins by induction of a luciferase reporter gene. HT1080 cells transfected with various BDAD-teneurin-2 fusion constructs and a construct expressing only BD (negative control) were analysed for luciferase activity of the co-transfected luciferase reporter plasmid. (B) Luciferase activity obtained by transfection of BDAD-ITEY (left bars) or BDAD-IT (right bars) into HT1080 control cells (bars a, HT-control) or cells stably expressing TEY (bars b, TEY cells). The ratio of the values obtained for each construct in TEY cells versus the values obtained in HT-control cells is given above the bars. (C) A model is proposed for the activation of the release of the intracellular domain by homophilic interaction between the C-terminal parts of the teneurin-2 extracellular domains on the basis of the data presented in this figure and in Table 1, showing a 6.5-fold induction of luciferase activity of BDAD-ITEY/BDAD-IT in TEY cells, whereas no difference is obtained when the same experiments are performed in TE cells (data of Table 1).

Table 1. Induction of the release of the intracellular domain of transfected teneurin-2 constructs in cells constitutively overexpressing the entire extracellular domain

Cell lines compared	Transfected construct	Ratio of luciferase activity	Induction of BDAD-ITEY/ BDAD-IT
TEY/HTcontrol	BDAD-ITEY	2.9±0.2	
TEY/HTcontrol	BDAD-IT	0.5±0.1	5.8
TE/HTcontrol	BDAD-ITEY	2.4±0.1	
TE/HTcontrol	BDAD-IT	2.3±0.5	1.0

The data presented are the average of five independent experiments for each condition.

Discussion

In this paper we show that teneurin-2 is a membrane-bound transcription regulator. This could explain the pair-rule phenotype of ten-m mutants in *Drosophila*, where ten-m was shown to be epistatic to opa and to regulate the expression of downstream target genes such as *prd*, *slp* or *gsb* (Baumgartner et al., 1994). The authors postulated that ten-m may influence the activity of opa. For this reason we investigated whether such an interaction could be shown for the respective vertebrate homologues. The zinc finger transcription factors zic-1 to -4 have been proposed to be the vertebrate homologues of *opa* (Aruga et al., 1996). It was shown that zic-1 and -2 are able to activate transcription from the apolipoprotein E promoter (Salero et al., 2001). We therefore tested whether teneurin-2 would influence the activation by zic-1 of a luciferase gene controlled by the ApoE promoter. We found that teneurin-2 repressed the zic-1 activity. At the same time the presence of zic-1 influenced the subnuclear localization of teneurin-2. Although teneurin-2 alone was present in PML nuclear bodies it became diffusely distributed in the nucleus in the presence of zic-1. This diffuse distribution coincided with a rapid degradation of teneurin-2. Thus on the one hand zic-1 causes downregulation of teneurin-2 expression and on the other hand teneurin-2 reduced zic-1-mediated transcriptional activation of the apolipoprotein E promotor.

The genetics in *Drosophila* imply that ten-m and opa interact to induce the transcription of the same downstream target genes (Baumgartner et al., 1994). Furthermore it is known that opa is expressed throughout the segments and ten-m only in the part of the segment where the downstream genes are induced (Baumgartner et al., 1994). On the basis of our results we

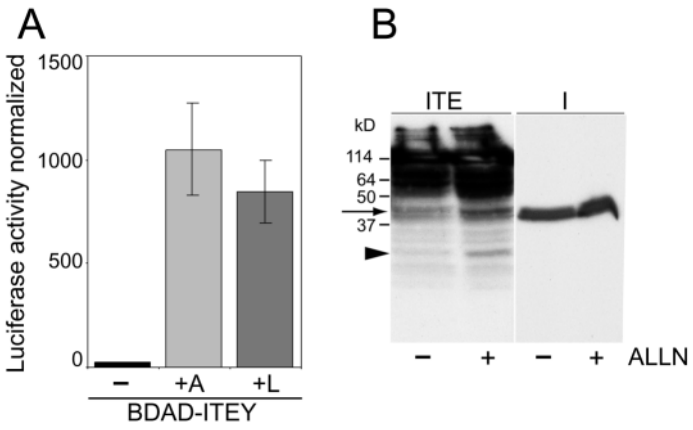

Fig. 7. Proteasome inhibitors stabilize the cleaved intracellular domain of teneurin-2. (A) Comparison of luciferase activities induced by transfection of BDAD-teneurin-2 (BDAD-ITEY) in the absence (–) or presence of protease inhibitors (A, ALLN; L, lactacystin). (B) Western blot analysis of ITE- and I-transfected COS-7 cells showing cleavage products of ITE, which were stabilized by ALLN. Each lane was loaded with the same amount of cell extracts from parallel cultures treated or not treated with ALLN to ensure equal protein loading. Proteins were detected by anti-VSV antibodies. One of the stabilized cleavage products corresponded to the size of the entire intracellular domain (arrow) and another one to a smaller fragment (arrowhead).

therefore speculate that opa could act as a transcriptional repressor and that the repressor function is interfered with by ten-m. Thus, both ten-m and opa are required to determine localized transcription of their target genes in segmental stripes. Also, in vertebrates, functional interaction in vivo of zic proteins with teneurin family members is not unlikely, since they may be co-expressed in many tissues and both appear to be involved not only in regulating neuronal development but possibly also limb pattern formation (Aruga et al., 2002a; Rubin et al., 2002; Nagai et al., 1997; Tucker et al., 2001).

For the transmembrane protein teneurin-2 to function as a transcription regulator the release of the intracellular domain is indispensable. To date, two types of proteolytic mechanisms have been shown to account for such a release of the intracellular domains involved in transcription control, namely regulated intramembranous proteolysis RIP by either γ-secretase or S2P (site-2-protease) or regulated ubiquitin/proteasome-dependent processing RUP (Hoppe et al., 2001). In the case of teneurin-2, RUP is unlikely to be responsible for the cleavage since proteasome inhibitors enhance the presence of the cleaved intracellular domain. However, levels of the intracellular domain of teneurin-2 seem to be tightly controlled by degradation through the proteasome pathway. Under conditions where the intracellular domain of teneurin-2 is localized in PML bodies the teneurin-2 protein is stable. In contrast, conditions leading to a diffuse nuclear expression, as is the case for the teneurin-2 I BDAD fusion protein or the soluble intracellular domain in the presence of zic, result in the degradation of the protein, which can be inhibited by the proteasome inhibitors ALLN and lactacystin. These findings are intriguing in the light of recent discoveries concerning the regulation of transcription by ubiquitin, which causes a rapid turnover of the ubiquitinylated transcription factors (Molinari et al., 1999; Salghetti et al., 2001; Conaway et al., 2002). Interestingly, one of the first proteins discovered to function through RIP, SREBP, is also subject to rapid degradation by the ubiquitin-proteasome pathway (Hirano et al., 2001). The same is true for Notch, which is the most intensely studied example of a membrane-anchored transcription factor functioning through RIP. Cleaved fragments of Notch could not be identified at first owing to rapid downregulation in proteasomes simultaneously with a low sensitivity in the detection methods (Chan and Jan, 1998; Annaert and De Strooper, 1999; Kopan and Goate, 2000; Schroeter et al., 1998). The same problem might be responsible for our inability to directly detect the cleaved intracellular domain of teneurin-2 in the nucleus by immunohistochemistry.

Cleavage of Notch is induced by interaction with its heterophilic ligand Delta (for a review, see Artavanis-Tsakonas et al., 1999). In the case of teneurin-2 we found that homophilic interactions can induce the release of the intracellular domain. This is interesting considering our previous observation that neurons making up functional circuits in the avian visual system express the same type of teneurin molecule (Rubin et al., 2002). Therefore, promotion of the release of the intracellular domain of teneurin-2 could be the mechanistic basis by which growing axons realize that they have reached a proper target, namely another neuron expressing teneurin-2. Since homophilic interaction leads to only a moderate activation of the release of the intracellular domain, we cannot exclude the possibility that more potent mechanisms exist by interaction with yet-to-be identified heterophilic ligands. The released intracellular domain could then turn on a gene expression program to stabilize the connection to differentiate and to build synapses. In this respect teneurin-2 would counteract the action of zic, which by itself was shown to promote the expansion of neuronal progenitors (Aruga et al., 2002b). This would fit our present observation that transcription from at least one zic target gene, namely *ApoE*, is downregulated by teneurin-2.

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References

Annaert, W. and de Strooper, B. (1999). Presenilins: molecular switches between proteolysis and signal transduction. *Trends Neurosci.* **22**, 439-443.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Aruga, J., Nagai, T., Tokuyama, T., Hayashizaki, Y., Okazaki, Y., Chapman, V. M. and Mikoshiba, K. (1996). The mouse zic gene family. Homologues of the *Drosophila* pair-rule gene odd-paired. *J. Biol. Chem.* **271**, 1043-1047.

Aruga, J., Inoue, T., Hoshino, J. and Mikoshiba, K. (2002a). Zic2 controls cerebellar development in cooperation with Zic1. *J. Neurosci.* **22**, 218-225.

Aruga, J., Tohmonda, T., Homma, S. and Mikoshiba, K. (2002b). Zic1 promotes the expansion of dorsal neural progenitors in spinal cord by inhibiting neuronal differentiation. *Dev. Biol.* **244**, 329-341.

Baumgartner, S. and Chiquet-Ehrismann, R. (1993). Tena, a *Drosophila* gene related to tenascin, shows selective transcript localization. *Mech. Dev*. **40**, 165-176.

Baumgartner, S., Martin, D., Hagios, C. and Chiquet-Ehrismann, R. (1994). Tenm, a *Drosophila* gene related to tenascin, is a new pair-rule gene. *EMBO J*. **13**, 3728-3740.

Ben-Zur, T., Feige, E., Motro, B. and Wides, R. (2000). The mammalian Odz gene family: homologs of a *Drosophila* pair-rule gene with expression implying distinct yet overlapping developmental roles. *Dev. Biol*. **217**, 107-120.

Brown, M. S. and Goldstein, **J. L.** (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331-340.

Brown, M. S., Ye, J., Rawson, R. B. and Goldstein, J. L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* **100**, 391-398.

Chan, Y. M. and Jan, Y. N. (1998). Roles for proteolysis and trafficking in notch maturation and signal transduction. *Cell* **94**, 423-426.

Conaway, R. C., Brower, C. S. and Conaway, J. W. (2002). Emerging roles of ubiquitin in transcription regulation. *Science* **296**, 1254-1258.

Doucas, V., Ishov, A. M., Romo, A., Juguilon, H., Weitzman, M. D., Evans, R. M. and Maul, G. G. (1996). Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. *Genes Dev*. **10**, 196-207.

Dyck, J. A., Maul, G. G., Miller, W. H., Jr, Chen, J. D., Kakizuka, A. and Evans, R. M. (1994). A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell* **76**, 333-343.

Ebinu, J. O. and Yankner, B. A. (2002). A RIP tide in neuronal signal transduction. *Neuron* **34**, 499-502.

Fascetti, N. and Baumgartner, S. (2002). Expression of *Drosophila* Ten-a, a dimeric receptor during embryonic development. *Mech. Dev*. **114**, 197-200.

Feng, K., Zhou, X. H., Oohashi, T., Morgelin, M., Lustig, A., Hirakawa, S., Ninomiya, Y., Engel, J., Rauch, U. and Fassler, R. (2002). All four members of the Ten-m/Odz family of transmembrane proteins form dimers. *J. Biol. Chem*. **277**, 26128-26135.

Gao, Y. and Pimplikar, S. W. (2001). The gamma-secretase-cleaved C-terminal fragment of amyloid precursor protein mediates signaling to the nucleus. *Proc. Natl. Acad. Sci. USA* **98**, 14979-14984.

Haass, C. and de Strooper, B. (1999). The presenilins in Alzheimer's disease–proteolysis holds the key. *Science* **286**, 916-919.

Haze, K., Yoshida, H., Yanagi, H., Yura, T. and Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol. Biol. Cell* **10**, 3787-3799.

Hirano, Y., Yoshida, M., Shimizu, M. and Sato, R. (2001). Direct demonstration of rapid degradation of nuclear sterol regulatory element-binding proteins by the ubiquitin-proteasome pathway. *J. Biol. Chem*. **276**, 36431-36437.

Hoppe, T., Rape, M. and Jentsch, S. (2001). Membrane-bound transcription factors: regulated release by RIP or RUP. *Curr. Opin. Cell Biol*. **13**, 344-348.

Horton, R. M. (1995). PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. *Mol. Biotechnol*. **3**, 93-99.

Kakizuka, A., Miller, W. H., Jr, Umesono, K., Warrell, R. P., Jr, Frankel, S. R., Murty, V. V., Dmitrovsky, E. and Evans, R. M. (1991). Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* **66**, 663-674.

Kidd, S., Lieber, T. and Young, M. W. (1998). Ligand-induced cleavage and regulation of nuclear entry of Notch in *Drosophila melanogaster* embryos. *Genes Dev*. **12**, 3728-3740.

Kopan, R. and Goate, A. (2000). A common enzyme connects notch signaling and Alzheimer's disease. *Genes Dev*. **14**, 2799-2806.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? *Cell* **85**, 951-961.

Lee, H. J., Jung, K. M., Huang, Y. Z., Bennett, L. B., Lee, J. S., Mei, L. and Kim, T. W. (2002), Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4, *J. Biol. Chem*. **277**, 6318-6323.

Levine, A., Bashan-Ahrend, A., Budai-Hadrian, O., Gartenberg, D., Menasherow, S. and Wides, R. (1994). Odd Oz: a novel *Drosophila* pair rule gene. *Cell* **77**, 587-598.

Levine, A., Gartenberg, D., Yakov, R., Lieberman, Y., Budai-Hadrian, O., Bashan-Ahrend, A. and Wides, R. (1997). The genetics and molecular structure of the *Drosophila* pair-rule gene odd Oz (odz) *Gene* **200**, 59-74.

Lin, R. J. and Evans, R. M. (2000). Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. *Mol. Cell* **5**, 821-830.

Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z. et al. (2002). A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J*. **21**, 1948-1956.

Mieda, M., Kikuchi, Y., Hirate, Y., Aoki, M. and Okamoto, H. (1999). Compartmentalized expression of zebrafish ten-m3 and ten-m4, homologues of the *Drosophila* ten(m)/odd Oz gene, in the central nervous system. *Mech. Dev*. **87**, 223-227.

Minet, A. D. and Chiquet-Ehrismann, R. (2000). Phylogenetic analysis of teneurin genes and comparison to the rearrangement hot spot elements of *E. coli*. *Gene* **257**, 87-97.

Minet, A. D., Rubin, B. P., Tucker, R. P., Baumgartner, S. and Chiquet-Ehrismann, R. (1999). Teneurin-1, a vertebrate homologue of the *Drosophila* pair-rule gene ten- m, is a neuronal protein with a novel type of heparin-binding domain. *J. Cell Sci*. **112**, 2019-2032.

Molinari, E., Gilman, M. and Natesan, S. (1999). Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency in vivo. *EMBO J*. **18**, 6439-6447.

Mu, Z. M., Chin, K. V., Liu, J. H., Lozano, G. and Chang, K. S. (1994). PML, a growth suppressor disrupted in acute promyelocytic leukemia. *Mol. Cell. Biol*. **14**, 6858-6867.

Nagai, T., Aruga, J., Takada, S., Gunther, T., Sporle, R., Schughart, K. and Mikoshiba, K. (1997). The expression of the mouse Zic1, Zic2, and Zic3 gene suggests an essential role for Zic genes in body pattern formation. *Dev. Biol*. **182**, 299-313.

Ni, C. Y., Murphy, M. P., Golde, T. E. and Carpenter, G. (2001). γ-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**, 2179-2181.

Niwa, M., Sidrauski, C., Kaufman, R. J. and Walter, P. (1999). A role for presenilin-1 in nuclear accumulation of Ire1 fragments and induction of the mammalian unfolded protein response. *Cell* **99**, 691-702.

Okamoto, I., Kawano, Y., Murakami, D., Sasayama, T., Araki, N., Miki, T., Wong, A. J. and Saya, H. (2001). Proteolytic release of CD44 intracellular domain and its role in the CD44 signaling pathway. *J. Cell Biol*. **155**, 755-762.

Oohashi, T., Zhou, X. H., Feng, K., Richter, B., Morgelin, M., Perez, M. T., Su, W. D., Chiquet-Ehrismann, R., Rauch, U. and Fassler, R. (1999). Mouse ten-m/Odz is a new family of dimeric type II transmembrane proteins expressed in many tissues. *J. Cell Biol*. **145**, 563-577.

Otaki, J. M. and Firestein, S. (1999). Neurestin: putative transmembrane molecule implicated in neuronal development. *Dev. Biol*. **212**, 165-181.

Raggo, C., Rapin, N., Stirling, J., Gobeil, P., Smith-Windsor, E., O'Hare, P. and Misra, V. (2002). Luman, the cellular counterpart of herpes simplex virus VP16, is processed by regulated intramembrane proteolysis. *Mol. Cell. Biol*. **22**, 5639-5649.

Rubin, B. P., Tucker, R. P., Martin, D. and Chiquet-Ehrismann, R. (1999). Teneurins: a novel family of neuronal cell surface proteins in vertebrates, homologous to the *Drosophila* pair-rule gene product Ten-m. *Dev. Biol*. **216**, 195-209.

Rubin, B. P., Tucker, R. P., Brown-Luedi, M., Martin, D. and Chiquet-Ehrismann, R. (2002). Teneurin-2 is expressed by the neurons of the thalamofugal visual system in situ and promotes homophilic cell-cell adhesion in vitro. *Development* **129**, 4697-4705.

Salero, E., Perez-Sen, R., Aruga, J., Gimenez, C. and Zafra, F. (2001). Transcription factors Zic1 and Zic2 bind and transactivate the apolipoprotein E gene promoter. *J. Biol. Chem*. **276**, 1881-1888.

Salghetti, S. E., Caudy, A. A., Chenoweth, J. G. and Tansey, W. P. (2001). Regulation of transcriptional activation domain function by ubiquitin. *Science* **293**, 1651-1653.

Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-386.

Seeler, J. S. and Dejean, A. (1999). The PML nuclear bodies: actors or extras? *Curr. Opin. Genet. Dev*. **9**, 362-367.

Struhl, G. and Adachi, A. (1998). Nuclear access and action of notch in vivo. *Cell* **93**, 649-660.

Tucker, R. P., Chiquet-Ehrismann, R., Chevron, M. P., Martin, D., Hall, R. J. and Rubin, B. P. (2001). Teneurin-2 is expressed in tissues that regulate limb and somite pattern formation and is induced in vitro and in situ by FGF8. *Dev. Dyn*. **220**, 27-39.

Tucker, R. P., Martin, D., Kos, R. and Chiquet-Ehrismann, R. (2000). The expression of teneurin-4 in the avian embryo. *Mech. Dev*. **98**, 187-191.

Yokota, N., Aruga, J., Takai, S., Yamada, K., Hamazaki, M., Iwase, T., Sugimura, H. and Mikoshiba, K. (1996). Predominant expression of human zic in cerebellar granule cell lineage and medulloblastoma. *Cancer Res*. **56**, 377-383.

Zhong, S., Salomoni, P. and Pandolfi, P. P. (2000). The transcriptional role of PML and the nuclear body. *Nat. Cell Biol*. **2**, E85-E90.