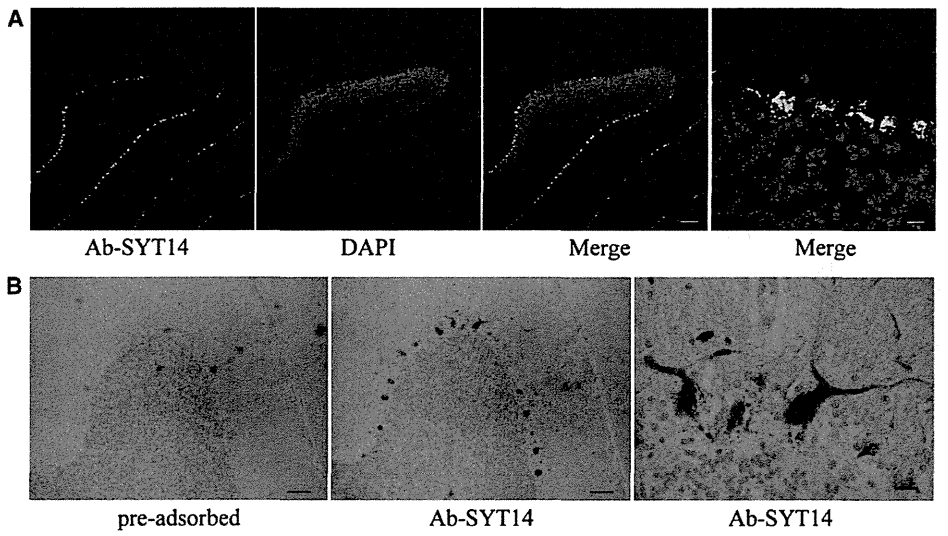


**Figure 3. Selective Localization of SYT14 in Purkinje Cells of the Cerebellum in Mice and Humans**
(A) Immunohistochemical analysis with the Ab-SYT14 antibody of the cerebellum from an adult mouse at 12 weeks of age. Nuclei were stained with DAPI (the scale bar represents 100 μm). A magnified image is shown in the first right panel (the scale bar represents 10 μm).The Ab-SYT14 antibody (0.9 mg/dl) was used at a dilution ration of 1:2000, and the Alexa-488-conjugated secondary antibody dilution was 1:1000.
(B) Immunohistochemical analysis with the Ab-SYT14 antibody of the cerebellum from the human control. Ab-SYT14 antibodies were preincubated with (left panel) or without (middle panel) peptide antigen before immunostaining. Nuclei were stained with hematoxylin (scale bars represent 100 μm). A magnified image is shown in the right panel (the scale bar represents 20 μm). The Ab-SYT14 antibody (0.9 mg/dl) was used at a dilution of 1:500.

performed with Ab-SYT14, as previously described.[19–21] Mouse brain sections were prepared at the RIKEN Brain Science Institute. Mouse experimental protocols were approved by the animal experiment committee of the RIKEN Brain Science Institute. The frozen brain of C57BL/6J mouse was mounted in Tissue-Tek and sliced to 10 μm sections with a freezing microtome. A human adult brain specimen was obtained through the postmortem examination of a brain from a control subject without neurodegenerative disorders. Informed consent was obtained from the family on the basis of the IRB-approved protocol of Yokohama City University School of Medicine. The human brain was fixed in 10% formalin and cut into 1-cm-thick slices. Sliced tissues were embedded in paraffin wax, and 5 μm sections were immunostained with primary antibodies and visualized with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Selective localization of SYT14/Syt14 in Purkinje cells of the mouse cerebellum (Figure 3A) and human cerebellum (Figure 3B) were recognized, indicating that SYT14 plays an important role in the cerebellum. These data are in agreement with a scenario in which the *SYT14* mutation causes cerebellar degeneration in this family.

In this study, only one p.Gly484Asp mutation of *SYT14* was identified in association with SCA. Quintero-Rivera et al.[16] previously described a 12-year-old female with cerebral atrophy, absence seizures, developmental delay with a WISC III score of 58 for full IQ, and de novo t(1;3)(q32.1;q25.1) disrupting *SYT14*. Her brain MRI showed diffuse cerebral atrophy, including that of the cerebellar hemisphere and vermis. Although the inheritance modes are different (recessive impact on our family and dominant on the female patient), mild to moderate mental retardation and cerebellar atrophy are common among patients with *SYT14* abnormalities. It will be important to assess the future phenotype of the female patient studied by Quintero-Rivera et al.[16]

Relatively common ARCAs in Japan include ataxia, early-onset; oculomotor apraxia, hypoalbuminemia/ataxia-oculomotor apraxia 1 (EAOH/AOA1 [MIM 208920]); ataxia-oculomotor apraxia 2 (AOA2 [MIM 606002]); spastic ataxia; Charlevoix-Saguenay type (SACS [MIM 270550]); ataxia with isolated vitamin E deficiency (AVED [MIM 277460]); and ataxia-telangiectasia (AT [MIM 208900]). (Friedrich ataxia 1 [FRDA (MIM 229300)] has never been described in the Japanese population.) In this family, patients never showed ocularmotor apraxia, spasticity, peripheral neuropathy, retinal abnormality, immunological abnormality, or other systemic involvements. As an adult-onset type of pure ARCA, SYNE1-related ARCA (also known as spinocerebellar ataxia, autosomal-recessive 8; SCAR8 [MIM 610743]) is found to be caused by mutations of the gene encoding synaptic nuclear envelope protein 1.[22] Furthermore, these patients were not associated with psychomotor retardation. Thus, SYT14-mutated ARCA, described here, should be categorized to a distinct type of ARCA.

SYTs is a large family of transmembrane proteins associated with exocytosis of secretory vesicles (including synaptic vesicles).[23] The mammalian SYT family is composed

of 17 members. SYTs are anchored to the secretory vesicles via a single transmembrane domain (TM) close to its N terminus and have tandem cytoplasmic domains, C2A and C2B.[24] Among SYTs, SYT1 (MIM 185605) is involved in neurotransmitter release and has been intensively studied. The crystal structure of the C2 domains consists of a compact eight-stranded β-barrel with two protruding loops (loops 1 and 3) that form the $Ca^{2+}$-binding pockets.[25] SYT1 binds three and two $Ca^{2+}$ ions via loops 1 and 3 of C2A and C2B, respectively. $Ca^{2+}$ binding triggers the rapid penetration of the C2 domains into membranes harboring negatively charged phospholipids. $Ca^{2+}$ also promotes SYT1 binding to t-SNAREs (target-membrane-soluble N-ethylmaleimide-sensitive factor attachment protein receptors). SYT1 is a key sensor for evoked and synchronous neurotransmitter release in many classes of neurons.[23] SYT14 also has TM, C2A, and C2B domains, but it has no conserved $Ca^{2+}$-binding motif that includes the conserved aspartic acid residues in loops 1 and 3 of C2A and C2B.[26] Although the roles of SYTs as $Ca^{2+}$ sensors have been studied extensively, little is known about $Ca^{2+}$-independent SYTs, which might inhibit the SNARE-catalyzed fusion in both the absence and presence of $Ca^{2+}$.[27] Recently, Zhang et al.[28] suggested that $Ca^{2+}$-independent SYT4 (MIM 600103) negatively regulates exocytosis, regardless of its inability to induce $Ca^{2+}$-dependent exocytosis.

SYT14 has phospholipid-binding activity that is $Ca^{2+}$ independent.[14] The glycine residue mutated in the family is located around the C2B domain loop 1, which plays an important role in binding to phospholipids in SYT1.[25] We confirmed that, compared to the wild-type, the mutation did not alter the binding activity of SYT14 to phospholipids. In an overexpression system, wild-type SYT14 as well as normal variants were distributed in the cytoplasm close to the plasma membrane, showing in-line accumulation along with the membrane. In contrast, the p.Gly484Asp mutant showed a different (reticular) distribution pattern. In the ER, several cotranslational and posttranslational modifications that are required for the correct folding of transmembrane and secretory proteins take place.[29,30] Incompletely folded proteins are generally excluded from ER exit sites.[29] The fact that the p.Gly484Asp was not properly transferred from the ER suggests that the mutant protein might not fold correctly. The lower yield of the mutant protein as compared to the wild-type in the bacterial expression system we performed also supports the improper folding of the mutant. Abnormal distribution in the ER might result in the loss of function of SYT14 or in ER dysfunction.

In conclusion we have shown that SYT14 is localized specifically in Purkinje cells of mouse and human cerebellum. The results strongly support the involvement of SYT14 in the pathogenesis of SCA and are consistent with the atrophy of the cerebellum seen in both patients. A possible relationship between SYTs and neurodegeneration has been suggested previously,[31] and here we provide data that support the idea that disruption of an SYT protein is involved in human neurodegeneration and that exocytosis machinery can be involved in one of the pathomechanisms of neurodegeneration.

## Supplemental Data

Supplemental Data include two figures and five tables and can be found with this article online at http://www.cell.com/AJHG/.

## Acknowledgments

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## Web Resources

The URLs for data presented herein are as follows:

Align GVGD, http://agvgd.iarc.fr/
Allen Human brain Atlas, http://human.brain-map.org/
Allen Mouse Brain Atlas, http://mouse.brain-map.org/
HomozygosityMapper, http://www.homozygositymapper.org/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
PolyPhen, http://genetics.bwh.harvard.edu/pph/
PolyPhen2, http://genetics.bwh.harvard.edu/pph2/
SIFT, http://blocks.fhcrc.org/sift/SIFT.html

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# Behavioral Alterations in Mice Lacking the Gene for Tenascin-X

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**Tenascin-X (TNX) is the largest member in the tenascin family of large oligomeric glycoproteins of the extracellular matrix (ECM). TNX is expressed in the leptomeningeal trabecula and connective tissue of choroid plexus in the brain as well as in muscular tissues. Interestingly, single nucleotide polymorphism (SNP) analysis in human showed that TNX is significantly associated with schizophrenia. Previously we generated TNX-deficient (TNX−/−) mice by homologous recombination using embryonic stem (ES) cells. In the present study, we analyzed behaviors relevant to affect, learning and memory, and motor control in TNX−/− mice. TNX−/− mice showed increased anxiety in light–dark and open-field tests and superior memory retention in a passive avoidance test. Also, TNX−/− mice displayed higher sensorimotor coordination than did wild-type mice in a rotorod test. However, TNX−/− mice did not differ from wild-type mice in locomotor activity in a home-cage activity test using telemetric monitoring. These findings suggest that TNX has diverse roles including roles in behavioral functions such as anxiety, emotional learning and memory, and sensorimotor ability.**

**Key words** tenascin-X; knockout mouse; behavioral analysis

The tenascin family constitutes a group of extracellular matrix (ECM) glycoproteins with a characteristic structure. Four members of this family [tenascin-C (TNC), tenascin-R (TNR), tenascin-X (TNX) (known as tenascin-Y in birds), and tenascin-W (TNW)] have so far been identified in vertebrates,[1] and they all have a cysteine-rich segment at the amino terminus followed by epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats, and a fibrinogen-like domain at the carboxy terminus.

TNX is the largest member of the tenascin family with a size of about 450 kDa. Complete deficiency of TNX in humans leads to a rare recessive form of Ehlers-Danlos syndrome (EDS), and TNX haploinsufficiency is associated with hypermobility type of EDS.[2—4] There are several lines of evidence suggesting that TNX participates in collagen fibrillogenesis,[5,6] collagen deposition,[7] modulation of collagen stiffness,[8] and development and maintenance of elastic fibers.[9] TNX-deficient (TNX−/−) mice generated by TNX gene targeting in murine embryonic stem (ES) cells showed progressive skin hyperextensibility, similar to individuals with EDS. Biomechanical analyses indicated reduced tensile strength of their skin.[10] Furthermore, TNX−/− mice showed enhanced tumor invasion due to activation of matrix metalloproteinase (MMP)-2 and MMP-9.[11] TNX−/− mice also displayed increased amount of triglyceride and altered composition of triglyceride-associated fatty acids.[12]

TNX is expressed much more widely than other tenascins.[13] TNX is also present in the leptomeningeal trabecula and connective tissue of choroid plexus in the brain[14] as well as in the peripheral nerves[15] and in developing spinal cord meninges.[16] Interestingly, single nucleotide polymorphism (SNP) analysis has revealed a strong association between the *TNX* locus and schizophrenia.[17,18] Previously, we investigated the distribution of TNX in sciatic nerves by immunohistochemical staining.[19] TNX was found to be localized in the perineurium and the endoneurium of sciatic nerve fibers. These results suggested that TNX plays an important role in neural functions.

In the present study, we analyzed behaviors relevant to affect, cognition, and motor control in the TNX−/− mice to better elucidate the role of endogenous TNX.

## MATERIALS AND METHODS

**Animals** TNX−/− mice were generated by TNX gene targeting in ES cells as described previously.[11] TNX−/− mice were further established by backcrossing original TNX−/− mice into a congenic line, C57BL/6J, for 10 generations. Male C57BL/6J mice (CLEA Japan, Tokyo, Japan) were used as wild-type (TNX+/+) mice. The animals were housed in the Department of Experimental Animals, Center for Integral Research in Science, Shimane University at room temperature of 23±2 °C, humidity of 55±10% and ventilation of 10—13 times per hour. The mice were kept on a 12:12 h light–dark schedule (lights on at 7:00 a.m.) with commercial chow (NMF, Oriental Yeast, Tokyo, Japan) and water given *ad libitum*. This study was approved by the Ethical Committee for Animal Research of Shimane University, and all of the experimental procedures were performed according to the institutional guidelines.

**Behavioral Testing** Male mice were tested at 8, 9, 10, 11 weeks of age for light–dark preference, open-field, passive avoidance, and rotorod tests and at 9, 13, 17 weeks of age for a home-cage activity test. All experiments were performed during the light period.

**Light–Dark Preference Test** The light–dark preference test measures the conflict between the tendency to explore a novel environment *versus* the aversive qualities of a lighted space. Longer time spent in the dark side of the apparatus is indicative of increase in anxiety-like behavior. Mice at 8 weeks of age were examined in a light–dark apparatus consisting of a light (illuminated) compartment (10 cm×25 cm×25 cm) with a 100 W bulb (luminescence: 2000 lux) connecting to a dark compartment (30 cm×30 cm×30 cm) separated by a board (Muromachi-Kikai Co., Ltd., Tokyo, Japan). Mice were placed in the center of the light box for 5 min to acclimate to the test environment. Then the separation board was removed and the connecting gate to the dark compartment

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was opened. The trials were each 10 min, and the percentage of cumulative time spent in the light compartment and the total number of transitions between the two compartments were scored live by an experimenter.

**Open-Field Test** The open-field test is used to evaluate exploratory behavior and measures of anxiety by counting small movements such as grooming, large movements such as ambulation, and rearing. Intense anxiety causes mice to suppress grooming, ambulation, and rearing. Each mouse at 10 weeks of age was placed in a 56.5 cm×56.5 cm×6 cm brightly lit open arena (300 lux) equipped with a near-infrared sensor interfaced with a computer (SCANET-MV-10, Toyo-Sangyo Co., Ltd., Okayama, Japan) for 35 min. By the near-infrared sensor, small movements such as grooming were detected at a minimum distance of 6.0 mm (Grooming). A square of 15.0 cm was set, and when mice moved out of the square, the movement was counted as a large locomotion (Ambulation). In addition, the number of rearing movements was counted by detecting movement over a height of 4 cm (Rearing). The apparatus was cleaned with 70% ethanol between trials.

**Rotorod Test** To assess sensorimotor ability, mice at 9 weeks of age were tested using a rotorod apparatus (SN-445, Shinano Manufacturing Co., Ltd., Tokyo, Japan). Each mouse was habituated on the rotating rod (3.0 cm in diameter) for 1 min before testing began. The trials were conducted at turn speed of 15 rpm. The number of turns before the mouse fell from the rotating rod was counted. Mice were tested once per day on three consecutive days.

**Passive Avoidance Test** The passive avoidance test was used to examine emotional learning and memory. On day 1 of testing, each mouse at 11 weeks of age was placed in a light compartment connected to a dark compartment separated by a board, the same apparatus as that used in the light–dark preference test (Muromachi-Kikai Co., Ltd.). After 5 min of acclimation, the separation board was removed and the connecting gate to the dark compartment was opened. A mouse preferring the darkened side moves quickly through the gate to the dark compartment. Upon doing so, the mouse received a 0.3 mA electrical shock (3 s in duration) from the grid floor (Shock Generator SGS-002T, Muromachi-Kikai Co., Ltd.). On day 2, the same procedure was used except for removal of the shock. Cumulative time spent in the light compartment was measured.

**Home-Cage Activity Test** Pentobarbital anesthesia (50 mg/kg intraperitoneally (i.p.)) was used for all surgical procedures. A battery-operated free-floating transmitter (model TA10TA-F20, Data Sciences International, St. Paul, MN, U.S.A.) was inserted into the abdominal cavity of the mice at 9, 13, 17 weeks of age. The peritoneal muscle and skin layers were closed with sutures. Immediately after surgery, each mouse was returned to its home cage. Locomotor activity was continuously monitored using the Dataquest A.R.T. system (Data Sciences International). Locomotor activity was obtained by counting the number of impulses, detected by changes in signal strength at 10-min intervals. The signal was received by an antenna under each mouse's cage and transferred to a computer. After 24 h of acclimation, the data of cumulative locomotor activity (counts) during light and dark periods were collected separately every 12 h.

**Statistics** Data were analyzed for statistical significance by Student's *t*-test or ANOVA with *post hoc* by Scheffé's test in six to ten experiments, where $p<0.05$ was considered statistically significant. These analyses were performed using StatView version 4.0 (SAS Institute Inc., Cary, NC, U.S.A.). Results are expressed as means±S.E.

## RESULTS

**TNX−/− Mice Show Increased Anxiety-Like Behavior** In the light–dark preference test, TNX−/− mice spent less time in the light compartment ($p<0.01$) (Fig. 1A) and moved less ($p<0.01$) (Fig. 1B). These results indicated that TNX−/− mice show more anxiety-like behavior than do wild-type mice.

Consistent with the increased anxiety-like behavior in the light–dark preference test, increased anxiety-like behavior in TNX−/− mice was also revealed in the open-field test. Small movements such as grooming ($p<0.01$) (Fig. 2A) and large movements such as ambulation ($p<0.05$) (Fig. 2B) as well as rearing ($p<0.01$) (Fig. 2C) in TNX−/− mice were significantly less frequent than those in wild-type mice.

**TNX−/− Mice Display Superior Sensorimotor Coordination and Emotional Learning and Memory** TNX−/− mice were superior to wild-type mice in the rotorod test as indicated by longer latency to fall from the rotorod. This tendency was strengthened as the number of trials increased ($p<0.05$) (Fig. 3A). The results indicated that TNX−/− mice have ability superior to sensorimotor coordination (Fig. 3B).

In the passive avoidance test of learning and memory, TNX−/− mice showed higher latency than did wild-type mice to re-enter the dark compartment ($p<0.01$) (Fig. 4).

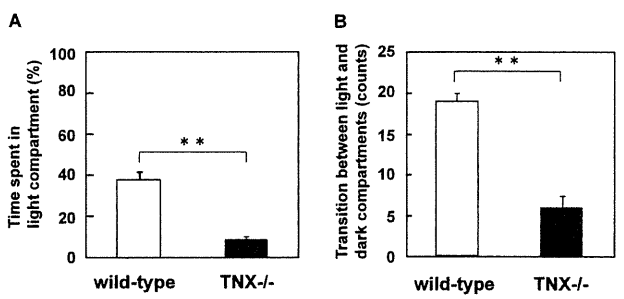


Fig. 1. Light–Dark Preference Test in TNX−/− and Wild-Type Mice

(A) Percentage of cumulative time spent in the light compartment during a period of 10 min. (B) Cumulative light and dark compartment transitions of mice during a period of 10 min. Data represent means±S.E.. $**p<0.01$ *versus* age-matched C57BL/6J wild-type mice, Student's *t*-test. $n=10$ mice per genotype.

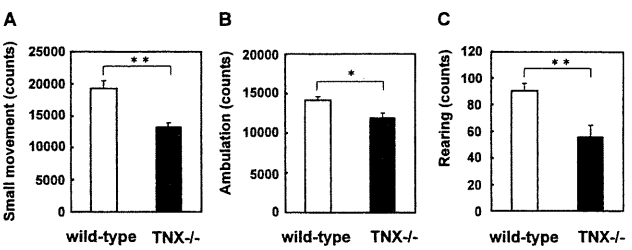


Fig. 2. Open-Field Test in TNX−/− and Wild-Type Mice

(A) Small movement. (B) Ambulation. (C) Rearing. Counts were done every 35 min. Data represent means±S.E. $**p<0.01$ and $*p<0.05$ *versus* age-matched C57BL/6J wild-type mice, Student's *t*-test. $n=10$ mice per genotype.

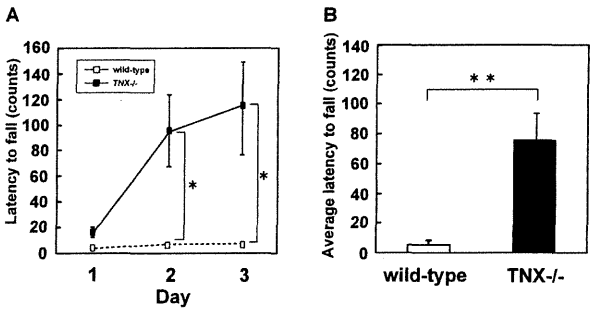


Fig. 3. Rotorod Test in TNX−/− and Wild-Type Mice

(A) Number of turns before mice fell from the rotating rod. Mice were tested once per day on three consecutive days. $*p<0.05$ *versus* age-matched C57BL/6J wild-type mice, Scheffé's test. (B) Average number of turns for three days. Data represent means±S.E. $**p<0.01$ *versus* age-matched C57BL/6J wild-type mice, Student's *t*-test. $n=10$ mice per genotype.

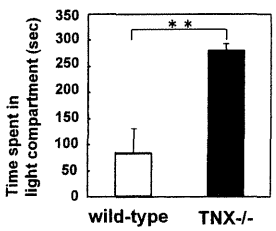


Fig. 4. Passive Avoidance Test in TNX−/− and Wild-Type Mice

After 24 h from electrical shock to mice, cumulative time spent in the light compartment is shown. $**p<0.01$ *versus* age-matched C57BL/6J wild-type mice, Student's *t*-test. $n=6$—10 mice per genotype.

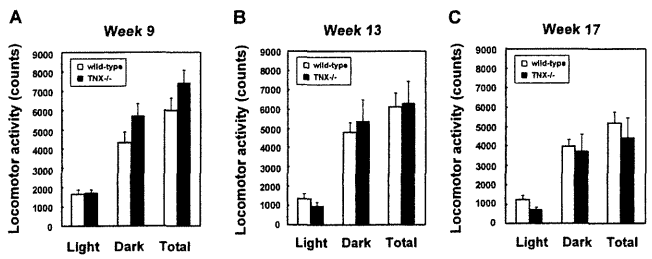


Fig. 5. Home-Cage Activity Test in TNX−/− and Wild-Type (C57BL/6J) Mice

Locomotor activities of free moving mice were counted using telemetry and a data acquisition system in the light and dark periods at the ages of 9 weeks (A), 13 weeks (B), and 17 weeks (C) for 12 h. Note that there is no genotype difference in locomotor activity at any age. $n=9$—10 mice per genotype.

This result indicated that TNX−/− mice have superior passive avoidance memory retention.

**Home-Cage Activity Test** To assess diurnal locomotor activity in free moving TNX−/− and wild-type mice, telemetry and a data acquisition system were used. All of the mice used for this test appeared lively throughout the study, and we observed no behavioral differences compared with mice without transmitters. In consideration of circadian rhythms in locomotor activity, locomotor activities were separately recorded during the light period (07:00 to 19:00 h) and during the dark period (19:00 to 07:00 h). Figure 5 shows the locomotor activity at indicated ages. Although circadian rhythms in locomotor activity were observed in both genotypes (low during the light period and high during the dark period), there was no difference in locomotor activity between the two genotypes at any age.

## DISCUSSION

Our data indicated that TNX deficiency alters measure of anxiety. TNX−/− mice showed hypoactivity and increased anxiety-like behavior in the two anxiety tests used (light–dark preference and open-field tests). The knockout mice also displayed superior emotional learning and memory in the passive avoidance test and superior sensorimotor ability in the rotorod test. However, no difference in diurnal locomotor activity was detected in TNX−/− mice in the home-cage test.

Among the tenascin family members, TNC has been reported to influence cerebellar granule cell migration and guide postnatal granule cell neurons from the external to the internal cell layer in the cerebellum.[20] Behavioral abnormalities such as hyperlocomotion have been observed in TNC−/− mice due to a decreased level of dopamine transmission in the brain.[21,22] Furthermore, TNR plays an important role in neurite outgrowth, axon targeting, neural cell adhesion, and migration and differentiation during nervous morphogenesis in the central nervous system.[23] TNR−/− mice showed alterations of the extracellular matrix and decreased axonal conduction velocities in the central nervous system.[24] On the other hand, although the localization of TNX in the cerebral cortex has not been disclosed, TNX is localized in the leptomeningeal trabecula and in the connective tissue of the choroid plexus in the brain.[14] In the peripheral nervous system, TNX is localized in the perineurium and endoneurium of nerve fibers.[19] Although we previously showed that individual axons in the sciatic nerves of TNX−/− mice do not differ from those of wild-type mice in ultrastructure,[19] morphological and biochemical analyses of the brains of TNX−/− mice have not been done. Interestingly, SNP analysis in human showed that TNX is significantly associated with schizophrenia.[17,18] This evidence indicates an important role of TNX in the central nervous system.

It is known that a hyperlocomotive and anxiolytic-like phenotype is characteristic of rodent models of schizophrenia[25] and could correspond to psychomotor agitation present in schizophrenic patients. In contrast to the behaviors exhibited in schizophrenia model animals, TNX−/− mice showed reverse phenotypes such as increased hypoactivity and anxiety-like behavior. SNP analysis in schizophrenia patients suggested an important non-synonymous substitution such as Glu2578Gly located in exon 23 of *TNX*.[17,18] Thus, such point mutation in *TNX* might be necessary for a factor of schizophrenic illness rather than the null mutation in the TNX-deficient mice. In TNX−/− mice, some abnormalities such as collagen deposition alteration,[10] enhanced activation of MMP,[12,26] triglyceride accumulation and altered composition of triglyceride-associated fatty acids,[12] have been reported. The behavior alterations in TNX−/− mice in this paper might appear as comprehensive outcome of some abnormalities to be seen in TNX−/− mice. Further biochemical and morphological studies are needed to elucidate the relationship between schizophrenia and function of TNX.

In conclusion, this study suggests an important role for TNX in anxiety-like behavior, emotional learning and memory, and sensorimotor ability. Future biochemical and pharmacological studies should be done to reveal the precise

mechanism underlying the effects of TNX on behavior. Morphological and biochemical analyses of the brains of TNX−/− mice would be also useful for this purpose.

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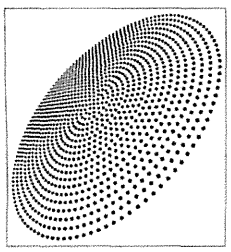

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# エーラス・ダンロス症候群の原因遺伝子の1つのテネイシンX

松本健一*

## はじめに

1980年代に細胞外マトリックス(ECM)の1つであるテネイシン(後のテネイシンC(TNC))は，サイトタクチンやヘキサブラキオン等の名前で，筋腱接合部，脳，乳腺などから同定された．その後1990年代に入りタンパク質のドメイン構造がTNCと共通のテネイシン様分子が相次いで同定された．TNC抗体との反応性によりテネイシンR(TNR／レストリクチン／J1-160/180)が，主要組織適合性抗原複合体(MHC)のクラスIII領域のゲノム解析よりテネイシンX(TNX／TNY)が，最後にEST解析よりテネイシンW(TNW／TNN)がクローニングされ，テネイシンファミリーが明らかとなった[1]．

我々のグループはTNXの発見当初から関わり[2]，それ以来，TNXの機能解明を目指して研究を行ってきた．その後1997年にBurchら[3]により，TNX遺伝子(*TNXB*)が，エーラス・ダンロス症候群(EDS)の原因遺伝子の1つとして同定された．本稿においては，TNX欠損によるEDSに関して説明し，これまでに明らかとなっているTNXの機能に関して概説する．

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## 1. テネイシンファミリーの基本構造と分子進化

TNファミリーの中で，TNXはもっとも分子量が大きいメンバーで，分子量約450 kDaを持つ．TNファミリーの各メンバーの基本構造は，N末端からC末端にかけて，システインに富む領域，ヘプタッド反復配列，EGF様反復配列，フィブロネクチンIII(FNIII)様反復配列，フィブリノーゲン(FG)様配列からなる共通のドメイン構造からなる(図1)．また，各メンバーは，FNIII様反復配列において，選択的スプライシングが報告されている．これまでに同定されたヒト，マウス，ニワトリのTNファミリー各メンバーのFG様配列のアミノ酸配列を基に，クラスタルWを用いて分子系統樹を作成した(図2)．その結果，同じメンバーに属するオーソログは，1つのクラスターを形成し，規則正しく種分化に従って分岐したことが明らかとなった．この分子系統樹より，原始テネイシンからまずTNRが分岐し，その後TNCとTNWとTNXが分岐したことが明らかとなった．

## 2. TNXの発現様式と反接着活性

TNXは，心臓や骨格筋などの筋組織で特に発現が高く見られるが，体全体にわたって発現が観察される．皮膚においては，真皮や皮下組

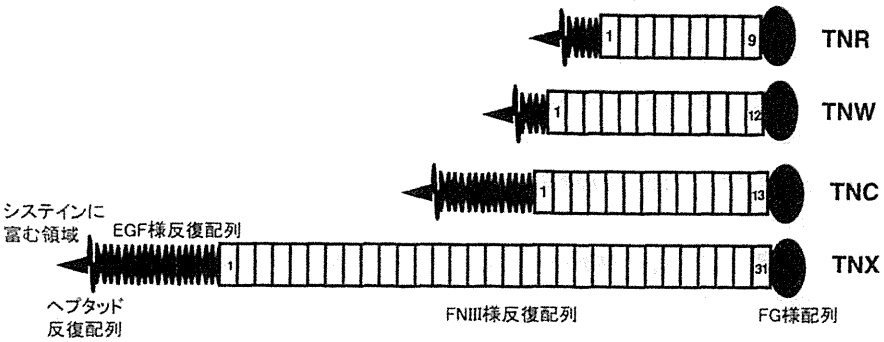


**図1　テネイシンファミリー**

マウスのテネイシンファミリーの各メンバーの特徴であるドメイン構造(N末端からC末端にかけて，システインに富む領域，ヘプタッド反復配列，EGF様反復配列，FNIII様反復配列，FG様配列)を示す．以下に，参考にした配列データベースのアクセションNo.を示す．
TNC, DDBJ No. D90343; TNR, GenBank No. NM_022312; TNW, DDBJ No. AF455756; TNX, DDBJ No. AB010266.

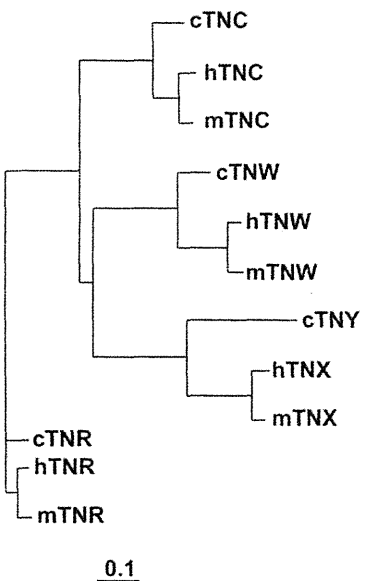


**図2　テネイシンファミリーの各メンバーの分子系統樹**

ヒト(h)，マウス(m)，ニワトリ(c)における，テネイシンファミリーの各メンバーのFG様配列を用いた，クラスタルW解析(http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja)により，分子系統樹を作成した．枝の長さは，進化的距離を表す．cTNYは，ニワトリのTNXに相当する．以下に，参考にした配列データベースのアクセションNo.を示す．
hTNC, DDBJ No. X56160; mTNC, DDBJ No. D90343; cTNC, DDBJ No. M23121; hTNR, DDBJ No. X98085; mTNR, GenBank No. NM_022312; cTNR, DDBJ No. X64649; hTNW, GenBank No. NM_022093; mTNW, DDBJ No. AJ580920; cTNW, DDBJ No. AM231718; hTNX, DDBJ No. X71937; mTNX, DDBJ No. AB010266; cTNY, DDBJ No. X99062.

織にその発現が見られる．またTNXとTNCの局在パターンは相補的で，特に皮膚や消化器系の組織でその傾向が強く見られる[4]．また，興味深いことに，ヒト神経膠腫においては，がんの悪性度が増すに従ってTNXの発現が減少する[5]．

またTNXは反接着活性を示す．その反接着活性にはp38 MAPキナーゼを介するシグナル伝達系が関与している[6]．なお，反接着活性はほかのテネイシンファミリーのメンバーにも共通に見られる特性である．

## 3．TNX欠損による Ⅰ型(古典型)EDSの発症

EDSは，主に，皮膚過進展，関節可動亢進，易出血性等の症状が見られ，皮膚や血管や関節等の結合組織に異常をきたす疾患である．症状の特徴から，主に6つの型の，Ⅰ型・Ⅱ型(古典型)，Ⅲ型(関節可動亢進型)，Ⅳ型(血管型)，Ⅵ型(後側彎型)，ⅦA型・ⅦB型(多発性関節弛緩型)，ⅦC型(皮膚弛緩型)に大別され，症状やその程度は型や個人により異なる[7](参照URL 日本エーラスダンロス症候群協会(友の会): http://ehlersdanlos-jp.net/)．また，原因遺伝子もすでに明らかにされ，Ⅰ型・Ⅱ型EDSはⅤ型コラーゲン遺伝子(*COL5A1*，*COL5A2*)やⅠ型コラーゲン遺伝子(*COL1A1*)が，Ⅳ型

EDSはIII型コラーゲン遺伝子(*COL3A1*)が，VI型EDSはコラーゲン修飾酵素の1つであるリシルヒドロキシラーゼ-1遺伝子(*PLOD1*)が，VIIA型・VIIB型EDSはI型コラーゲン遺伝子(*COL1A1*，*COL1A2*)が，VIIC型EDSはコラーゲン修飾酵素の1つであるプロコラーゲン-1-N-プロテイナーゼ遺伝子(*ADAMTS2*)が原因遺伝子として報告されている．なお，III型EDSの原因遺伝子はこれまで同定されていなかった(次章参照)．

1997年にBurchら[3]は，*TNXB*遺伝子が不等交差等により遺伝子重複や欠失が頻発する主要組織適合性抗原複合体(MHC)のクラスIII領域(*RB2-C4B-CYP21A2-TNXB*)(RCCXモジュール)に存在することから[8]，*CYP21A2*の欠損である21水酸化酵素欠損症により先天性副腎過形成(CAH)を発症し，かつ結合組織疾患を併発している患者の有無を調べた．その結果，CAHとI型EDSを同時に発症している1名の患者を見出した．その患者のゲノム解析により，*C4B*遺伝子，*CYP21A2*遺伝子，*TNXB*遺伝子の3'領域(29番目のFNIII様配列からC末端まで)を含む約30kbに及ぶ欠失変異があることが明らかとなった．なお，本稿では，ヒトとマウスのTNXのEGF様反復配列およびFNIII様反復配列の番号は，Ikutaら[9]の論文に基づく．その後，2001年にSchalkwijkら[10]により，5名の新たなTNX欠損I型EDS患者が見出された．これらの患者の*TNXB*遺伝子の解析より，*TNXB*遺伝子の3'領域の欠失ホモ変異のほか，*TNXB*遺伝子の第17番目のEGF様配列内，第5番目のFNIII様配列内でのホモ変異が明らかとなった．これらのホモ変異は，TNXのC末端の欠失体の生成や，フレームシフト後のナンセンス変異を引き起こすことが明らかとなった．以上のことから，TNX欠損I型EDSの遺伝様式は，常染色体劣性遺伝であることが明らかとなった．これまでに，15名程度のTNX欠損I型EDS患者が報告されている．またKoppensら[11]の解析により，CAH患者のうち約10%の患者において遺伝子欠失領域が*TNXB*遺伝子に及び，CAHのほかにTNX欠損I型EDSを併発する可能性が指摘されている．

TNX欠損I型EDS患者は，関節可動亢進，皮膚過進展，易出血性等の，これまでのI型EDS(*COL1A1*，*COL5A1*，*COL5A2*が原因遺伝子)と共通の臨床所見を示す一方，萎縮性瘢痕の欠如，正常な創傷治癒等のこれまでのI型EDS患者においては見られない所見も示す．その他，関節脱臼，僧帽弁逸脱，慢性閉塞性肺疾患(COPD)，多発性憩室症等の症例も報告されている[12]．

さらに，TNX欠損EDS患者の皮膚の電子顕微鏡解析により，コラーゲン線維の密度低下や配列の不均一性が指摘されている．しかしながら，これまでのI型EDS患者の皮膚でよく観察されるカリフラワー様コラーゲン線維は，TNX欠損I型EDS患者の皮膚においては観察されていない[13]．また，TNX欠損I型EDS患者のエラスチン線維は断片化され，ミクロフィブリル欠損のエラスチン線維の存在も明らかとなっている[14]．

## 4. TNXハプロ不全による III型(関節可動亢進型)EDSの発症

III型EDSの原因遺伝子は明らかになっていなかったが，2003年にZweersら[15]により，*TNXB*遺伝子のヘテロ変異によるハプロ不全によるIII型EDSの発症が報告され，初めてIII型EDSの原因遺伝子が明らかにされた．これまでに報告された変異は，*TNXB*遺伝子の3'領域(29番目のFNIII様配列からC末端まで)のヘテロ欠失や第17番目EGF様配列内，第5番目FNIII様配列内でのフレームシフト後のヘテロナンセンス変異である．さらには，システインに富む領域内，第5番目のFNIII様配列内，33番目のFNIII様配列内でのヘテロミスセンス変異によるIII型EDSの発症も報告されている[16]．また，TNXのハプロ不全が見られる約20人の患者のうち，約45%の患者がIII型EDSを発症し，その発症は女性が圧倒的に多いことが明らかとなった．また，他のコフォート研究においては，80人のIII型EDS患者(すべて女性)のうち，TNXハプロ不全を示す患者は全体の7.5%いた．また，TNXハプロ不全III型EDSは，常染色体

優性の遺伝様式に従うことが明らかとなった.

TNXハプロ不全III型EDS患者においては,関節可動亢進と柔らかいビロード状皮膚が特徴として見られる.また,関節弛緩による亜脱臼や脱臼が頻繁に起こり,慢性疲労や疼痛が見られる場合もある[7].また,TNX欠損I型EDSとの相違は,皮膚過進展性や易出血性の欠如があげられる[15].さらには,TNXハプロ不全III型EDS患者の皮膚の電子顕微鏡観察により,エラスチン線維の異常が明らかとなったが,コラーゲン線維の異常は見出されていない[16].なお,III型EDSと類似の所見を示す疾患に,良性家族性過剰運動症候群(BJHS)が知られているが,BJHS患者における*TNXB*遺伝子のヘテロ変異の有無を調べたところ,これまでのところTNXハプロ不全III型EDS患者で同定されているTNXB遺伝子の3'領域の欠失は見つかっていない[17].

## 5. TNXの機能

以上,TNX欠損によるI型EDSの発症とTNXハプロ不全によるIII型EDSの発症について述べた.次にこれまでに報告されているTNXの機能について述べたい.TNXの機能に関する報告の多くは,TNXのコラーゲン線維の形成の制御分子としての機能である.まず始めに,コラーゲン線維間の橋渡し(アダプター)分子としての機能である[18,19].TNXは,コラーゲン線維結合分子であるデコリンやXII型コラーゲンとの結合を介してコラーゲン線維と結合し[20,21],コラーゲン線維間の繋ぎ止めを担い,コラーゲン線維の組織形成に関与する.実際に,EDS様病態を示すTNX欠損マウスにおいては,コラーゲン線維の密度の低下が報告されている[22].2つ目として,TNXが直接コラーゲン線維と結合し[23],コラーゲン線維形成を制御する機能である[24,25].*In vitro*コラーゲン線維形成系にTNXを添加すると,コラーゲン線維形成が重合速度,重合量とも促進することが明らかとなっている.3つ目として,TNXがコラーゲン線維の力学的特性に関与する機能である[26].4つ目として,TNXが,非線維性コラーゲンで細線維の成分であるVI型コラーゲンの発現を制御する機能である[27].このことは,VI型コラーゲン遺伝子(*COL6A1*, *COL6A2*, *COL6A3*)の変異により発症することが知られている,ウールリッヒ型先天性筋ジストロフィー(UCMD)やベスレムミオパチー(BM)の症状とEDSの症状が一部オーバーラップしていることと関連があるのかもしれない[28].

また,TNXの結合分子として血管内皮増殖因子(VEGF)が同定され,TNXとVEGF-Bの共存下では,VEGF-Bのみの存在下に比べ,血管内皮細胞の増殖促進が明らかとなっている.このことは,TNXの血管形成への関与を示唆する[29].

## 6. TNX欠損マウスに見られる異常

TNX欠損マウスは,Maoら[22]と我々のグループ[30]により,別々に遺伝子ターゲティングの手法により作成された.Maoら[22]は,生体力学測定により,TNX欠損マウスの皮膚の脆弱性を示した.また,Eggingら[31]は,TNX欠損マウスの皮膚の解析により,エラスチン線維の形態や発現パターンに異常は見られないものの,野生型マウスに比べエラスチン線維の密度の増加を明らかにした.また,TNX欠損マウスの創傷治癒の解析により,創傷治癒部位での破断強度の減少が明らかとなった[32].さらには,Voermansら[33]は,筋原性疾患と遺伝性結合組織疾患との関連から,TNX欠損マウスの筋肉組織を調べたところ,生体力学測定により,野生型マウスに比べて,TNX欠損マウスにおけるマイルドな筋肉の脆弱化とミオパチー様特徴を明らかにした.また,TNX欠損マウスの座骨神経の神経内膜のコラーゲン線維の密度を野生型マウスのそれと比較したところ,マイルドな減少が明らかとなった.このことは,TNX欠損I型EDS患者に見られる,多発ニューロパチーと関連があるのかもしれない[34].

そのほかに,我々の研究室において,これまでにTNX欠損マウスの解析により明らかにしたいくつかを紹介する.前章で述べたように,TNX欠損マウスにおいてはコラーゲン線維形

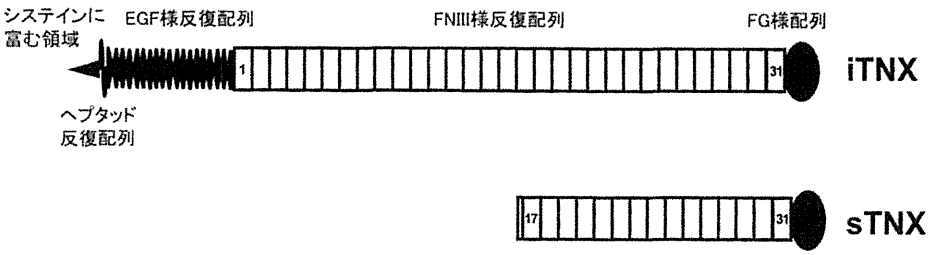


**図3　マウスにおける完全長TNXと血清型TNX**

マウスの場合，完全長TNX(iTNX)は約450 kDaの分子量を持つ．一方，血清型TNX(sTNX)は約200 kDaの分子量を持つ．

成に異常が生じるため，その結果細胞に負荷される張力(メカニカル・ストレス)の変化が考えられる．また，ECMの変異は，がん細胞の増殖，浸潤，転移に深く関与していることが知られている．そのため，B16-BL6メラノーマ細胞を用いて，TNX欠損マウスにおけるがんの増殖，浸潤，転移の解析を行ったところ，野生型マウスに比べTNX欠損マウスでは，原発部位での増殖が1.2倍，浸潤が2.2倍，肺への転移が6.8倍と，有意に高いことが明らかとなった[30]．これは，TNX欠損マウスにおけるマトリックスメタロプロテアーゼ(MMP)(特にMMP-2)の発現亢進によることが明らかとなった[35]．さらに，TNX欠損マウスにおいては，トリクリセリド(TG)の増加による皮下の脂肪層の肥厚が明らかとなった[36]．またTG結合性の脂肪酸組成の変化を調べたところ，TNX欠損マウスにおいて飽和脂肪酸(C16:0)は野生型のそれに比べ減少し，不飽和脂肪酸(C16:1やC18:1)は増加していることが明らかとなった．

## 7. TNXの他の疾患への関与

次にTNXの他の疾患への関与について述べる．一塩基多型(SNP)と疾患との関連を調べるためにゲノムワイドな解析がなされたところ，*TNXB*遺伝子座に位置する2つのSNP(rs1009382，rs204887)と統合失調症との相関が明らかとなった[37]．また，*TNXB*遺伝子座内のSNP(rs3130342)と全身性エリテマトーデス(SLE)との関連も示された[38]．さらにYuanら[39]は，マイクロアレイ解析により，悪性中皮腫においてTNXの発現が著しく上昇することを見出し，TNXが悪性中皮腫の診断マーカーとなり得る可能性を示した．

## 8. 血清型TNX(sTNX)の機能

興味深いことに，結合組織のみならず血清中にもTNXが存在することが明らかとなった[10]．血清中のTNXであるsTNXは，ヒトの場合は約150 kDaの分子量を持ち，完全長のTNX(iTNX)の第23番目FNIII様配列内のアミノ酸番号(aa)2,999付近からC末端までを[40]，またマウスの場合は約200 kDaの分子量を持ち，iTNXの第16目FNIII様配列内のaa 2,356からC末端までを持つ[41](図3)．なお，アミノ酸番号はIkutaら[9]の論文に基づく．さらに，sTNXは完全長のiTNXの分解産物であることが明らかとなった．そのため，sTNXはTNX欠損I型EDS患者の血清中[10]やTNX欠損マウスの血清中[41]には存在しない．

これまでに，プロテアーゼの分解により生じたECMの一断片(例えばXVIII型コラーゲンα1鎖から生じた20 kDa断片であるエンドスタチン[42]等)が，強力な血管内皮細胞増殖阻害等の，重要な生理作用を担っていることはよく知られている．そこで，我々は組換え体sTNXを作成，発現，精製後，血管内皮細胞の増殖への影響を調べた[43]．sTNXは，iTNXに比べ培養上清に分泌されやすく，VEGF-AやVEGF-Bとの結合も，iTNXに比べより強いことも明らかとなった．さらに，sTNXのみでも弱いながらも血管内皮増殖活性を示すが，VEGF-AとsTNXの共

存により，その活性がより促進されることが明らかとなった．

## 9. sTNXの血清濃度の測定系の開発

我々は，TNX欠損I型EDSやTNXハプロ不全III型EDSを始めとして種々の疾患の診断への応用を目指し，血清中のsTNXの濃度測定のためのサンドイッチ酵素免疫定量(ELISA)法を開発した．捕捉用抗体として，ヒトTNXの第30番目のFNIII様配列に対する抗体を用い，一方，ペルオキシダーゼ(HRP)標識を行った第31番目のFNIII様配列に対する抗体を検出用抗体として用いた．

これまでに，腹部・胸部大動脈瘤組織におけるTNXの発現亢進が明らかとなっていたが[44,45]，腹部・胸部大動脈瘤手術を受ける患者の血清中のsTNX濃度をサンドイッチELISA法により測定した．その結果，腹部・胸部大動脈患者と健常人の血清中のsTNX濃度の差は，ほとんど見られなかった[45]．

## おわりに

テネイシンファミリーの中で，遺伝性疾患の原因遺伝子として同定されたのは*TNXB*遺伝子が初めてである．TNX欠損によるI型EDSやTNXハプロ不全によるIII型EDSの発症など，臨床上重要な知見が明らかとなってきた．我々の研究室では，前章に述べたように，疾患の診断に利用可能な鋭敏な血清TNX濃度のELISA系を開発した．今後このELISA系が，原因不明のI型EDSやIII型EDSの発症の原因究明に役立つことを期待する．また，ウールリッヒ型先天性筋ジストロフィーやベスレムミオパチー等の筋原性疾患におけるTNX欠損の関わりの解明など，今後の研究の発展が待たれる．

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## Ehlers-Danlos syndrome due to tenascin-X deficiency

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