Identification, cloning and characterization of the novel tenascin family member tenascin-N

and

Conditional gene targeting of tenascin-R

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Introduction

1 The extracellular matrix

To form and maintain the function of a complex organ such as the nervous system, an interplay between different cellular mechanisms is required. Cells within an organism recognize their surrounding, targets and pathways by interactions with the molecules in their neighborhood. These molecules can either be diffusible like transmitters or hormones, or nondiffusible like cell surface molecules or the content of the extracellular matrix. The extracellular matrix is composed of a complex network of macromolecules, including laminin, fibronectin, collagen, members of the tenascin family, proteogylcans and polysaccharides. These provide support to organs in terms of mechanical strength and scaffolding. Furthermore, they participate in cell proliferation, migration, differentiation and survival. In the nervous system, the extracellular matrix additionally regulates axonal pathfinding, synapse formation and regulation of synaptic efficacy.

2 The tenascin family

The tenascin family is part of the extracellular matrix. So far five members of the family are known, named tenascin-C (TN-C), tenascin-R (TN-R), tenascin-W (TN-W), tenascin-X (TN-X) and tenascin-Y (TN-Y). The first part of this work describes the identification, cloning and characterization of an additional family member, which was named tenascin-N (TN-N). The second part of this work focuses on the construction and analysis of a conditionally targeted TN-R mouse.

2.1 The molecular structure of tenascin family members

Tenascins are large multimeric proteins and contain common structural modules. The arrangement of these domains is characteristic within this family. Each member has an amino-terminal cysteine-rich domain, followed by arrays of epidermal growth factor-like (EGF) domains and fibronectin type III-like (FN III) domains. The EGF array always starts with one truncated EGF repeat. At the carboxy-terminus a single fibrinogen-like (FBG) domain is located. In contrast to the other family members, TN-X und TN-Y contain serine- and proline-rich parts, which are inserted in the array of FN III domains. An overview of the tenascin family members and their multimeric structure is given in Fig. 1.

Multimerization of TN-C and TN-R has been shown by either Western blot analysis or rotatory shadowing images (Erickson and Inglesias, 1984). The latter technique is capable to visualize the structure of large molecules in purified protein preparations. The images show that TN-C can form hexamers, consisting of six arms emanating from a central core, each arm representing a single molecule. The linear EGF portion appears thin and rigid, whereas the array of FN III domains is thicker and flexible. A globular domain is present at the distal end of the structure and shows the compact appearance of the FBG domain (Jones et al., 1989; Spring et al., 1989). For TN-R, only dimeric and trimeric structures have been found (Norenberg et al., 1992). The assembly of the TN-C hexamer is a two step process, involving formation of trimers and the linkage of trimers into hexamers. Trimers and hexamers are stabilized by disulfide bonds of cysteines at the amino-terminal part of the tenascins (Kammerer et al., 1998). Because all known tenascins contain a domain that mediates mulimerization of TN-C and TN-R, it is likely that all family members can form at least trimers.

The EGF arrays in tenascins are encoded by a single exon and consist of 31 amino acids (aa). A single domain contains 6 cysteines in conserved positions, which are capable of forming intrachain bonds. These bonds contribute to an exceptionally compact structure, that lacks the acidic residues to bind calcium ions, as seen in EGF domains from other proteins, e.g. coagulation factors and Notch (Handford et al., 1990; Lendahl, 1998).

The FN III domains consist of approximately 90 aa with a flexible structure composed of seven antiparallel beta-strands arranged in two sheets. FN III domains of TN-C, TN-R, and TN-X are known to be altered by differential RNA splicing. For TN-R only a single splice variant is known. In contrast, for TN-C at least 27 different FN III variants are

generated from 9 spliced FN III domains, as shown by RT-PCR (Joester and Faissner, 1999). Some splice variants are preferentially expressed during development (Prieto et al., 1990). Mechanisms that determine the selection of splice sites in tenascins and their regulation during development are currently unknown.

The FBG domain contains polypeptide loops formed by two intrachain disulfide bonds (Doolittle, 1984). It represents the most conserved region during evolution and shows the ability to bind calcium ions. Evolutionary studies suggest that proteins containing FBG domains already played roles in the development of invertebrates before the emergence of the domain in the vertebrate lineage (Xu and Doolittle, 1990).

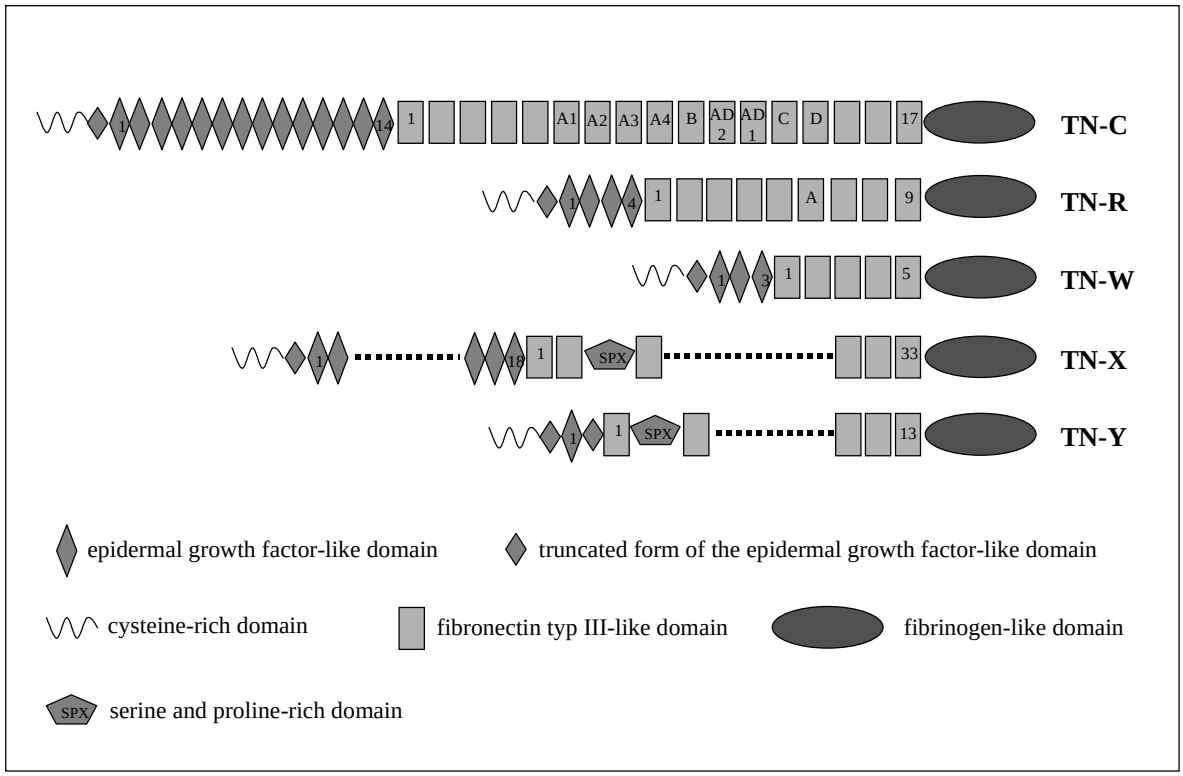

Fig. 1: Tenascin family members and their structural motifs. The family consists of five members with a typical arrangement of domains. The amount of similar domains is indicated by numbers. Alternatively spliced FN III repeats are marked by letters.

2.2 Expression profile of tenascin family members

Tenascin family members are expressed in a variety of cell type lineages and tissues. The expression is often regulated with respect to both localization and timing.

Expression in the CNS has been documented for TN-C, TN-R, and TN-Y. TN-C is spatially and temporally regulated in the developing CNS (Bartsch, 1996) and is first detected at gastrulation and somite formation (Crossin et al., 1986) and later during invasion of neural crest cells (Tan et al., 1987). It is mainly secreted by immature and reactive astrocytes, but also by subsets of radial glia cells (Kawano et al., 1995) and by subpopulations of neurons localized in the hippocampus, spinal cord, and retina (Bartsch, 1996). TN-C immunoreactivity is often located in a boundary-like expression pattern, which correlates with the functional subdivision of neuroanatomical systems (Faissner and Steindler, 1995; O'Brien et al., 1992). Although TN-C is absent from most regions of the adult brain, the protein persists in areas known to retain a high degree of plasticity, e.g. the olfactory bulb and hypothalamus (Theodosis et al., 1997; Gonzalez and Silver, 1994). TN-C is expressed in the mature PNS at the nodes of Ranvier and at the neuromuscular junction. In addition to the expression in CNS and PNS, TN-C is present during morphogenesis of the skeleton and vasculature, and occurs in cartilage, tendons, connective tissue, spleen, and in the renal interstitium of the kidney (Chiquet and Fambrough, 1984; Liakka and Autio-Harmainen, 1992; Truong et al., 1996).

In contrast to TN-C, TN-R is expressed later during development. TN-R, previously designated J1-160/180 or janusin in rodents and restrictin in chicken (Norenberg et al., 1992; Fuss et al., 1993; Schachner et al., 1994), is expressed predominantly in the central nervous system, but a recent publication described TN-R as a minor, transiently expressed component of the peripheral nervous system (Probstmeier et al., 2001). It is mainly synthesized by oligodendrocytes with highest expression during the period of active myelination (Bartsch et al., 1993; Wintergerst et al., 1993) and is detectable at contact sites between unmyelinated axons, at the interface between axons and myelinating processes of oligodendrocytes, and in myelin sheaths (Bartsch et al., 1993). TN-R is also expressed by subpopulations of neurons of retina, hippocampus, and spinal cord and highly accumulates at the nodes of Ranvier in the adult (Bartsch et al., 1993; ffrench-Constant et al., 1986). Furthermore, TN-R is a component of the perineuronal nets surrounding neuronal cell bodies of inhibitory interneurons of e.g. cortex and hippocampus (Weber et al., 1999). These observations suggest that TN-R is involved in

interactions between oligodendrocytes and neurons during myelin formation and axonal regeneration (Schachner et al., 1994).

TN-W has been identified in the zebrafish and is expressed during ontogenetic development in embryos, larvae, and juvenile animals. It is present in the lateral plate mesoderm and most conspicuously in the presumptive sclerotome (Weber et al., 1998). Migrating cells of sclerotomal and neural crest origin also show high levels of expression. Furthermore, non-neuronal cells in dorsal root ganglia express detectable levels of TN-W. The expression partially overlaps with that of TN-C mRNA, suggesting an involvement of both molecules in neural crest and sclerotome migration and in the formation of the skeleton.

As the largest member of the tenascin family, TN-X is predominantly expressed in connective tissue, in heart and skeletal muscle, tumor tissues, skin, and in the vicinity of blood vessels (Lethias et al., 2001; Hasegawa et al., 1997; Ikuta et al., 2000; Sakai et al., 1996). Furthermore, a weaker expression was found in the digestive tract, e.g. oesophagus, gut, and stomach. The localization of TN-X to blood vessels probably explains the occurrence of TN-X mRNA and protein in most tissues examined, whereas in brain, lung, and mammary gland no TN-X was detectable (Matsumoto et al., 1994a). The TN-X gene shares its chromosomal localization with the major histocompatibility complex class III (Bristow et al., 1993; Matsumoto et al., 1994; Rupert et al., 1999).

TN-Y was identified in chicken and is expressed by different types of muscles, but also in lung, kidney, skin and in the nervous system. In the CNS it shows a partially overlapping, but also complementary expression pattern with TN-C (Fluck et al., 2000; Hagios et al., 1999; Hagios et al., 1996; Tucker et al., 1999).

2.3 *In vitro* functions of tenascin family members

The restricted expression of TN-C in boundary-like structures suggested a function for this tenascin to form or maintain borders within the CNS. This observation lead to experiments which aim to test this notion *in vitro*. TN-C was initially examined for anti-adhesive and inhibitory properties, which became the first characterized functions of a tenascin (Faissner and Kruse, 1990; Dorries et al., 1996). Interestingly, TN-R has been shown to exhibit similar properties. In the presence of homogenous TN-C or TN-R substrates, cells

do not attach to the culture plate or strongly aggregate to minimize their attachment points. Furthermore, the anti-adhesive properties of TN-C and TN-R become apparent in experiments where outgrowing neurites or migrating cells can choose between tenascin-containing substrates and substances which support neurite outgrowth and cell migration, e.g. laminin or polycations. For example, explants from postnatal day 1 hippocampus send out neurites after a few days in culture. The neurites generally avoid the tenascin containing substrate and follow the borderline between the permissive and the anti-adhesive substrate. Thus, the anti-adhesive properties of tenascins induce growth cone turning in choice situations (Dorries et al., 1996; Xiao et al., 1996). In addition to effects on neurons, TN-C inhibits the adhesion of oligodendroglia and migration of their O2A precursor cells (Kiernan et al., 1996). By recombinant expression of selected regions of TN-C and TN-R, functionally specialized domains have been identified suggesting an interaction of tenascins with many types of receptors. Distinct domains have been identified for anti-adhesion and growth cone deflection, for stimulation of axon growth, and for the influence on migratory behavior of oligodendrocyte precursors (Joester and Faissner, 2001).

2.4 *In vivo* functions of tenascin family members

TN-C is the best characterized member of the family and has been implicated in different morphogenetic processes during development, axonal regeneration, synaptic plasticity, tumorigenesis, wound healing, and in kidney diseases. However, mice with an inactivated TN-C gene have been described as surprisingly normal (Saga et al., 1992, Forsberg et al., 1996). It was even suspected, that TN-C might be redundant or that TN-R and TN-X are upregulated and compensate for TN-C in the knockouts. So far the hypothesis of redundancy and compensating mechanisms has not been confirmed. By more detailed analysis subtle abnormalities have been observed in the TN-C knockout, demonstrating a precise function of the molecule in different tissues. Moreover, the ablation of TN-C influences the behaviour. Normal C57BL/6N mice can swim for minutes in a water tank without training, whereas TN-C knockouts fail the test and occasionally have to be rescued from drowning. Furthermore, they show hyperactive behaviour in open-field test and during the day cycle (Fukamauchi et al., 1996). In spite of the normal CNS

architecture in these animals, the brain chemistry is altered on the level of neurotransmitters (Fukamauchi and Kusakabe, 1997). A recent publication described a deficiency in the coordinated migration of neural crest cells, when the translation of TN-C mRNA is drastically reduced by knockdown studies in avian embryos (Tucker, 2001). TN-C also has functions in the mature peripheral nervous system. It was found that TN-C deficient mice have a decreased level of properly myelinated axons compared to control animals, and these axons often are in direct contact to other unmyelinated fibers, rather than Schwann cells (Cifuentes-Diaz et al., 1998). The end plates are abnormal in knockout mice and the terminal sprouting of peripheral nerves, as induced by injections of botulinum-A toxin, is reduced to 70 % in the TN-C knockouts (Cifuentes-Diaz et al., 1998). Some of the observed phenotypes in the peripheral nervous system are in contrast to findings of other groups (Moscoso et al., 1998). TN-C is also expressed in many non-neuronal tissues, where expression is often associated with pathological conditions such as neoplasia and inflammation. Studies on TN-C null mutants in inflammatory and regenerative processes revealed striking phenotypes. In kidneys, TN-C is expressed predominantly in embryonic stages, but also in the adult inner medulla in the vicinity of the tubules. Its expression is strongly induced in glomerulonephritis. In the animal model for this kidney disease, wildtype mice are able to recover from Habu snake venom induced glomerulonephritis. In TN-C knockout mice of three different genetic backgrounds with induced glomerulonephritis striking differences have been found in the degree of the phenotype depending on the genetic background. In the most extreme case (TN-C deficient mice with GRS/A background) the progression of the disease is irreversible and all animals died within four months because of renal failure (Nakao et al., 1998). The phenotype could be assigned to a defect in mesangial cells of the kidney, a cell type which is phagocytic and removes trapped residues from the glomerular filters.

The expression of TN-R appears to be restricted to the central nervous system, although a recent publication described TN-R as a minor, transiently expressed component of the peripheral nervous system (Probstmeier et al., 2001). In the central nervous system, TN-R is synthesized by oligodendrocytes with the highest expression at postnatal day 14 and it accumulates at the nodes of Ranvier. In this context it was shown that TN-R deficient mice display a two times decreased axonal conduction velocity in the optic nerve, as shown by electrophysological measurements. The morphological analysis of the optic nerves of TN-R deficient mice on the ultrastructural level revealed no difference in myelination and structure of nodes of Ranvier (Weber et al., 1999). The interaction of TN-

R with the immunglobulin superfamily adhesion molecule F3/F11/contactin (Pesheva et al., 1993) led to the notion that TN-R might also interact with the β2 subunit of the voltage gated sodium channels, since a significant homology was found between F3/F11/contactin and this β2 subunit (Isom et al., 1995). This postulated interaction has been demonstrated by incubating cell cultures expressing the Na^+ channels with recombinant fragments of TN-R. In these studies an increase in Na^+ current by a factor of two has been observed (Xiao et al., 1999), whereas morphological and immunocytochemical investigation of the distribution, clustering, and number of voltage gated Na^+ channels at the nodes of Ranvier detected no differences between knockout and wildtype (Weber et al., 1999). In addition, the TN-R knockout shows alterations in the extracellular matrix by a disturbed distribution of the chondroitin sulfate proteoglycan phosphacan. Furthermore, the perineuronal nets of GABAergic interneurons in TN-R deficient mice were clearly different in terms of distribution and shape, indicating a role of TN-R in the coordinated formation of these mesh-like structures.

TN-X has been associated to a disease, called the Ehler-Danlos syndrome (Burch et al., 1997). Due to a deletion arising from a recombination event of TN-X gene with its partial duplicate gene, patients were deficient for TN-X expression and suffered from the Ehlers-Danlos disorder. Most striking findings are the abnormal elastin bodies beneath the dermal-epidermal junction, an increase in the perivascular matrix, and uneven packing of myelin sheaths of peripheral nerves. The patient's phenotype is characterized by skin hyperextensibility, vascular fragility, and poor wound healing, whereas the disturbed myelin formation in the PNS did not lead to an obvious peripheral neuropathy (Burch et al., 1997).

In comparison to other tenascins only little is known about TN-Y and TN-W, and their *in vivo* function still remains to be elucidated. Nevertheless, the overlapping expression of TN-W and TN-C in migrating neural crest cells lead to the speculation that TN-W might also be relevant for the target finding of neural crest cells as shown for TN-C.

3 Conditional gene targeting in mice

3.1 The construction of gene deficient mice

Gene targeting can be defined as the introduction of site specific modifications into the mouse genome by homologous recombination. Since the first description of the homologous recombination in mouse embryonic stem (ES) cells (Thomas and Capecchi, 1987; Doetschman et al., 1988), gene targeting has become a powerful technology to study gene function *in vivo*. The technology utilizes pluripotent murine ES cell lines which can be manipulated *in vitro*. Using these cells, the selection of rare, homologous recombination events can be accomplished. In homologous recombination a mutated DNA replaces the genomic sequences of the target gene. When such genetically modified ES cells are introduced into preimplantation embryos, they can contribute even after extensive *in vitro* manipulation to all cell lineages of the resulting genetically chimeric animal. The breeding of germline chimeras results in the transmission of the ES cell derived mutant chromosome to their progeny. This allows the establishment of animals heterozygous for the desired genetic alteration and by further breeding a homozygous mutant mouse strain. The major steps of the method are illustrated in Fig. 2.

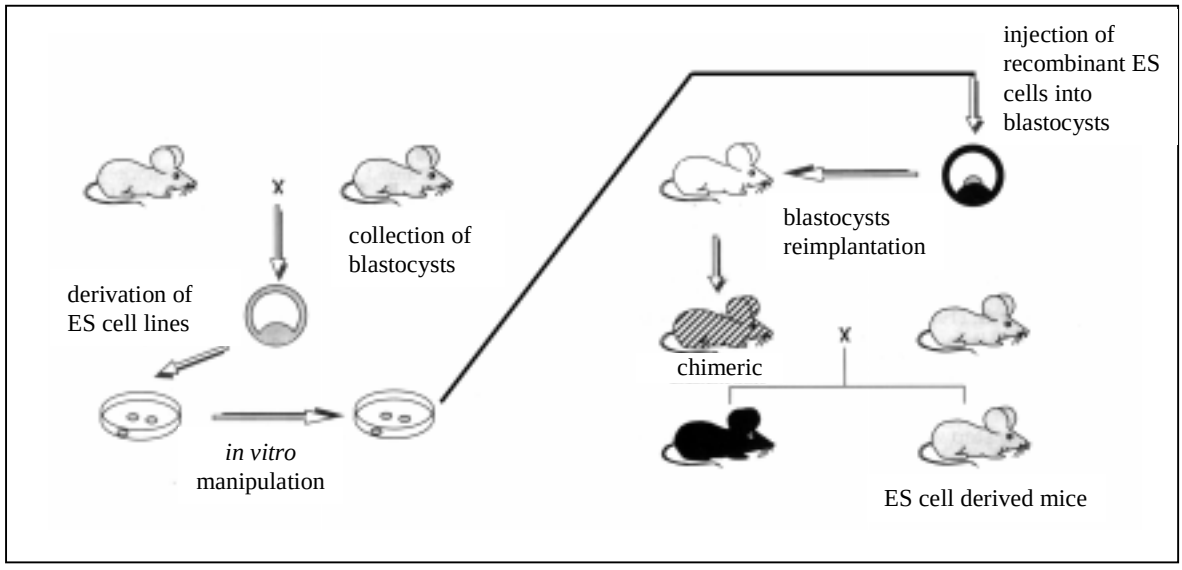

Fig. 2: Construction of targeted mouse mutants. Embryonic stem (ES) cells are derived from preimplantation embryos and manipulated *in vitro* by homologous recombination with a targeting vector. Recombined ES cells are injected into blastocysts and contribute to the developing offspring to generate genetically chimeric mice. Breeding of these mice leads to heterozygous and homozygous mice with the desired mutation.

3.2 ES cell culture

Pluripotent ES cells were first established directly from blastocysts in 1981 (Martin, 1981; Evans and Kaufman, 1981) and were later shown to efficiently produce germline chimeras even after transfection (Gossler et al., 1986; Robertson et al., 1986). Since then, a fairly large number of ES cell lines have been generated which are mainly derived from 129 mouse substrains or 129 hybrids. There is a smaller number of less frequently used ES cell lines derived from other mouse strains, e.g. C57BL/6. All ES cell lines are male, thus the germline transmission is usually through male chimeras. The success of a targeting approach depends on the ability of the ES cells to colonize the germline at a high frequency. To achieve this goal, the ES cells have to be maintained in the state of pluripotency by optimal treatment. Mistreated ES cells will easily differentiate from pluripotency. Consequently, they may be unable to participate in germline transmission. To prevent the differentiation process, ES cells are usually cultured in the presence of leukaemia-inhibitory factor (LIF) and on a layer of mitotically inactive feeder cells. These feeder cells are derived from embryonic fibroblasts and are treated with mitomycin. In spite of the poisoning with mitomycin the cells are able to survive for several days *in vitro*. Feeder cells have to possess the bacterial aminoglycoside phosphotransferase gene conferring neomycin/G418 resistance, since this selection marker is used to identify ES cells which have undergone recombination with the targeting vector (Muller et al., 1991).

3.3 Recombination systems and conventional targeting vectors

The production of a gene targeted mutant strain is a laborious and technically demanding effort which often takes more than a year to complete, even longer if technical problems are encountered. Therefore, extensive planning and foresight should be given to the strategy chosen to elucidate the desired gene function by targeting experiments. The construction of the targeting vector, which introduces the mutation into the ES cell genome by homologous recombination, is probably the most important step to ensure that the gene modification will fulfill the experimental demands. In this context, a flexible strategy improves the chance to reach the aim. Most frequently used are conventional gene targeting approaches which inactivate the gene function from the beginning of the development. Because the potential of each targeting construct is greatly expanded by the use of LoxP and/or FRT, these systems should be generally considered for all targeting vectors even if the initial goal is simply to inactivate gene function.

The P1 bacteriphage Cre recombinase is a member of the λ integrase superfamily of site-specific recombinases and belongs to the few members which do not require cofactors for recombination (Argos et al., 1986). It recognizes and mediates site-specific recombination between 34 bp sequences referred to as locus of crossover in P1 (loxP). The loxP sequence consists of two 13 bp inverted repeats interrupted by a 8 bp non-palindromic sequence which specifies the orientation of the overall sequence (Hoess et al., 1982). When two loxP sites are placed in the same orientation on a linear DNA molecule, a Cre-mediated intramolecular recombination results in the excision of the loxP-flanked (floxed) sequence as a circular DNA, which contains the single loxP site. If the two loxP sites are places in opposing orientation the floxed sequence will be inverted (Abremski et al., 1983). The yeast-derived FLP recombinase shares many features with the Cre recombinase and recognizes the so-called FRT sequence. The FLP recombinase operates optimally at 30 °C, whereas the Cre recombinase has an optimum at 37 °C. In spite of the thermo-instability of the FLP, its specific recombination has been successfully used to remove a FRT flanked selection marker in ES cells.

In general, the aim of conventional targeting experiments is to replace endogenous wildtype sequences which results in complete inactivation of a specific gene. Conventional targeting vectors are constructed from a cloned genomic sequence of the gene of interest and contain two regions of homology to the genomic locus. Virtually all

targeting vectors possess a long and a short arm of homology which flank the region of the selectable marker gene, e.g. the bacterial aminoglycoside phosophotransferase (Neo) gene. The Neo cassette is often used to replace parts of the coding region of the targeted gene with the aim to ablate its expression. Furthermore, the incorporation of the neo gene into the genome enables the ES cell to survive the presence of neomycin/G418 in the culture medium and thus selects all cells with a randomly integrated or homologously recombined targeting vector. The homologous recombinants have to be differentiated from the random integrants by PCR or Southern blot analysis. In conventional approaches the recombination system is mainly used to remove the selectable marker from the ES cell genome prior to injection into blastocysts, which restricts this approach in flexibility.

3.4 Conditional gene targeting

Major problems of conventional gene targeting experiments are the lack of temporal control and tissue specificity, possible adaptation and compensation mechanisms for the missing gene function during embryogenesis and ontogenesis, as well as embryonic and neonatal lethality. The combination of gene targeting techniques with site-specific recombination systems such as the Cre/loxP system of the bacteriophage P1 or the yeast derived FLP/FRT system allows the development of strategies to circumvent these problems by restricting the mutation to certain cell types and/or a specific time period, but also to introduce large genomic alterations.

The applicability of the Cre/loxP system for conditional, tissue-specific gene targeting was first demonstrated by Gu et al. (1994). Tissue specific gene inactivation can be achieved by crossing a mouse line containing floxed alleles at the gene of interest with a mouse strain expressing the Cre recombinase under the control of a well characterized tissue-specific promoter. The number of transgenic mouse lines expressing the Cre recombinase under a tissue specific promoter is rapidly growing and is a prerequisite for tissue restricted gene inactivation (Nagy, 2000). Additionally, time-controlled expression of Cre and time-controlled gene inactivation is achieved by expressing Cre under the control of the tetracycline-regulated expression system (Kistner et al., 1996; Shin et al., 1999, Yamamoto et al., 2000) or by using ligand-dependent fusion proteins of the Cre

recombinase with a mutated hormone binding domain of steroid receptors (Sauer and Henderson, 1988; Kellendonk et al., 1996; Buchholz et al., 1998).

Another well characterized and similar site specific recombination system that has been applied to conditional gene targeting is the FLP/FRT system derived from *Saccharomyces cerevisiae* (Andrews et al., 1985; Dymecki, 1996; Koch et al., 2000; Meyers et al., 1998; Vooijs et al., 1998). In contrast to the Cre/loxP system, inducible systems have not been reported for the FLP/FRT system so far. The use of FLP is more restricted, due to the relatively low activity of FLP at 37° C (Buchholz et al., 1996b). Meanwhile, this problem has been overcome: a FLP recombinase with improved properties at 37°C has been evolved by cycling mutagenesis and termed FLPe (Buchholz et al., 1998). Recently, a transgenic mouse line expressing FLPe was shown to be an efficient alternative to the Cre/loxP system (Rodriguez et al., 2000), and in the future, broader use and applicability of the FLPe/FRT system will be expected.

Construction of the targeting vector needs detailed information about the gene of interest and very often tedious and complicated cloning steps. For conditional gene targeting, the vector usually consists of a positive selection cassette flanked by the recognition sequences for the site specific recombinase (loxP or FRT) placed in an intron and a third recognition sequence placed upstream or downstream in the next intron, thereby marking the exon in-between for excision. After identifying homologously recombined ES cell clones the selection cassette can be removed by transfection and transient expression of a Cre (or FLP) expressing plasmid *in vitro*. However, three alternative recombination products are possible: 1) The so called type I deletion between the outer loxP sites removes the selection cassette including the floxed exon, which results in a constitutive gene inactivation. This type of clone can be used directly to generate a constitutive knockout. 2) The type II deletion which leads to the desired removal of the selection cassette leaving the floxed exon. 3) The third type of deletion, which removes only the floxed genomic sequence and leaves the selection cassette, can be counterselected by screening for neomycin/G418 sensitivity.

4 Aim of this work

The tenascin family consists of extracellular matrix molecules and in the past decade five members of this family have been identified and characterized. They are expressed in many types of tissues and contribute to a variety of processes. Two of the tenascin family members, namely tenascin-R and tenascin-C, are expressed in the central nervous system and show repellent functions on outgrowing neurites and migrating neural cells. In contrast to this striking function, gene deficient mice of these molecules have rather disappointing phenotypes in the central nervous system.

The aim of this work was to identify possible mechanisms which compensate for the lacking target molecule in these knockouts and thus contribute to the unexpectedly mild phenotypes.

The missing link could be a yet not identified tenascin family member, which has similar functions as tenascin-R or tenascin-C. Therefore, the major aim of this study was to search for a novel tenascin and to characterize its expression pattern and function.

A frequently discussed problem in conventionally gene targeted mice is the lack of the target molecule from the beginning of the development, which might provoke compensatory mechanisms. This problem can be overcome by the newly developed method of conditional gene targeting. Therefore, it was aimed to construct a conditionally targeted tenascin-R mouse line and to ablate its expression at selected time points and in specific cell types.

Materials and methods

1 DNA analysis and manipulation

1.1 Preparation of competent bacteria

Bacteria were grown at 37 °C in 100 ml LB medium (10 g/l Bacto-tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7.4) until an optical density of 0.5 was reached at 600 nm. The culture was cooled on ice for 5 minutes and transferred to a sterile plastic centrifuge tube. Cells were collected by centrifugation at 4000 g for 5 minutes at 4 °C and gently resuspended in ice-cold buffer 1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15 % glycerol, pH 5.8, sterile-filter). The suspension was kept on ice for 90 minutes. The cells were centrifuged again and resuspended in 4 ml ice-cold buffer 2 (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15 % glycerol, pH 6.8, sterile-filter). Aliquots were snap frozen in liquid nitrogen and stored at –70 °C for no longer than 6 months.

1.2 Transformation of bacteria

Aliquots of competent bacteria were thawn on ice for 10 minutes. Fifty ng plasmid DNA was supplemented to the bacteria suspension and incubated for 20 minutes at 4 °C. After a heat shock at 42°C for 1 minute and successive incubation on ice for 2 minutes, 800 µl LB medium were added and incubated at 37 °C with constant agitation. Cells were collected by centrifugation at 1000 g for 2 minutes at room temperature. The transformed bacteria were plated on selective agar plates (20 g/l agar in LB medium with antibiotics) and incubated overnight to allow single bacteria colonies to grow.

1.3 Plasmid propagation in bacteria

The following two bacteria strains were used to propagate plasmid DNA prior to DNA isolation procedures: *Escherichia coli* DH5α (New England Biolabs, Frankfurt am Main, Germany) and *Escherichia coli* XL1-blue (Stratagene, La Jolla, California, USA). Between 3 and 15 ml of LB medium (10 g/l Bacto-tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7.4) were inoculated by a single bacteria colony and incubated at 37 °C overnight with constant agitation. One of the following antibiotics was supplemented prior to inoculation to select for plasmid containing bacteria: 100 µg/ml ampicillin, 25 µg/ml kanamycin, or 25 mg/ml tetracycline. For large scale preparations of DNA a 10 ml overnight bacteria culture was used to inoculate 500 ml LB medium. Further incubation at 37 °C for 4 h resulted in an optical density of 0.6 at 600 nm.

1.4 Maintenance of bacterial strains

Selected bacterial strains which contain plasmids of interest were stored in a mixture with 25 % glycerol and LB medium at –70 °C for up to one year or at –20°C for up to 2 months. Bacteria grown on agar plates containing an appropriate antibiotic were stored up to 6 weeks at 4 °C.

1.5 Plasmid DNA purification

The methods used to purify plasmid DNA from bacteria employ alkaline cell lysis of bacteria, denaturation of protein by chaotropic salts, and use a glass fiber matrix to separate the DNA from contaminants.

For small scale preparations, bacteria of a 3 ml overnight culture were pelleted by centrifugation at 12000 x *g* for 1 minute at room temperature. Plasmids were isolated from the bacteria according to the manufacturer's protocol (Amersham Pharmacia Mini

preparation kit). The DNA was eluted from the glass fiber matrix column by addition of 50 µl 10 mM Tris-HCl and subsequent centrifugation at 12000 x *g* for 1 minute. The concentration of the eluted DNA was about 0.25 µg/µl as determined by UV-spectrometry.

To obtain higher concentrations of DNA, the plasmids from 15 ml bacterial culture were purified according to the protocol provided with the Nucleospin Kit (Macherey-Nagel), with the exception that the suggested volume of buffers were doubled and the elution buffer was warmed to 70 °C prior to usage. Up to 80 µg total DNA per preparation in a concentration of 1.5 µg/µl were obtained by the method.

For large scale preparations with about 500 µg total plasmid DNA, the Maxiprep Kit (Qiagen) was used according to the manufacturer's protocol.

Further purification of plasmid DNA was achieved by phenol/chloroform protein extraction and successive precipitation and washing steps of the DNA in 70 % ethanol at 4 °C (Sambrook et al., 1989).

1.6 Determination of DNA concentration and purity

DNA molecules absorb UV light of a wavelength of 260 nm, whereas proteins absorb strongest at 280 nm. Thus, the absorption at 260 nm of purified DNA solutions can be converted into DNA concentrations and the ratio of A_{260}/A_{280} is a measure of the purity of the DNA (Sambrook et al., 1989). DNA concentration and purity was determined spectrometrically using an Amersham-Pharmacia spectrometer. Absorbance at 260 nm need to be between 0.1 and 0.6 for reliable results. DNA preparations with A_{260}/A_{280} ratios of 1.8 to 1.9 were considered to be sufficiently pure for most applications.

1.7 Endonuclease restriction analysis

Endonuclease restriction enzymes were used to digest and analyze the sequence of plasmids or DNA fragments. The restriction endonucleases were obtained from New England Biolabs together with their appropriate buffer sets. The enzyme concentration

recommended by the manufacturer's was doubled and the reaction mixture was incubated at 37 °C for 2 h prior to analysis by agarose gel electrophoresis. DNA samples were digested successively with two different enzymes that were incompatible with each other.

1.8 Dephosphorylation and polishing of plasmid DNA ends

Linearized plasmid DNA was treated with shrimp alkaline phosphatase (SAP, Boehringer Ingelheim) to remove the 5' phosphate group. Fifty ng plasmid DNA was incubated with 1 u of the enzyme at 37 °C for 2 h. SAP was inactivated by incubation at 65 °C for 15 minutes. The dephosphorylated plasmid DNA was used for ligation without further purification.

Non-compatible sticky ends of linearized plasmid DNA were converted into blunt ends for ligation using the Klenow enzyme (Boehringer Mannheim) according to the manufacturer's instructions. The reaction was terminated by incubation at 70 °C for 10 minutes.

1.9 Ligation of DNA fragments

Ligation of DNA fragments into linearized plasmid DNA was performed by mixing 50 ng vector DNA with a threefold to fivefold molar excess of insert DNA. The ligation was catalyzed by the T4 ligase (Boehringer Mannheim) in its appropriate buffer set. The reaction was incubated overnight at 16 °C for sticky end ligation and at 4 °C overnight for blunt end ligation (Sambrook et al., 1989).

For the ligation of PCR fragments and inserts with a low DNA concentration, the TOPO-TA cloning Kit (Invitrogen) was used according to the manufacturer's instructions.

1.10 Preparation of genomic DNA

To distinguish between the different genotypes of mice, the tailtip of a mouse was cut off and incubated in 400 µl Boston buffer (50 mM Tris-HCl, 50 mM KCl, 2.5 mM EDTA, 0.45 % NP40, 0.45 % Tween 20, pH 8, 0.1 mg/ml proteinase K) overnight at 55 °C. The lysate of the tailtip was vortexed to shear the genomic DNA and centrifuged for 2 minutes at 12000 x *g* at room temperature. One µl of the supernatant, containing a crude preparation of genomic DNA, was subjected to a standard PCR with appropriate primer combinations under stringent conditions.
A standard PCR was also used to identify ES cells which had undergone homologous recombination or Cre mediate recombination, although the preparation of genomic DNA was different: ES cell clones were grown for several days in 96 well plates, washed with PBS, and lyzed with 100 µl Boston buffer (see above) overnight at 55 °C. Ten µl 8 M LiCl and 100 µl isopropanol was added and incubated overnight at 4 °C with constant agitation. The 96 well plate was centrifuged at 3000 x g and the pellet was washed with 70 % ethanol and dried. The purified genomic DNA was resolved overnight in 100 µl 10 mM Tris (pH 8) at 65 °C. One µl was subjected to PCR analysis.

1.11 PCR

Amplification of linear DNA fragments was performed by polymerase chain reaction (PCR). Thin-walled PCR tubes were used in MJ PCR cyclers with a 50 µl reaction mixture. The amplification of DNA was catalyzed by the proofreading Turbo-Pfu polymerase (Stratagene) to minimize the incorporation of point mutations due to an error rate of the polymerase reaction. The quality of the PCR product was analyzed by gel electrophoresis.

1.11.1 Standard PCR

The following reaction mixture was used for standard PCR:

template	0.5 to 10 ng DNA per reaction
primer A (5 µM)	3 µl
primer B (5 µM)	3 µl
nucleotides (10 mM)	1 µl
buffer (10x)	5 µl
Turbo-Pfu polymerase	2.5 units
ddH_2O	ad 50 µl

The following cycling program was used to amplify the desired DNA:

1) denaturation	94 °C	5 minutes
2) denaturation	94°C	1 minute
3) annealing	55 – 68 °C	1 minute
4) elongation	72 °C	2 minutes per kb DNA
5) termination	72 °C	10 minutes
6) cooling	4 °C	

The amplification procedure (steps 2-4) was repeated 25-35 times.
When the product length exceeded 3 kb, the elongation time was increased by 1 or 2 additional minutes.
The annealing temperature was estimated by calculation of the melting temperature of the primer oligonucleotide minus 5 °C, although the annealing temperature often needed further optimization. The melting temperature of the primers depends on the GC content and was calculated by the following formula:

T_m = 4 x (amount of purine bases) + 2 x (amount of pyrimidine bases)

If the two primers had different melting temperatures, the lower of both was used to determine the annealing point.

1.11.2 Single colony PCR

The ligation of DNA fragments with low concentrations is a rare event and it can be time consuming to identify the desired clone. To screen large amounts of bacterial colonies for the desired insert, single colonies were picked from a transformation plate with a sterile tooth pick and dotted on a new LB plate. The rest of the colony on the tooth pick was lyzed in 30 µl Boston buffer lacking proteinase K (50 mM Tris-HCl, 50 mM KCl, 2.5 mM EDTA, 0.45 % NP40, 0.45 % Tween 20, pH 8) at 55 °C for 1 h. To test for the presence of the insert DNA, 2 µl of this lysate were subjected to a standard PCR with appropriate primers. For plasmid preparation, the colony that contained the desired vector was inoculated into LB medium with the colony dotted on the LB plate.

1.11.3 Nested PCR

Nested PCR involves two rounds of amplification reactions, in which the product of the first round is used as the template DNA of the second round. The second-round PCR is performed with two new primers that hybridize to sequences internal to the first-round primer-target sequences. If the PCR sensitivity is too low, a nested PCR can increase the product yield and specificity.

Nested PCR was used to specifically amplify the desired product from a mixture of amplified DNA fragments obtained after RT-PCR.

The reaction conditions were as follows: An aliquot of a standard PCR mixture (only 10-15 cycles) was diluted 1:100 with ddH_2O and subjected to the second standard PCR reaction as template DNA with different primers for 30 cycles.

1.11.4 Touchdown PCR

Touchdown PCR uses a cycling program where varying annealing temperatures increase the specificity of PCR. The annealing temperature in the initial cycle should be 5 to 10 °C above the T_m of the primers. In subsequent cycles, the annealing temperature was

decreased in steps of 1 to 2 °C per cycle until a temperature was reached that is equal to the T_m of the primers.
The touchdown method was used to enhance the specificity of the final PCR product amplified from RT-PCR. The applied PCR cycling program started at annealing temperatures of 72 °C for two cycles, followed by 2 °C reduction in annealing temperatures every second cycle until the desired temperature was reached. From there the cycles were repeated 25 to 30 times without annealing temperature changes.

1.11.5 Genotyping by PCR

To distinguish between the different genotypes of mice or ES cells, 1 µl of genomic DNA was subjected to a standard PCR with appropriate primer combinations under stringent conditions. For genomic DNA preparation see section 1.10 of Materials and methods. To distinguish between the three possible genotypes (homozygous, heterozygous, and wildtype), two primers were constructed to flank the mutated region. Thus, the PCR gave rise to three different band patterns, as visualized by agarose gel electrophoresis, and each pattern represented one genotype.

1.12 Purification of DNA from polymerase chain reactions

For direct purification of DNA fragments from the PCR reaction mixture, the Rapid purification kit (Life Technologies) was used according to the manufacturer's instructions. The DNA was eluted from the column by addition of 50 µl prewarmed (70 °C) 10 mM Tris-HCl (pH 8).

1.13 Agarose gel electrophoresis

The integrity and quality of DNA fragments were analyzed in horizontal electrophoresis chambers (BioRad) using agarose gels (Gibco). The agarose solution was prepared by heating 0.8-2 % (w/v) agarose in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) until the powder was completely dissolved. For analysis of smaller DNA fragments, 1.5 or 2 % agarose gels were used, whereas larger DNA fragments were analyzed in 0.8 or 1 % agarose gels. DNA sample buffer (50 % glycerol in TAE, 0.025 % Orange G dye (Merck)) was added to the probes, the gel was covered with TAE buffer and DNA samples were pipetted into the gel pockets. The gel was run at the constant voltage of 10 V/cm gel length until the Orange G dye had reached the end of the gel. The separated DNA fragments were visualized under UV light after incubation of the gel in ethidiumbromide staining solution (0.5 µg/ml TAE) for 30 minutes. Gels were documented with the E.A.S.Y. UV light system (Herolab, Wiesloh, Germany).

1.14 Purification of DNA from agarose gels

Gel electrophoresis of DNA fragments provided not only a method to analyze the quality or integrity of DNA, but was also a powerful method to purify DNA fragments of different lengths by separation. Separated DNA fragments were excised from the agarose gel after ethidiumbromide staining. The DNA was purified with the Rapid Gel Extraction Kit (Life Technologies) according to the manufacturer's instructions. The fragments were eluted from the glass fiber matrix column by addition of 50 µl of pre-warmed (70 °C) 10 mM Tris-HCl (pH 8.0). DNA concentration was determined by UV light spectrometry.

1.15 Sequencing of DNA

DNA sequencing was performed by the sequencing facility of the ZMNH using a Abi Prism Sequencer 100, model 377. Vector DNA (1 µg) or PCR product (0.1 µg) were mixed with 10 µM appropriate primer before analysis.

1.16 Targeting vector construction

To generate a conditionally TN-R deficient mouse line, a targeting vector of the replacement type was constructed from parts of the 5' genomic sequence of the TN-R gene. A genomic clone, which contained the 5' end of tenascin-R, was kindly provided by P. Weber. The clone was isolated from a isogenic λGEM11 genomic library constructed from 129/SV(ev) embryonic stem cell DNA (Muller et al., 1994) by hybridization with a 500 bp cDNA fragment that recognized the EGF domains in tenascin-R.
This genomic clone was characterized by systematic restriction enzyme analysis. Different enzymes were tested for their ability to cut the DNA only once. Afterwards, the position of single cutters on the genomic clone was determined by double digestion with a restriction endonuclease from the multiple cloning site of the vector backbone. A 11 kb fragment flanked by EcoRI was identified and subcloned in the vector pBlueAmp-KS. From this 11 kb fragment, two subfragments flanked by either EcoRV/EcoRV or EcoRV/XhoI restriction sites, were both isolated and ligated into pBlueCam-KS. This cloned genomic DNA (7 kb) contained the 5' end of TN-R including exon 1 and 2 and was used as the targeting vector backbone. The targeting vector backbone was then equipped with a neo selection gene, loxP, and FRT sites at appropriate positions.

1.17 Southern blot analysis

To identify ES cells which had undergone a desired homologous or Cre mediated recombination, Southern blot analysis was performed. The protocol resembles the classical capillary blot (Sambrook et al., 1989).
From each ES cell clone 5-10 µg genomic DNA was digested with 50 to 100 u of an appropriate restriction endonuclease and separated by electrophoresis in a 0.8 % agarose gel. The DNA was depurinated by incubation of the gel in 0.25 M HCl (10 minutes, room temperature) and denaturated 30 minutes in a solution containing 1.5 M NaCl and 0.4 M NaOH. The capillary blot procedure was used to transfer the DNA onto Hybond-N(+)

membranes (Amersham Pharmacia) by 0.4 M NaOH solution. The genomic DNA was hybridized with a radioactively labelled 400 bp probe in hybridization solution (0.5 ng labelled probe, 5x Denhardts, 5x SSPE, 0.5 % SDS, 100 µg/ml denaturated salmon sperm DNA) overnight at 65 °C. The probe was derived from the 3' part outside of the targeting vector. Unspecific bound probe was washed from the membrane by two washing steps with 2x SSPE/0.1% SDS for 15 minutes followed by 1x SSPE / 0.1 % SDS for 30 minutes at 65 °C. Signals were detected by overnight exposure of the membrane to Kodak-Xomat-AR film in a cassette equipped with amplifier screens.

2 *In silico* analysis of DNA and protein

DNA sequence data were processed and assembled using the Lasergene biocomputing software package (DNAStar) including the programs Editseq, Seqman II, Primerselect, Mapdraw, Megalign, Protean, and Genquest.
Protein structures were predicted with the internet accessible programs SMART and PFAM tools (http://smart.embl-heidelberg.de, http://pfam.wustl.edu/), which helped to identify domains by conserved amino acids. Putative target regions of posttranslational modifications were analyzed with the PSORT program (http://psort.nibb.ac.jp/).

3 RNA analysis and manipulation

3.1 mRNA preparation

The described method is designed for rapid isolation of messenger RNA (mRNA) directly from crude extracts of animal tissues without a prior extraction of total RNA. The method relies on the base pairing between the poly $(A)^+$ residues of mRNA and the oligo(dT)

covalently bound to the purification matrix surface. The matrix consists of superparamagnetic beads, which can be collected by magnetic force.

Dissected tissues from adult mice were immediately frozen in liquid nitrogen and ground to powder in frozen state. Fifty mg of tissue powder was further processed with the Dynabeads mRNA Direct Kit (Dynal) to yield pure mRNA. The tissue was resuspended in 1.25 ml lysis-buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1 % SDS, 5 mM DTT) and genomic DNA was sheared by repeated passages through a hypodermic needle with a diameter of 0.9 mm to achieve complete homogenization. After 2 minutes of centrifugation at 14,000 $\times$ *g* and room temperature the supernatant was mixed with superparamagnetic Oligo(dT)$_{25}$-Dynabeads and incubated for 5 minutes at room temperature to allow binding of polyadenylated RNAs to the beads. A magnetic device collected the beads and contaminants were removed by successive washing steps with a modified buffer set (buffer I: 100 mM Tris, pH 8, 500 mM LiCl, 5 mM EDTA, 0.5 % SDS; buffer II: 50 mM Tris, pH 8, 500 mM LiCl, 5 mM EDTA, 0.5% SDS; buffer III: 50 mM Tris, pH 8, 500 mM LiCl, 2.5 mM EDTA; buffer IV: 10 mM Tris, pH 8, 150 mM LiCl, 1 mM EDTA) at room temperature. The mRNA was eluted in 10 mM Tris, pH 8, at 65°C, aliquoted, and immediately frozen in liquid nitrogen. Aliquots were thawn at 4°C directly before application to reverse transcription or Northern blot analysis. The concentration of mRNA was tested by UV-light-spectrometry. Possible degradation products of the mRNA were detected by agarose gel electrophoresis.

3.2 Determination of RNA concentration and quality

RNA molecules absorb UV light at wavelength of 260 nm. In contrast, the absorption at 280 nm is more specific for proteins. This can be utilized to determine the RNA concentration and purity in solution. Thus, the conversion of RNA absorption into concentrations and the determination of the ratio A_{260}/A_{280} provides a simple method to measure the quantity and quality of RNAs (Sambrook et al., 1989). RNA concentration and purity was determined spectrometrically using an Amersham-Pharmacia spectrometer. Absorbance at 260 nm need to be higher than 0.1 but less than 0.6 for reliable results. RNA preparations with A_{260}/A_{280} ratios of 1.9 to 2.0 were considered to be sufficiently

pure for most applications. The integrity of mRNA preparations was further visualized by agarose gel electrophoresis.

3.3 Reverse transcription

The reverse transcriptase (RT) is a multifunctional enzyme with three distinct enzymatic activities: a RNA-dependent DNA polymerase, a hybrid-dependent exoribonuclease (RNase H), and a DNA-dependent DNA polymerase. Since reverse transcriptases are generally derived from retroviruses, the combination of these three activities *in vivo* allows transcription of the single stranded RNA into double stranded DNA for retroviral infection. For reverse transcription *in vitro*, the first two activities are utilized to produce single-stranded cDNA.

Purified mRNA was transcribed to first strand cDNA using the RNase H deficient SuperScript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Briefly, 500 µg/ml oligo $(dT)_{18}$ or sequence specific primer was annealed to 50-500 ng of mRNA by initial heating to 70 °C and slow cooling to room temperature. The first strand cDNA synthesis was performed at 42 °C for 1 h in the presence of DTT. Amplification of some PCR targets required the removal of the RNA complementary to cDNA by adding 2 units of RNase H (Life Technologies).

For cDNA synthesis of samples with very low RNA concentration (1-50 ng), the Sensiscript reverse transcriptase (Qiagen) was used according to the manufacturer's instructions. Since the Sensiscript enzyme has an endogenous RNase H activity, the cDNA could be directly applied to PCR reactions.

3.4 5'-RACE

Rapid amplification of cDNA ends (RACE) is a PCR-based technique which facilitates the cloning of full-length cDNA sequences when only a partial sequence is available. cDNA clones frequently lack the 5' ends due to incomplete reverse transcription. Classical 5'RACE protocols are designed to add adaptor sequences to all 5' cDNA ends including

the truncated or degenerated cDNAs. The major disadvantage of these protocols is that no selection for the full length 5' end is included. These limitations of the classical 5'-RACE method can be overcome by RNA ligase mediated RACE (RLM-RACE). RLM-RACE is designed to ligate RNA adaptor sequences to the capped and thus full length RNA only. The protocol removes the 5' phosphate group of incomplete or fragmentized mRNA molecules by phosphatase treatment and thus inactivates these for ligation. The following decapping of the full length mRNA by pyrophosphatase leaves a 5‘ monophosphate group at complete mRNAs. During the ligation of the synthetic RNA adaptor most of the full length decapped RNA acquires the adaptor sequence, whereas the dephosphorylated RNA is inactivated for this ligation step. Using one gene specific primer in addition to an adaptor specific primer for PCR amplification (after cDNA synthesis), the complete 5' end of the desired cDNA can be obtained.

To clone the 5‘ end of TN-N, FirstChoice RNA Ligase Mediated RACE (RLM-RACE kit, Ambion) was performed according to the manufacturer's recommendations. The mRNA was prepared from kidney and brain and the TN-N specific primer sequence was: 5‘-agcaagccggtgatgtcatag-3‘. Cycling conditions for 5'-RACE were: 94 °C for 30 seconds, followed by annealing at 60 °C for 30 seconds, and 2.5 minutes of elongation at 72°C. These steps were repeated 30 times to amplify a single band specific for the complete 5' end of TN-N.

3.5 3'-RACE

In 3' RACE experiments a defined sequence is added to the 3' end of all mRNA molecules. This modification is achieved by priming the cDNA synthesis with an oligo (dT) primer which contains a specific adaptor sequence. PCR experiments using an adaptor specific primer together with a gene specific primer can amplify the complete 3' end of the desired cDNA.

For 3‘RACE, the first strand cDNA was synthesized from purified mRNA using Superscript II (Life Technologies) reverse transcriptase and an modified adaptor primer (5'-atcgatggtcgacgcatgcggatccaaagcttgaattcgagctc$(t)_{17}$-3'). Subsequent standard PCR with an adaptor-specific primer (5'-atcgatggtcgacgcatgcggatcc-3') and a TN-N specific primer (5'-gccggggatgctctcac-3') yielded a product of 1300 bps that was gel purified, cloned into

pCR2.1, and subjected to sequence analysis. The 3' clone of TN-N contained the FBG domain and a 993 bp untranslated region (UTR).

3.6 Discovery and identification of a cDNA encoding TN-N

Screening of the EST database at the National Center for Biotechnology Information using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) revealed EST sequences with significant homology to known tenascins. One of these EST clones (GenBank accession number W29478) was ordered from the Resource Center/Primary Database (Berlin, Germany) and sequenced to obtain the complete sequence of the 1kb cDNA insert. To connect the sequences of the ESTs W29478 and W35807, two primers were derived (primer1: 5'-atggcaggacagaaattgatggac-3', corresponding to EST W29478; primer2: 5'-tggaaaaggggcagttaaaaatagc-3', corresponding to EST W35807) and applied in a standard PCR experiment to amplify a 4 kb fragment from brain and kidney cDNA. The fragment was gel purified, cloned into pCR2.1 (Invitrogen), and sequenced. The sequence contained a large portion of the novel tenascin including 3 EGF domains and 10 FN III domains. To obtain the complete 5' and 3' ends, RACE experiments were performed.

3.7 Discovery of a splice product of TN-N

An alternatively spliced cDNA of TN-N was identified by RT-PCR that utilized mRNA preparations from hippocampi and kidneys of 3-month-old mice. In these experiments, reverse transcription was primed with the oligonucleotide $(dT)_{18}$. Subsequent PCR with a primer corresponding to FN III domain 1 (5'-accctgcgcaacgtgaagaaagac-3') and a primer corresponding to FN III domain 10 (5'-gacagtgctgctcttctccttcctca-3') simultaneously amplified the full length cDNA and a shorter splice variant of TN-N. The splice variant lacked the third FN III domain of TN-N. All PCR experiments were carried out with Pfu*Turbo* polymerase (Stratagene) according to the manufacturer's instructions to minimize the frequency of point mutations. Cycling conditions were: 95 °C for 1 minute,

followed by annealing at 60 °C for 1 minute, and followed by 4 minutes of elongation at 72 °C. These steps were repeated 40 times.

3.8 Northern blot analysis

Northern blot analysis was performed to detect the size and tissue distribution of mRNA molecules and to detect developmental differences in mRNA levels. The protocol described here resembles the classical capillary blot (Sambrook et al., 1989) to transfer size separated mRNA onto a membrane. The mRNA was detected by hybridization with a radioactively labelled gene specific probe. Briefly, 2 µg of different mRNA samples were resolved in formaldehyde and formamide containing sample buffer and were separated in a formaldehyde agarose gel at 80 V for 3 - 4 h at room temperature. The capillary blot was assembled to transfer the ribonucleotides overnight by 10 × SSC at room temperature to Hybond XL nylon membrane (Amersham Pharmacia). Ribonucleotides were crosslinked to the membrane by UV light exposure. The mRNA was hybridized with two specific cDNA probes coding either for the first and second FN III domain of TN-N or for the second exon of TN-R. The membranes were stripped before reuse by incubation with a boiling 0.1 % SDS solution for 5 minutes.

The distribution of TN-N transcripts in 3-month-old mouse tissues was evaluated using the Mouse Multiple Tissue Northern Blot (Clontech). A 350 bp PCR fragment comprising the region encoding FN III domains 1 and 2 of TN-N was labelled with ^{32}P (1×10^8 cpm/µg) by random priming (Megaprime-Kit, Amersham Pharmacia Biotech) and hybridized to the membrane in ULTRAhyb buffer (Ambion) at 42°C overnight. The blot membrane was washed twice with a high salt solution (2 × SSC, 0.1 % SDS) at 68 °C for 15 minutes and once with a low salt solution (0.1 × SSC, 0.1 % SDS) at 42 °C for 5 minutes. Kodak X-Omat AR films were used to detect the signals after overnight exposure at –70 °C in a cassette equipped with amplifier screens.

3.9 *In situ* hybridization analysis

For *in situ* hybridization analysis, ^{35}S labelled riboprobes corresponding to the TN-N specific 350 bp PCR fragment described above were generated from plasmid templates with the Maxiscript Kit (Ambion) according to the manufacturer's instructions. Cryosections of the respective tissues were fixed with 4 % paraformaldehyde in PBS for 10 minutes at 4 °C, washed twice with PBS for 5 minutes at room temperature, dehydrated by successive incubations in 60 %, 80 %, 90 %, 95 % and 100 % ethanol, chloroform, and 100 % ethanol, and air dried. After 3 hours of incubation with prehybridization solution (750 mM NaCl, 25 mM PIPES, 25 mM EDTA, pH 6.8, 0.1 % Ficoll, 0.1 % polyvinylpyrrolidone, 0.1 % BSA, 0.2 % SDS, 10 mM DTT, 50 % deionized formamide, denatured yeast RNA and sperm DNA, each 250 µg/ml) at 50 °C, the cryosections were transferred to hybridization solution (prehybridization solution with 10 % dextran sulfate and 1×10^7 cpm/ml of the labelled probe) and incubated overnight at 50 °C. Samples were washed in 4 × SSC for 5 minutes at room temperature, incubated in RNase A containing solution (500 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 40µg/ml RNase A) at 37 °C for 30 minutes and washed with the same solution lacking RNase A at 37 °C for 30 minutes. The samples were washed twice with 2 × SSC at 50 °C prior to signal detection by overnight exposure to Kodak X-Omat AR films. Autoradiographic signals in brain were detected by exposure to film for 4 days. For more detailed detection of the signal, the hybridized cryosections were dipped in Kodak-NTB3 emulsion, incubated for 3 weeks, developed, and counterstained with Mayer's hemalum reagent (Merck). Bone and cartilage were visualized by staining with Alizarin-Red S / Toluidine-Blue O (Sigma).

4 Protein analysis

4.1 Protein extraction

Protein extracts were prepared from snap frozen tissues. High protein concentrations were obtained by homogenization of 30 % (w/v) tissue in RIPA buffer (50mM Tris, pH 7.5, 150

mM NaCl, 1 mM EDTA, pH 7.5, 0.2 % NP-40, 1 × Complete Protease Inhibitors (Roche Diagnostics)) at 4 °C. The homogenate was centrifuged at 20,000 × *g* and 4 °C for 30 minutes to remove insoluble components. The protein content of the cleared supernatant was determined and appropriate amounts were subjected to Western blot analysis.

4.2 Determination of protein concentration

To ensure comparable protein concentrations in Western blot analysis, the protein extracts were subjected to protein concentration determination. Protein concentration was determined in 96 well plates using the Micro BCA Reagent (Pierce) according to the manufacturer's instructions.

4.3 Protein analysis by Western blot

Separation of proteins was performed by discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) using the Mini-Protean III system (Biorad). The different proteins were focused on top of the running gel by a 0.8 cm stacking gel containing 5 % (v/v) acrylamide (3,77 ml deionized water, 0.32 ml 1 M Tris (pH 6.8), 0.05 ml 10 % SDS, 0.83 ml 30 % acrylamide:bis (29:1), 0.025 ml 10 % APS, 0.007 ml TEMED), followed by separation of the proteins in a 4.5 cm running gel with 10 % acrylamide (3.92 ml deionized water, 5.26 ml 1 M Tris (pH 8.8), 0.14 ml 10 % SDS, 4.7 ml 30 % acrylamide:bis (29:1), 0.07 ml 10 % APS, 0.007 ml TEMED). The protein samples were boiled for 10 minutes in sample buffer (0.312 M Tris-HCl, pH 6.8, 10 % SDS, 5 % β-mercaptoethanol, 50 % glycerol, 0.15 % bromphenol blue) prior to analysis. The samples were then loaded in amounts of 1 ng to 100 µg per lane and the gel was run at constant 80 V for 10 minutes followed by constant 140 V for approximately 1 h until the bromphenol blue reached the end of the gel.

The separated proteins were transferred to a nitrocellulose membrane (Protean nitrocellulose BA 85, Schleicher&Schüll) using the Mini Transblot apparatus (Biorad) according to the manufacturer's instructions. Proteins were blotted electrophoretically at 4

°C in blot buffer (25 mM Tris-HCl, 192 mM Glycin) at constant voltage (80 V for 2 h or 30 V overnight). A pre-stained marker (BenchMark, Gibco) was used to determine the molecular weight of the separated proteins and to monitor the electrophoretic transfer.
For immunological detection of proteins, nitrocellulose membranes were washed once in TBS (10 mM Tris-HCl, pH 8, 150 mM NaCl) and incubated in 8 ml milk powder solution (2 % in TBS) for 1 h at room temperature. The antibody was diluted in milk powder solution and incubated for 2 h at room temperature with constant agitation. Unspecifically bound primary antibody was removed by washing the membrane 5 times in TBS for 5 minutes each. Secondary antibody was applied in milk powder solution for 1 h and the membrane was washed again 5 times with TBS. Signals were detected using horseradish peroxidase coupled species Ig specific secondary antibodies (Santa Cruz Biotechnology) and ECL reagent (Amersham Pharmacia) on Biomax ML film (Kodak).

4.4 Coomassie staining of polyacrylamide gels

Following the SDS-PAGE separation of proteins, the gels were stained by Coomassie blue (40 % ethanol, 10 % acetic acid, 0.1 % Serva blue R250) for 1 h at room temperature. Unspecific staining was washed out in stripping buffer (0.5 M NaCl, 0.5 M acetic acid) for 2 h at room temperature or until the gel became transparent.

4.5 Densitometric evaluation of band intensities

Band densities were quantified to compare protein amounts present in a single band which were detected by coomassie staining. The Scion Image program (Scion Corporation, USA) was used to analyze the relative band density from a digitized scan of the coomassie staining pattern according to the manufacturer’s instructions.

4.6 TN-N antibody production and immunoblot analysis

A polyclonal TN-N antibody was generated by immunization of rabbits with a peptide (TVPKSRDPKSRYDITC) that corresponded to the second FN III domain of TN-N. The peptide was coupled to key-hole limpet hemocyanin as a carrier protein (Eurogentec). Serum was taken after the third boost and stored at –20 °C.

To determine all bands detected by the antibody, crude protein extracts were prepared for Western blot analysis from snap frozen tissues and the homogenate was centrifuged at 10,000 × *g* and 4 °C for 5 minutes to remove large insoluble components only. Equal amounts (10 to 100 µg) of the supernatants, which contained soluble and insoluble proteins, were subjected to SDS-PAGE (Laemmli, 1970). TN-N was detected with a 1:500 dilution of the polyclonal TN-N antibody.

4.7 Evaluation of the specificity of the TN-N antibody

To evaluate the specificity of the antibody, a 1:500 dilution was incubated 15 minutes at room temperature with 50 µg of the TN-N peptide used for immunization. This incubation abolished the detection of the TN-N specific band at 170 kD. Furthermore, the preimmune serum was used as a control in 1:500 dilutions.

Recombinant TN-N fragments (10 ng purified and concentrated protein) were subjected to Western blot analysis under denaturing conditions using the TN-N antibody. These experiments confirmed the specificity of the antibody for the peptide sequence located in the FN III domain 2 of TN-N.

4.8 Expression and purification of recombinant TN-N fragments

To address the question whether the two splice variants of TN-N exhibit different functions in cell culture assays, a library of recombinant proteins from the FN III region of TN-N was generated.

Subfragments of the TN-N protein were expressed in *E.coli*: the corresponding regions of the cDNA were amplified by standard PCR procedures and cloned into the expression vector pQE30 (Qiagen) to provide the recombinant fragments with an amino-terminal His-tag. Primers were designed to amplify the desired FN III domains of TN-N and to contain appropriate restriction sites to facilitate directional cloning of the amplicons into pQE30. Primer sequences were:

FN III-2-forward: 5'-atatgcatgccctcagcatctacttgccacc-3';
FN III-2-reverse: 5'-tccccccgggtcattctgtcctgccattcaggag-3';
FN III-4-reverse: 5'-tccccccgggtcagctctcccgatcgcctttctc-3';
FN III-5-forward: 5'-atatgcatgcgacatcgacagccccaaaaactt-3';
FN III-7-reverse: 5'-gggaagctttcatggggctttggtgctggtcttc-3'.

TN-N cDNA fragments encoding the second FN III domain were amplified with primers FN III-2-forward and FN III-2-reverse, a fragment corresponding to FN III 5 to 7 was obtained by PCR with primers FN III-5-forward and FN III-7-reverse, and the combination of primers FN III-2-forward and FN III-4-reverse yielded a PCR fragment encoding either FN III 2 to 4 (when full length cDNA was used as a template) or FN III 2 + 4 (when the splice variant cDNA served as a template).

Expression and purification of the recombinant protein fragments in bacteria were achieved following the manufacturer's instructions (The QIAexpressionist, Qiagen). Briefly, recombinant plasmid clones were used to transform competent M15[pREP4] *E.coli* cells. To determine the target protein solubility 10 ml LB medium was inoculated from the glycerol stocks and grown until the OD_{600} was 0.6. Protein expression was induced by adding isopropyl-D-thiogalactopyranoside (IPTG) to 1mM. After 4 h of growth at 37 °C, the bacteria were harvested and lyzed by sonification in buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and centrifuged at 10,000 × *g*. The supernatants and the pellet fraction were analyzed by 12 % SDS-PAGE under reducing conditions and Coomassie staining. All FN III domains were purified from supernatants of large scale preparations as soluble proteins. Fragments were purified by affinity chromatography on a nickel-chelate-resin (Ni-NTA beads, Qiagen). The purified proteins were extensively dialyzed against PBS and concentrated with Centricon filter devices (Millipore). Aliquots were stored at –20 °C and thawn only once at 4 °C directly before use.

5 Cell culture

5.1 Preparation of dissociated hippocampal neurons

The preparation of hippocampal neuron cultures was essentially based on the method described elsewhere (Dityatev et al., 2000) and was performed in cooperation with G. Dityateva. Briefly, the hippocampi of 1 to 3 day old mice were isolated, cut into small pieces, and treated with trypsin (6 mg / 1.8ml) and DNase I (1.5 mg / 1.8 ml) in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution for 10 minutes at room temperature. The cells were dissociated by pipetting up and down with three fire-polished Pasteur pipettes with sequentially smaller diameter. After the dissociation, the cells were centrifuged twice at 100 x *g* for 15 minutes at 4 °C to remove cellular debris and plated at a density of 700 cell/mm^2 on glass cover slips (Assistant, Germany) coated with poly-L-lysine (100 µg/ml) and laminin (20 µg/ml). For the first two days, the neurons were maintained in culture medium (Eagle's MEM containing 5 g/l glucose, 2 mM glutamax I, 100 µg/ml bovine transferring and insulin, 5 µg/ml gentamycin, and 10 % tested horse serum). Starting from day 3, gentamycin was omitted, and the concentration of serum was reduced to 5 %. The culture medium was supplemented with 5 µM cytosine-arabinofuranoside and 2 % B-27 supplement (Life Technologies).

5.2 Indirect immunofluorescence

Dissociated hippocampal neurons (Dityatev et al., 2000) were cultured for 5 to 6 days. Cultures were fixed in 4 % paraformaldehyde for 10 minutes, washed extensively with PBS, and incubated with 1 % BSA for 1 h. The TN-N antibody and the preimmune serum (both diluted 1:500) were incubated with the cultures for 1.5 h at room temperature. Antibody binding was detected after washing with PBS by immunofluorescence labelling

with a Cy3 conjugated secondary antibody (Dianova). Images were taken with the Zeiss laser scanning microscope 510.

5.3 Hippocampal explant culture

Hippocampi were prepared from newborn C57BL/6J mice in dissection medium (powder medium Sigma H 2387 without Ca^{2+} and Mg^{2+}, 4.2 mM $NaHCO_3$, 5.8 mM $MgSO_4$, 10 mM HEPES, 33.3 mM D-glucose, 5 µg/ml gentamycin, 0.3 % BSA, fraction V) at 4 °C. From 400 µm hippocampal slices CA3 region explants were prepared in culture-medium (MEM Eagle Medium, 28 mM D-glucose, 0.1 mg/ml transferring, 0.025 mg/ml insulin, 2µM glutamax I, 5 µg/ml gentamicin, 10% fetal calf serum).
The explants were oriented around the substrate border which was prepared as described elsewhere (Becker et al., 2000). Briefly, glass bottom cell culture plates (MatTek) were successively coated with poly-D-lysine (PDL; 2.5 mg/ml), nitrocellulose (0.8 cm^2/ml dissolved in methanol), and PDL (2.5 mg/ml). TN-N fragments were spotted onto a defined area at a concentration of 100 µg/ml in a mixture with 100 µg/ml rhodamine-coupled BSA (Molecular Probes). Prior to placement of CA3 region explants around the protein coated area, the complete surface was coated with laminin at concentrations of 1.7 µg/ml. The explants were maintained in culture for 4 days before analysis. The substrate border was detected by the fluorescence of rhodamine-coupled BSA (Molecular Probes). Phase contrast microscopy was used to analyze neurite outgrowth and cell migration. Experiments were repeated at least 3 times.

5.4 Fibroblast cell culture

Fibroblasts were cultured to serve as a feeder cell layer for ES cells. Fibroblasts were isolated from 13-day-old mouse embryos which contained a neomycin resistance gene. The blood organs of the embryo were removed and the rest was minced in PBS. The minced tissue was treated with trypsin/EDTA (10 % (w/v) trypsin, 1 mM EDTA in PBS) for 10 minutes at 37 °C. The digestion was stopped by addition of 1 volume MEF medium

(DMEM with glucose and glutamax I, 9 % FCS, 1 x MEM non essential amino acids, 50 IU/ml penicillin, 50 µg/ml streptomycin, all ingredients from Gibco/BRL). The mouse embryonic fibroblasts (MEF) were collected from the supernatant by centrifugation at 350 x *g* for 5 minutes at room temperature, resuspended in MEF medium, and propagated by repeated passages every 3 days (splitted 1:3) for maximally 6 times. Aliquots were stored in liquid nitrogen.

Mitotically inactivated feeder cells were prepared for ES cell culture: MEF cells were grown on gelantinized culture dishes (incubate 0.1 % gelantine in PBS for 1 h at room temperature on the culture dish) until a confluent cell density was reached. An incubation with 10 µg/ml mitomycin C for 3 h at 37 °C in humidified atmosphere blocked the mitosis of the MEFs. The cells were resuspended and seeded at cell densities of 5 x 10^4 cells/cm^2 to serve as feeder cell layers for ES cell culture.

5.5 ES cell culture

Targeted ES cells were used as pluripotent vehicles to deliver their genome which contained the desired mutation. ES cells were received in cooperation with the Nagy Lab (http://www.mshri.on.ca/nagy). The ES cells were prepared and propagated from 3.5-day-old blastocysts of the 129/SV mouse line and cultured at 37 °C in humidified atmosphere on mitotically inactive feeder cells. The culture medium (DMEM with glucose and 25 mM hepes, 15 % ES qualified FCS, 1 x glutamine, 1 x non essential amino acids, 1 mM pyruvate, 50 IU/ml penicillin, 50 µg / ml streptomycin, 0.1 mM mercaptoethanol, 1000 u / ml LIF) was changed every second day in parallel with passage procedures.

After transfection of ES cells with the targeting vector and selection for neomycin/G418 resistance, only cell clones of round shape with sharp borders were selected for further culture procedures and blastocyst injection. This selection is essential to increase the chance of pluripotency of the ES cell clones. Furthermore, the myeloid leukaemia inhibitory factor (LIF) was supplemented to the culture medium to prevent the differentiation of ES cells.

5.6 Transfection of ES cells

The transfection of ES cells was done by electroporation of $1*10^7$ cells with 50 to 100 µg highly pure and linearized targeting vector in electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0,7 mM Na_2HPO_4, 6 mM Dextrose). Two parallel procedures were tested and gave similar results:

1) a single pulse protocol with 250 V and 500 µF
2) a double pulse protocol with 800 V / 3 µF followed by 250 V / 500 µF

Directly after the electroporation, the ES cells were incubated on ice for 5 minutes and 5 x 10^5 cells/cm^2 were seeded on prewarmed culture plates in co-culture with a feeder cells. After 2 days in culture G418 (200-250 µg/ml) was added to the culture medium to select for neomycin resistant clones. ES cell clones were picked after 5-7 days in culture.

5.7 Maintenance of ES cell clones

ES cells which have undergone homologous recombination were stored in liquid nitrogen tanks for more than one year. Approximately $1*10^6$ ES cells were completely resuspended in fresh ES cell culture medium and mixed with 1 volume of freeze medium (50 % FCS (ES qualified), 40 % ES cell medium, 10 % DMSO). The tubes were overlaid with mineral oil and slowly frozen to –80 °C overnight. The following day the tubes were placed into the liquid nitrogen tank.

5.8 Blastocyst injection

Homologously recombined ES cells were identified by Southern blot analysis and PCR. To generate chimeric offspring, 10 to 12 ES cells were injected into pre-implantation blastocysts of C57BL/6J mice and transferred to a host mother. The injections were done in cooperation with Dr. M. Bösl.

Results

The results section is divided into two independent, however thematically related parts.
The first results section describes the full length cloning, analysis of mRNA and protein expression, and functional characterization of a novel tenascin family member, designated tenascin-N (see pages 40-62).
The second part of the results describes the construction and analysis of a mouse line conditionally deficient in tenascin-R (TN-R) expression. (see pages 63-77).

1 Tenascin-N: characterization of a novel member of the tenascin family

1.1 Identification and cloning of TN-N, a novel member of the tenascin family

Mouse *Expressed Sequence Tags* (EST) databases were screened with TN-R, TN-C, and TN-W cDNA sequences to detect EST clones displaying significant homology with members of the tenascin family. A set of promising ESTs was identified and one of them (W29478) was available from a clone collection. The clone was sequenced to determine the complete insert size and sequence. The deduced amino acid sequence of the approximately 1 kb cDNA insert was analyzed with appropriate structure prediction programs and revealed EGF and FN III domains. The cDNA sequence of the putative new tenascin gene was extended by PCR using a primer corresponding to the already characterized clone W29478 and a second primer derived from sequences of other ESTs that showed homology to tenascins (Fig. 3). One combination yielded a PCR product of

4039 bps that was cloned and sequenced. Finally, the missing 5‘ and 3‘ ends of the cDNA were obtained by RACE experiments.

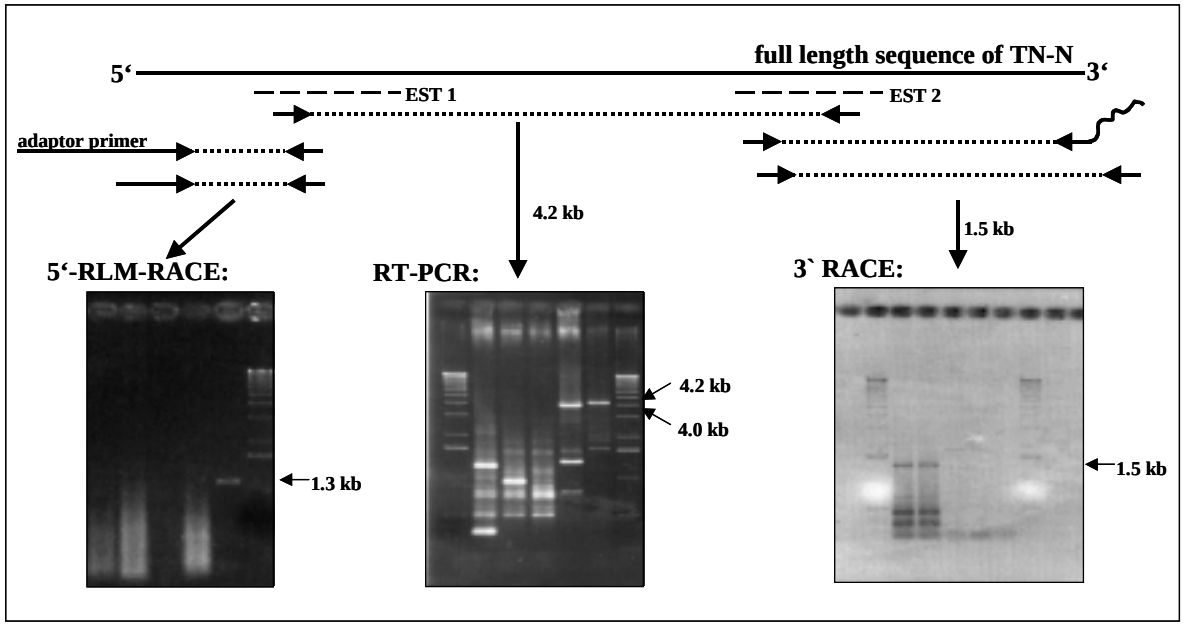

Fig. 3: Identification and cloning of TN-N. Two EST sequences (EST1: W29478; EST2: W35807) are depicted in their relative position to the full length TN-N sequence. RT-PCR experiments with two primer combinations derived from these ESTs resulted in a 4000 bp and 4200 bp band, whereas other primers gave unspecific DNA amplifications. The missing 5’ (1300 bp) and 3’ (1500 bp) ends of the TN-N sequence were cloned by 5’-RLM-RACE and 3’RACE, respectively.

3‘RACE amplified a 1500 bps fragment including the polyA tail and 5’RACE gave rise to a 1300 bps fragment containing the putative start codon and almost 100 nucleotides of 5‘ UTR (Fig. 3).

The combined sequence of the cloned EST insert, RT-PCR and RACE products comprised a total of 5773 bps (Fig. 4). To exclude the possibility that the combined sequence was artefactual, we designed primers to amplify the entire coding region in a single PCR experiment and cloned the respective 4.7 kb fragment to confirm its identity with the assembled sequence (data not shown). The open reading frame of the TN-N cDNA consists of 4683 bps (Fig. 4). The ATG start codon (nucleotides 95-97) is flanked by nucleotides resembling the eukaryotic translation initiation signal (Kozak, 1991). In

addition, an in frame stop codon upstream of the ATG suggests that the full length 5' end has been cloned.

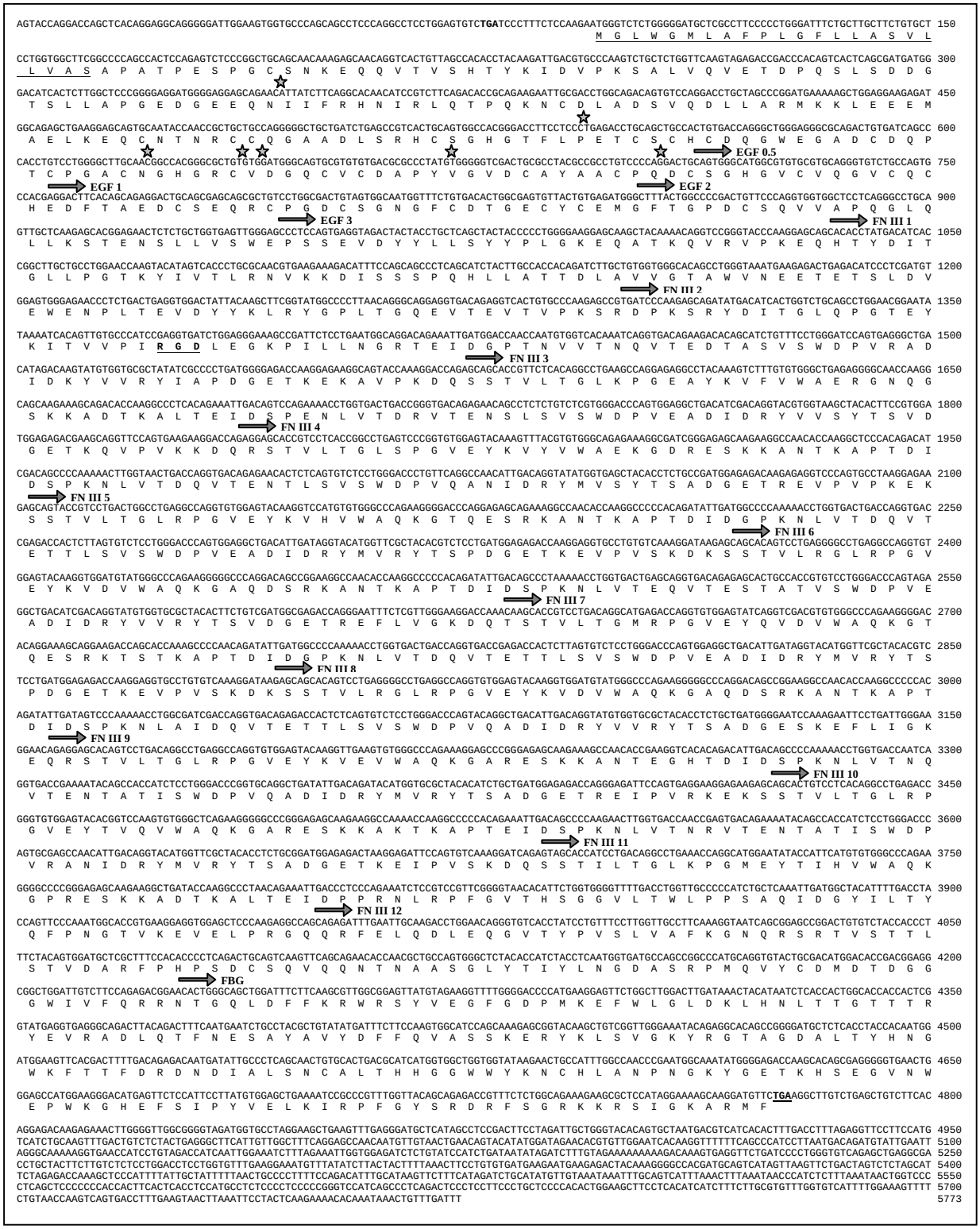

Fig. 4: The cDNA and deduced amino acid sequence of the murine TN-N gene. The open reading frame is preceded by an in-frame stop codon (printed in bold letters). Specific domains and features of the protein are indicated: The hydrophobic signal sequence predicted by the van Heijne method (von Heijne, 1986) is underlined, and conserved cysteine residues at the amino-terminal cysteine-rich region are marked with asterisks; arrows indicate the start of modular domains, which are epidermal growth factor-like domains (EGF 0.5 to 3), fibronectin type III-like domains (FNIII 1 to 12), and a globular domain homologous to fibrinogen (FBG). The RGD motif located in domain FNIII 2 and the TGA stop codon of the open reading frame are printed in bold and underlined. The sequence data have been deposited in GenBank/EMBL/DDBJ under the accession number AF455756.

The TN-N open reading frame is terminated by a TGA stop codon (nucleotides 4775-4777) shortly after the region coding for FBG (Fig. 4).The 3' UTR contains a polyadenylation signal (AATAAA) at position 5757-5762 bp, indicating that the clone covers the complete 3' end of TN-N. The 5' and 3' UTR are 94 bp and 993 bp in length, respectively.

1.2 Analysis of the protein structure of TN-N

The protein encoded by the TN-N mRNA comprises 1560 amino acid residues with a calculated molecular mass of 173 kD. From the deduced amino acid sequence, a number of protein domains can be predicted. The 23 amino-terminal residues constitute a hydrophobic region (Fig. 4) which is predicted to be a signal peptide with a putative cleavage site at position 23 according to McGeoch's algorithm and von Heijne's method (McGeoch, 1985; Nakai and Kanehisa, 1991; von Heijne, 1986). Thus, amino acid 24 is likely to be the first residue in the processed protein. Similar signal peptides have been described for TN-R (Fuss et al., 1993) and TN-W (Weber et al., 1998) and can also be predicted for TN-C with the algorithms mentioned above.

The amino-terminus of the mature protein consists of a cysteine-rich segment of 131 residues that contains 7 cysteine residues at conserved positions when compared to TN-C, TN-R, and TN-W (Jones et al., 1989; Fuss et al., 1993; Weber et al., 1998). The region of the EGF repeats begins with a single incomplete EGF domain of 16 residues followed by

3 complete EGF domains with a length of 31 residues each. Twelve FN III domains are positioned carboxy-terminally of the EGF repeats, and a FBG domain is present at the carboxy-terminus of the TN-N protein (Fig. 4). All of these modular elements are found in other tenascin family members (Fig. 5) (for review see: Jones and Jones, 2000).

Nine out of the 12 FN III domains in TN-N show homologies of approximately 70 % and thus are significantly more related to one another than to FN III domains of other tenascins. A special feature of TN-N which is unique among the tenascin family is the occurrence of two identical FN III domains at positions 6 and 8 (Table I and Fig. 5).

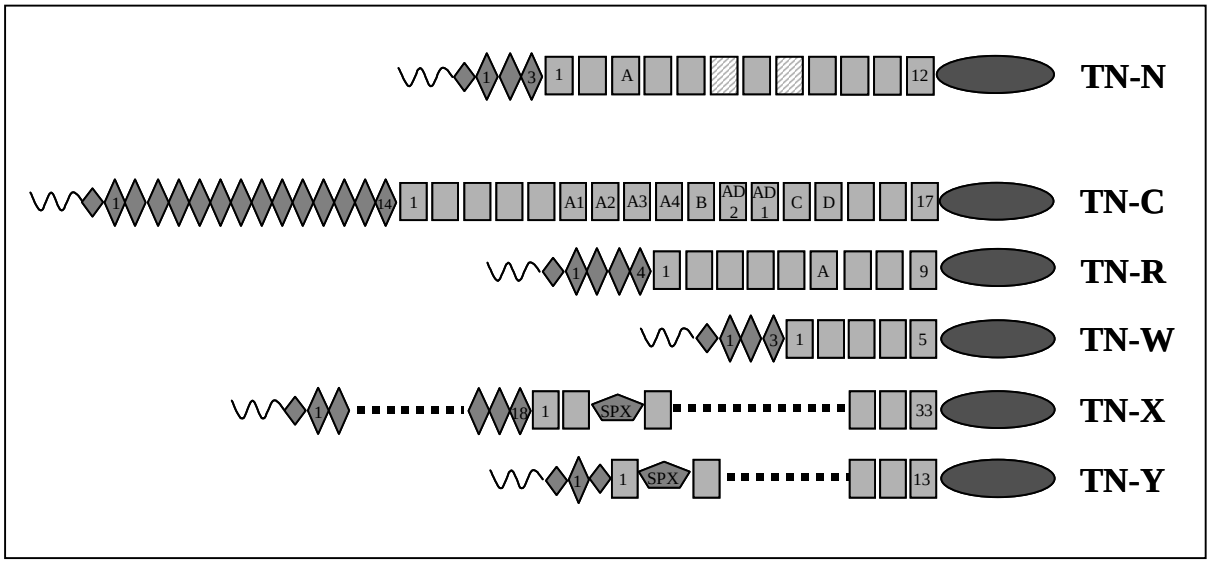

Fig. 5: Schematic representation of the domain structure of TN-N compared to known tenascins. The depicted modular structure is characteristic for tenascins. All tenascin proteins share an amino-terminal cysteine-rich region (curved line), EGF repeats (filled rhombi), FNIII domains (rectangles), and a carboxy-terminal FBG domain (oval). Similar domains are indicated by numbers, whereas letters denote alternatively spliced domains. The identical FN III domains 6 and 8 in TN-N are marked by striped rectangles. In TN-X and TN-Y the pentagons symbolize serine and proline rich regions (SPX) that are present only in these tenascins.

Table I: Sequence homologies of the FN III domains of TN-N

FN III domain	1	2	3	4	5	6	7	8	9	10	11	12
1	100	47	38	42	41	34	32	34	35	34	34	30
2		100	37	38	37	39	30	39	32	35	33	<30
3			**100**	**65**	**65**	**70**	**66**	**70**	**62**	**64**	**65**	36
4				**100**	**76**	**75**	**73**	**75**	**74**	**70**	**70**	35
5					**100**	**83**	**76**	**83**	**77**	**84**	**75**	36
6						**100**	**76**	**100**	**76**	**78**	**71**	38
7							**100**	**76**	**76**	**78**	**71**	35
8								**100**	**76**	**78**	**71**	38
9									**100**	**76**	**67**	36
10										**100**	**83**	33
11											**100**	33
12												100

Listed are the percentages of identical amino acid residues between each pair of FN III domains of TN-N as determined by the BLAST 2 sequences program. Bold numbers show that homologies between FN III domains 3 to 11 are significantly increased compared to FN III domains 1, 2, and 12. The underlined value highlights the identity of FN III domains 6 and 8.

1.3 Phylogenetic relationship between TN-N and other tenascins

Comparison of the entire amino acid sequence of the mouse TN-N protein with mouse TN-C, rat TN-R, zebrafish TN-W and chicken TN-Y revealed only low homologies to these tenascins. The general homology of TN-N domains with those of TN-W is slightly higher than the homologies with other tenascins (Table II). This is confirmed by the analysis of the ancestral relationship of the tenascins by the Clustal 5 method (Higgins and Sharp, 1989). The phylogenetic tree resulting from this analysis is shown in Fig. 6 and presents TN-N on a separate branch closer to TN-W than to TN-C and TN-R, whereas TN-X and TN-Y seem to be the most distant relatives of TN-N.

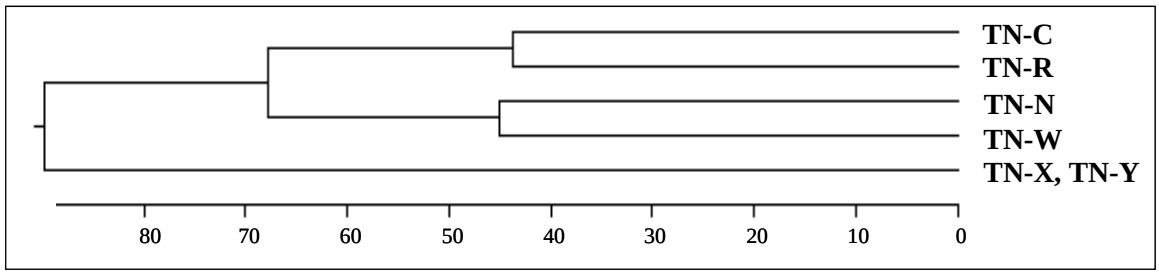

Fig. 6: Phylogenetic relationship of the tenascin protein family. A phylogenetic tree illustrates the ancestral relationship of the tenascin family members. The scale beneath the tree measures the phylogenetic distance between sequences, and the units indicate the number of substitution events. The tree was generated based on a multiple sequence alignment of amino acid sequences using the Clustal 5 method (Higgins and Sharp, 1989).

In conclusion, TN-N is not an orthologue of any other known tenascin protein. This is confirmed by the comparison of the TN-N specific protein domains with those of the other tenascin family members (Table II). When subdomains of TN-N are compared to corresponding arrays of domains in other tenascins, no striking similarities are found. The highest level of similarity regarding the EGF domains is seen with TN-C. In comparison, the EGF domains of orthologues of TN-C from mouse and chicken share higher values of up to 85% identical and up to 96% similar amino acids (not shown). The homologies of the EGF domains of TN-N compared to those of other tenascins are significantly lower (Table II). However, sequence similarity of the FN III domains again suggests a closer relationship between TN-N and TN-W rather than between TN-N and TN-C or TN-R. Finally, the FBG domain of the tenascins is the most conserved domain and the mouse and chicken TN-C orthologues show 83 % identical and 94 % similar amino acids in this domain. The FBG domains of TN-N and TN-W share only 64 % identical residues (Table II). All these data confirm that TN-N is a novel homologue of the tenascin family. Furthermore, the number of EGF and FN III domains varies between tenascin molecules, but is more or less constant between orthologues if the alternatively spliced FN III domains are not taken into account (Erickson, 1993; Chiquet-Ehrismann et al., 1994; Erickson, 1994; Chiquet-Ehrismann et al., 1995). Although TN-N displays the same number of EGF domains as TN-W, the number of FN III domains is unique among the tenascins.

Table II: Amino acid sequence comparison of TN-N domains with TN-C, TN-R, TN-W, and TN-Y.

TN-N domains	TN-C	TN-R	TN-W	TN-Y
Cys.-rich	26	37	33	n.s.
EGF-1	44-70	48-59	51-62	n.s.
EGF-2	48-66	48-59	48-55	51
EGF-3	48-63	44-59	51-59	59
FN III - 1	28-34	28-35	31-46	27
FN III - 2	30-39	30-38	35-44	n.s.
FN III - 3	28-38	32-36	33-52	n.s.
FN III - 4	25-42	30-38	30-55	26-31
FN III - 5	23-41	28-38	32-52	24-27
FN III - 6	25-39	29-39	26-53	24-27
FN III - 7	22-42	27-37	32-52	22-24
FN III - 8	25-39	29-39	26-53	24-27
FN III - 9	23-40	28-37	30-52	24-25
FN III - 10	25-43	25-38	30-56	24
FN III - 11	25-43	25-39	30-55	22-24
FN III - 12	26-38	28-34	36-43	41
FBG	56	58	64	48

Listed are the percentages of identical amino acids between single TN-N domains and protein sequence of a tenascin family member as calculated by the BLAST 2 sequences program. A single number indicates only one match between the TN-N domain and the respective member of the tenascin family. Where the TN-N domain matched more than one region of the tenascin molecules, the lowest and highest values are given. No significant similarity is indicated by n.s..

1.4 The genomic structure of the human TN-N

The existence of TN-N in other species than mouse was confirmed by BLAST data base searches. The complete coding sequence of the mouse TN-N was used to screen for homologous sequences in human genomic data bases. Since the human genome has been sequenced to completion, it was expected to find a human orthologue to the mouse TN-N. The search revealed a highly homologous genomic area at chromosome 1 in position q23.3-24.3 with a size of approximately 71000 bp (Fig. 7). The genomic structure is composed of 18 exons and 17 introns. The exon structure allowed to deduce a potential mRNA sequence which contained a total length of 4750 bp with an open reading frame of

3885 bp and thus converted into an 1295 aa protein. From this protein sequence 3.5 EGF domains, 9 FN III domains and a single fibrinogen-like sequence could be predicted with up to 84 % similar amino acids to the mouse TN-N protein.

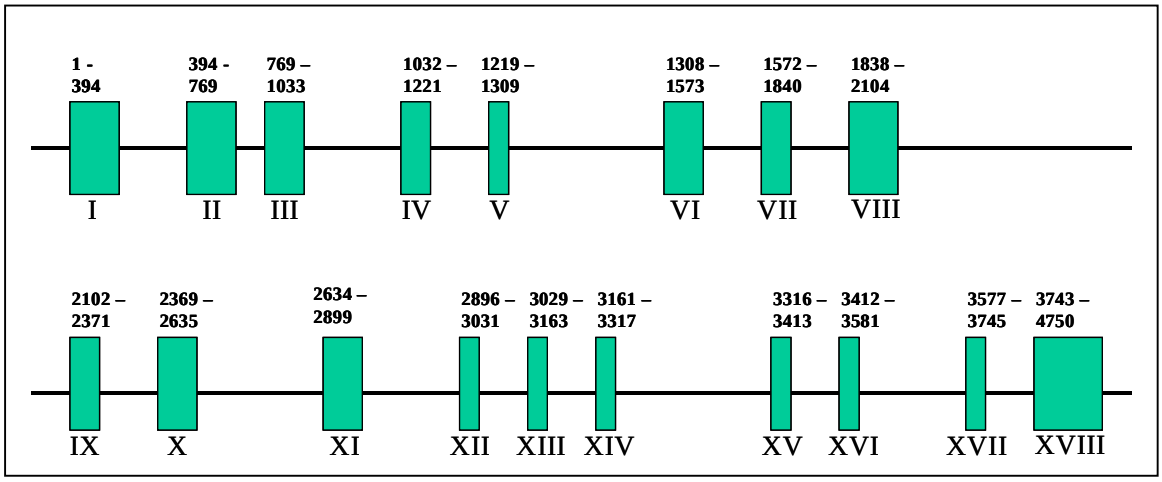

Fig. 7: Schematic representation of the genomic structure of the putative human TN-N. The *in silico* predicted genomic architecture of the human TN-N contains 18 exons (I to XVIII) and 17 introns. The deduced mRNA can be predicted from this structure, has a total length of 4750 bp, and shows an open reading frame of 3885 bp.

1.5 Northern blot analysis and discovery of a splice variant of TN-N by RT-PCR

The distribution of TN-N specific transcripts was investigated by a mouse multiple tissue Northern blot. A 350 bp cDNA probe corresponding to the first and second FN III domain of the mouse TN-N was used for hybridization. A mRNA species of approximately 6 kb was strongly detected in adult kidney and to a lower extent in adult spleen after overnight exposure to film (Fig. 8A). In brain, a hybridization signal was hardly detectable after overnight exposure. In a comparable Northern blot analysis of brain tissues a TN-N signal could be detected after 5 day exposure (not shown), suggesting a low transcript level compared to kidney and spleen. To confirm the Northern blot signal of TN-N in the brain,

RT-PCR was performed with adult hippocampal mRNA and primers designed to amplify a 2300 bp fragment of the cDNA encoding FN III domains 1 to 10. The RT-PCR yielded also a second product of 2000 bp (Fig. 8B), which was cloned and sequenced. It was identified as a splice variant of the larger size TN-N cDNA. This shorter isoform lacked the region encoding the third FN III domain.

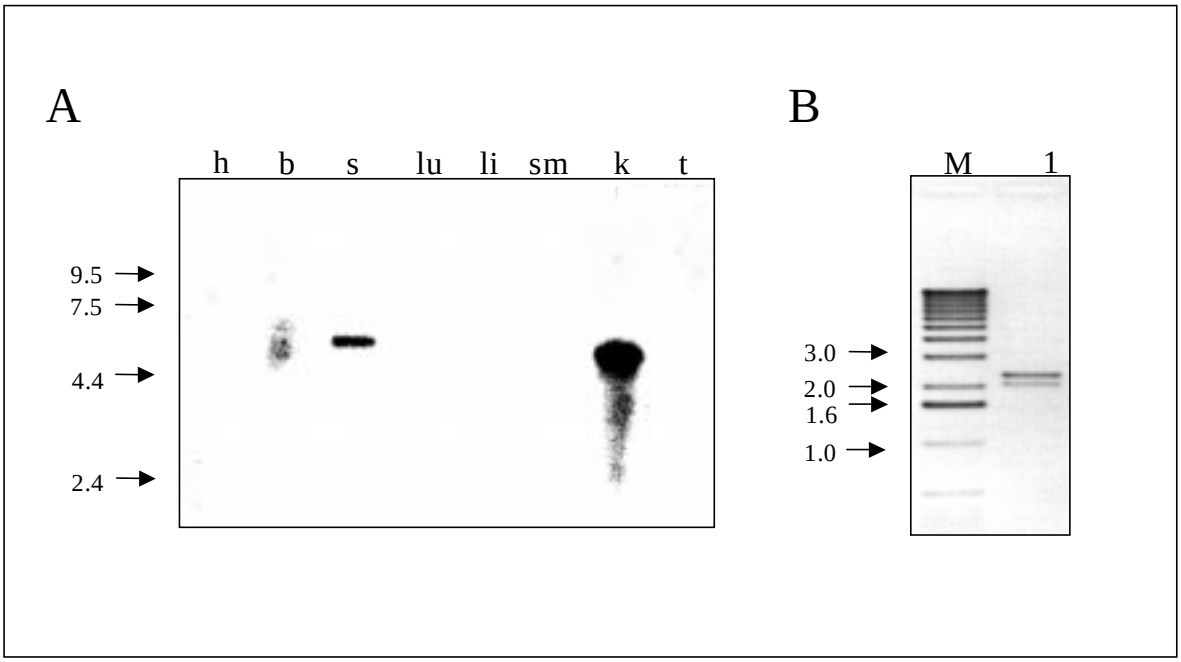

Fig. 8: Expression profile of TN-N mRNA and detection of an alternatively spliced TN-N transcript. (A) The distribution of TN-N specific transcripts was analyzed with a multiple tissue Northern blot containing mRNA preparations from heart (h), brain (b), spleen (s), lung (lu), liver (li), skeletal muscle (sm), kidney (k), and testis (t). In each lane 2 µg mRNA was loaded. (B) The amplification of two TN-N specific products obtained by RT-PCR of adult hippocampus mRNA (1) demonstrates the presence of two alternatively spliced TN-N transcripts. The shorter form lacks the third FN III domain. The size of DNA marker fragments (M) is indicated in kb.

1.6 Immunoblot analysis of TN-N expression

To investigate TN-N expression at the protein level, a polyclonal antibody was generated. The TN-N antibody was directed against a peptide which corresponded to the second FN III domain of the TN-N protein. Immunoblots of crude tissue extracts from brain and

kidney of adult mice detected a protein both in kidney and in brain with a mass of approximately 170 kD (Fig. 9A), which is in accordance with the calculated molecular weight of TN-N.

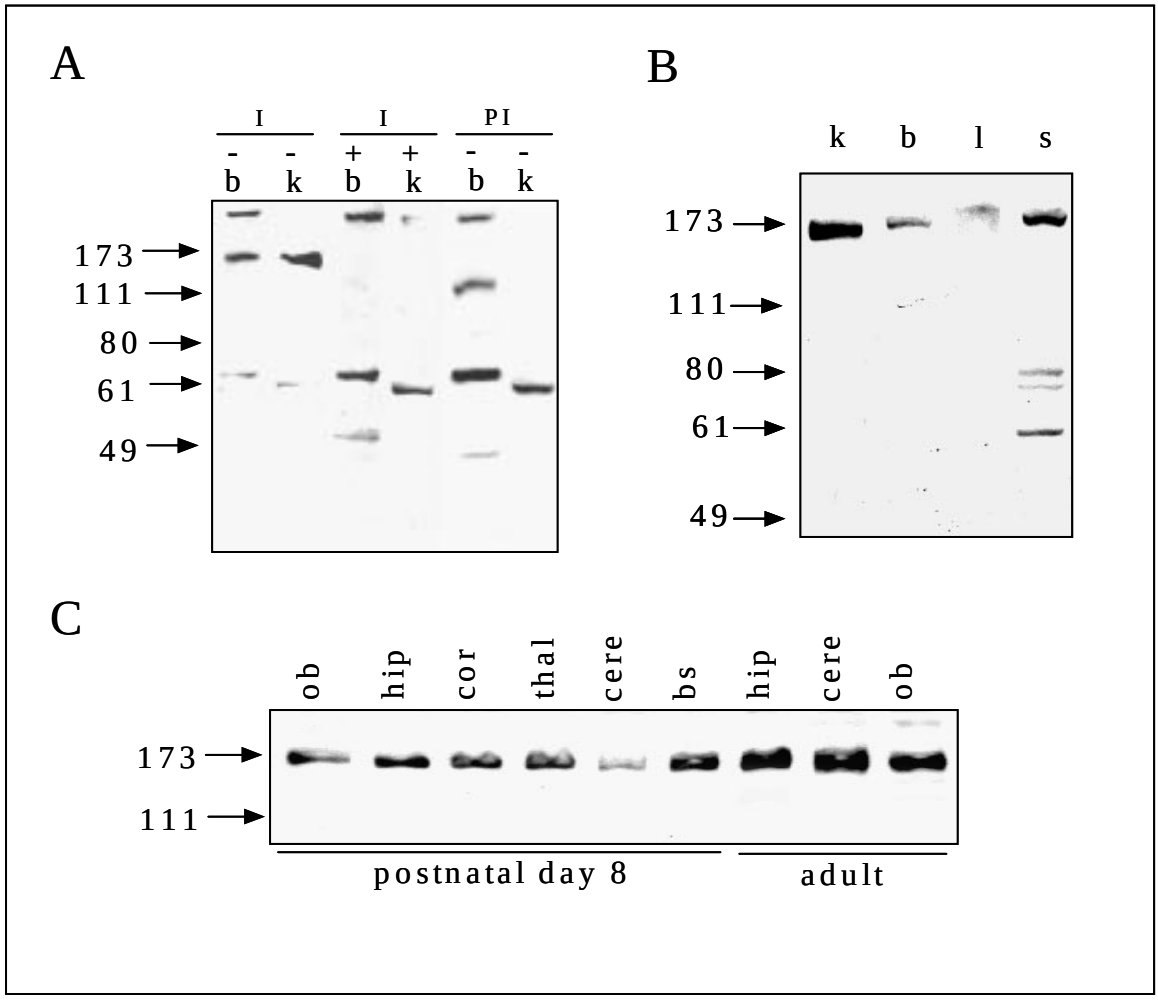

Fig. 9: Characterization of the TN-N antibody and immunoblot detection of the TN-N protein in several mouse tissues. (A) 50 µg of crude protein extracts from brain (b) and kidney (k) were subjected to Western blot analysis using a polyclonal antibody against a TN-N derived peptide. Except for the 170 kD band, all other bands obtained with the antibody (-) were not related to TN-N, since preincubation of the antibody with 50 µg of the TN-N peptide used for immunization (+) did not abolish these signals. (B) Detection of TN-N in 50 µg of fractionated proteins from kidney (k), brain (b), liver (l), and spleen (s). (C) Fractionated protein extracts (100 µg) of different regions of the brain (ob, olfactory bulb; hip, hippocampus; cor, cortex; thal, thalamus; cere, cerebellum; bs, brain stem) from 8-day-old and adult mice.

The reactivity of the TN-N antibody with two other proteins of higher and lower molecular weights could not be eliminated by preincubation of the antibody with the peptide used for immunization. This preincubation completely abolished the TN-N specific signal at 170 kD, even after prolonged exposure to film (Fig. 9A left and middle panel). All non-specific bands and an additional 120 kD protein were also detected with the preimmune serum (Fig. 9A, right panel) and could be removed by an additional high speed centrifugation step of the crude tissue extracts (Fig. 9B). The immunoblot analysis confirmed the Northern blot analysis: strongest TN-N expression was detectable in kidney followed by spleen and brain. TN-N expression was also examined in subregions of the brain from adult and 8-day-old mice. Highest expression was found in hippocampus, cerebellum and olfactory bulb of adult brains, whereas the TN-N protein was generally less abundant in 8-day-old mice (Fig. 9C). The most pronounced age difference could be observed in the cerebellum.

1.7 *In situ* hybridization analysis of the TN-N expression

To investigate the tissues expressing TN-N in more detail, *in situ* hybridization experiments on cryosections of adult kidney, spleen and brain were performed. Furthermore, complete embryos of different developmental stages were analyzed.

1.7.1 *In situ* analysis of the mouse kidney

The analysis of hemisected adult kidneys revealed strong expression of TN-N in the outer medulla and a weaker one in the inner medulla while the cortical zone remained completely negative (Fig. 10A to C). More specifically, in the inner and innermost stripe of the outer medullary zone multiple distinct areas with a strong hybridization signal were separated by tissue areas with a much weaker silver grain development in the emulsion. Microscopic inspection of successive unlabelled frozen sections which were stained with hemalum clearly revealed that the heavily labelled areas correspond to tubular structures of descending and ascending limbs of long loops of Henle and collecting ducts, while the

much weaker labelled areas between them are related to vascular bundles and descending limbs of short loops of Henle (Fig. 10C) (Kriz and Koepsell, 1974). The localization of autoradiographic hybridization signals to epithelial tubular structures of the outer medullary zone was substantiated by hybridization experiments with DIG labelled probes (data not shown). First expression of TN-N in the medulla could be detected in postnatal day 10 longitudinal murine kidney sections. In postnatal day 7 mice, however, the outer medulla is negative for TN-N transcripts. In experiments designed to find differences in the expression profiles of the two splice variants, we used a probe that detected all TN-N splice variants (the probe coded for the first and second FN III domain) and compared it to a probe detecting only the full length TN-N (this probe coded for the third FN III domain only). It turned out that there was no obvious difference in the expression profiles of the two TN-N isoforms (data not shown).

1.7.2 *In situ* analysis of mouse spleen tissues

In situ hybridization analysis of the adult spleen showed a specific occurrence of TN-N in the white pulp (Fig. 10D to F) consisting of lymphoid tissue. Predominantly the periarterial lymphatic sheath (PALS) of the white pulp, representing the T-cell-zone, showed a spotted expression pattern (Fig. 10F). This spotted pattern suggested a cell type specific localization of TN-N transcripts, probably in macrophages or dendritic cells of the PALS. Consecutive cryosections were stained with hemalum to detect cell nuclei and enabled the identification of lymphocytes and lipopigment-rich macrophages. The latter celltype was characterized by the brownish colour after hemalum staining (Fig. 10H). The distribution pattern of PALS specific macrophages was similar to the signal detected by the ^{35}S labelled probe (Fig. 10G). To confirm this observation we compared the number of PALS specific macrophages with the amount of signal spots visible from radioactively labelled cryosections and found similar values. We thus conclude that the TN-N mRNA is predominantly expressed in resident macrophages of the PALS. In B-cell lymphoid follicles, as well as in the splenic red pulp, no signal was detectable. Furthermore, the lack of signals in the white pulp at postnatal day 7 (not shown) supports the idea that the TN-N expression is associated with a mature and functioning immune system. In comparison to

the signal in the kidneys, the overall intensity of the labelling appeared weaker in the spleen, which is in accordance with the Northern blot analyzes (Fig. 8A).

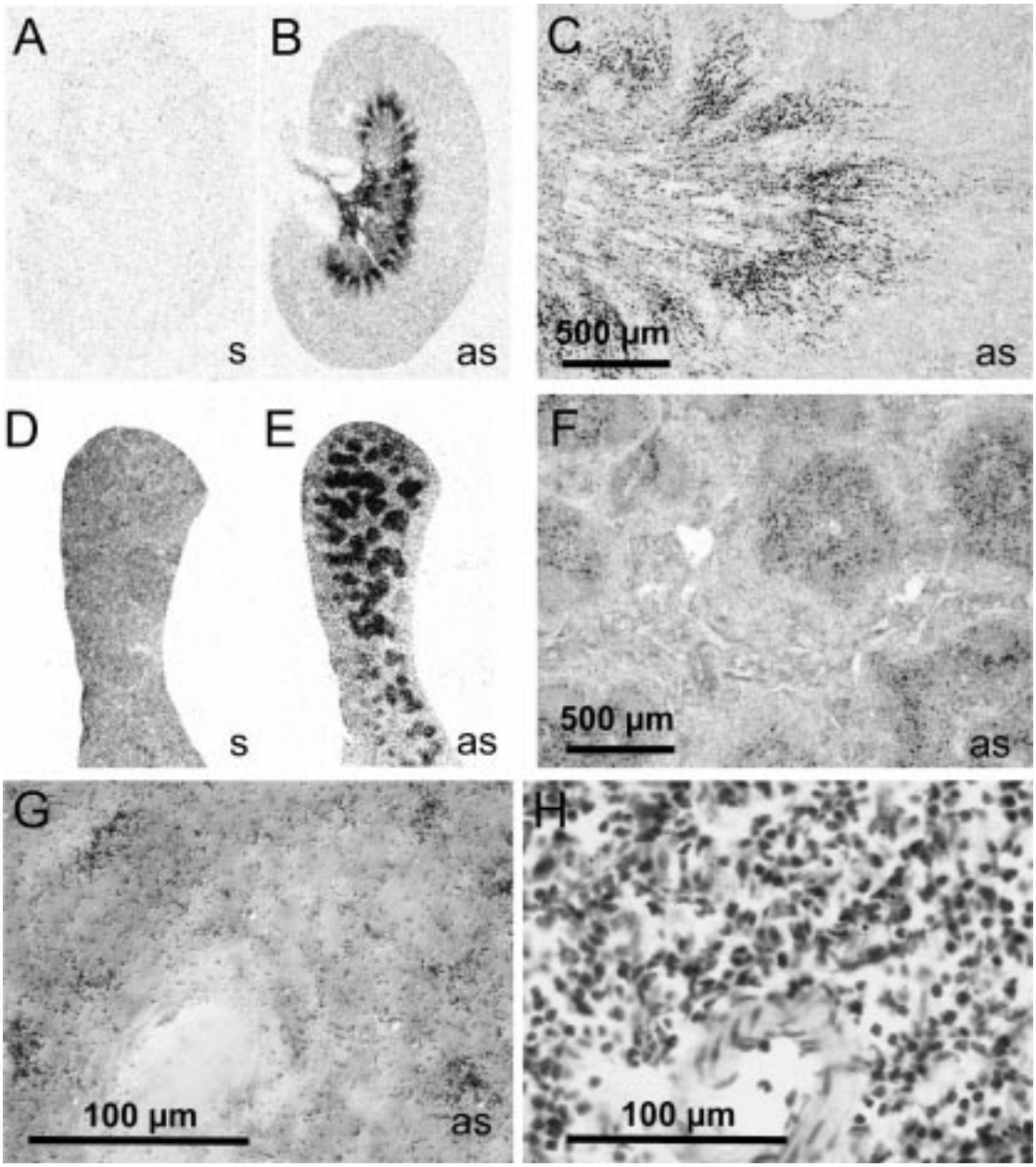

Fig. 10: *In situ* hybridization analysis of TN-N expression in kidney and spleen. ^{35}S labelled sense (s - A, D) and antisense (as - B, C, E, F, G) riboprobes were hybridized to cryosections of tissues from 3-month-old mice. Sections shown in C, F, G, and H were counterstained with hemalum to visualize cell nuclei. In the kidney (A - C) hybridization signals are restricted to structures of the outer and inner zone of the medulla. In the spleen (D - H) the white pulp shows a strong hybridization signal (E) which can be recognized as a dotted pattern in the periarterial lymphatic sheath (PALS) at higher magnification (F).

The distribution of silver grains in the PALS (G) corresponds to that of lipid-rich PALS macrophages (brownish cells, H).

1.7.3 *In situ* analysis of prenatal developmental stages of the mouse

The investigation of prenatal stages of mouse development revealed expression of TN-N mRNA in several unrelated anatomical structures. A TN-N specific signal was first detected in the developing skeletal system at embryonic day 14 (E14) when densely packed silver grains were obviously related to the initial appearance of ossification centers. On E16 the vertebral column, thoracal ribs, the clavicles, part of the sternum, the diaphyses of long bones such as humerus and femur, mandibular and maxillary primordia, primordia of teeth, and the temporal and parietal bone primordia of the skull were strongly labelled (Fig. 11A to C). At higher magnification the hybridization product could be assigned to either mesenchymal / perichondral tissue surrounding skeletal elements, e.g. ribs (Fig. 11D and E), sternum, humerus and vertebral column, or mesenchymal tissue structures which subsequently undergo membranous ossification such as maxilla and premaxilla as well as os frontale and parietale of the skull. On postnatal day 0 (P0), the expression pattern of the skeletal system remained principally unchanged, but at these time points the labelling signal was strongly intensified due to the to progression of the ossification process. In addition to the predominantly periostal pattern of the earlier stages (E14, E16), now more interior parts adjacent to the cortical layer of the diaphyses of long bones (Fig. 11H and I), vertebral bodies, and ribs exhibited a clear hybridization signal. This signal corresponded to the beginning formation of cancellous bone tissue (Fig 11J). Thus, TN-N expression reflects the general progression of ossification irrespective of membranous or enchondral modes of bone tissue formation. In addition to the skeletal system, a consistent yet less intense hybridization signal was detected at E16 and P0 along the intestinal tract including oesophagus and large bowel. Microscopical inspection allowed to assign the label to the smooth muscle layer of the intestinal tract, and accordingly a comparatively weak labelling was seen along the wall of large blood vessels such as the aorta and pulmonary artery. At P0, a further intestinal TN-N localization emerged in the apical parts of the now finally differentiating villi of the small intestine

presenting a dense silver grain accumulation in the lamina propria beneath the apical enterocytes. Apart from these more systemic expression patterns, distinct and localized foci with a strong hybridization signal could be observed during the murine fetogenesis in the primordia of the upper and lower lips, in the developing nostrils, and at the dorsal surface of the tongue (Fig. 11J and K). Here, the hybridization pattern was reminiscent of the formation of filiform, fungiform, foliate and vallate papillae of the tongue epithelium. Also remarkable was a moderate hybridization signal in the ectoderm of the immature cornea of the eye. Perinatally, when cornification of the epidermis starts, a pronounced signal appeared in the epidermal stratum spinosum of the skin of the back.

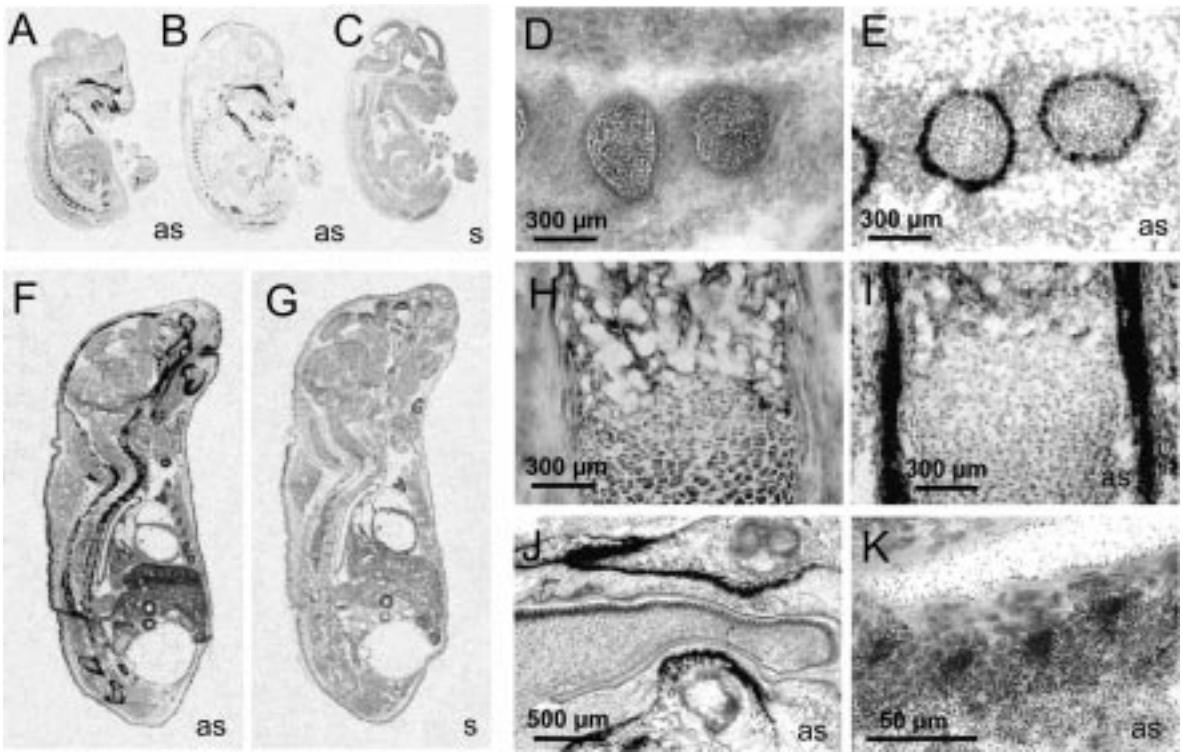

Fig. 11: *In situ* hybridization analysis of TN-N expression during embryonic development. TN-N specific, ^{35}S labelled antisense (as - A, B, E, F, I, J, K) and sense (s - C, G) riboprobes were hybridized to cryosections of complete embryos from day 16 (A to E) and 19.5 (F to K) post conception. Alizarin red S / toluidine blue O staining was used to visualize differentially cartilagnous and osseous tissues (D, H). **E16**: Sagittal and parasagittal sections show strong positive hybridization in skeletal elements of the vertebral column, thorax, cranium and extremities (A, B). A strong hybridization signal is also present in the region of the oral orificium. A longitudinal section of the oesophagus (A) and several cross sections of intestines (A,B) show a less intense hybridization signal. Higher magnifications of cross sections of rib primordia (E) demonstrate intensive hybridization of perichondrial tissue, while the cartilage rib models remain negative.

E19.5: Areas of compact and cancellous bone tissue formation of a sternal sternebrum are positively labelled (I). Beside positive labelling of mandibular and maxillary bones, the mucosa of the dorsal tongue surface exhibits a distinct hybridization signal (J). Higher magnification demonstrate dense accumulations of hybridization label in the connective tissue beneath the epithelium of the developing papillae (K).

1.7.4 *In situ* analysis of the mouse brain

In situ hybridization analysis of cryosections of adult mouse brain revealed a distinct expression pattern in regions and layers with high neuronal cell density. In frontal sections of the forebrain, predominantly the regions rich in neuronal cells, such as cerebral cortex, gave strong signals in contrast to the white matter of the corpus callosum (Fig. 12A). This observation was confirmed by examination of more posterior sections including the hippocampus and part of the midbrain (Fig. 12B) or in sections through the hindbrain with cerebellum and medulla oblongata (Fig. 12C). At higher magnifications of the cerebral cortex and corpus callosum (Fig. 12I) a predominant occurrence of TN-N transcripts was again found in neuronal cell bodies, but not in the white matter of corpus callosum. The autoradiographs also revealed pronounced signals in the cerebellum and choroid plexus, which was strongly labelled compared to e.g. medulla oblongata. TN-N mRNA was most abundant in the Purkinje and granule cell layers and not detectable in the molecular layer of the cerebellum (Fig. 12J). The Purkinje and granule cell layers were as intensely labelled as the choroid plexus (Fig. 12J and K). The autoradiographs of brain were obtained after exposure to film for 4 days, indicating a significantly weaker mRNA level of TN-N compared to kidney and spleen for which overnight exposures gave sufficient signal intensities.

1.8 Immunocytochemical analysis of TN-N expression in neuronal cell cultures

The results of *in situ* hybridization experiments suggested that TN-N transcripts were abundant in neuronal cells (Fig. 12A to D). To confirm this observation by *in vitro* investigations, we cultured dissociated hippocampal neurons of newborn mice for 5 to 6 days and applied the TN-N antibody to visualize the TN-N protein on non-permeabilized

neurons by laser scanning microscopy. The TN-N antibody labelled neurons and their processes, whereas astrocytes showed only very faint background staining (Fig. 12G and H). The specificity of the TN-N antibody was confirmed by negative staining obtained with the preimmune serum (Fig. 12E and F). The specific localization of TN-N to cultured neurons confirmed the *in situ* analysis.

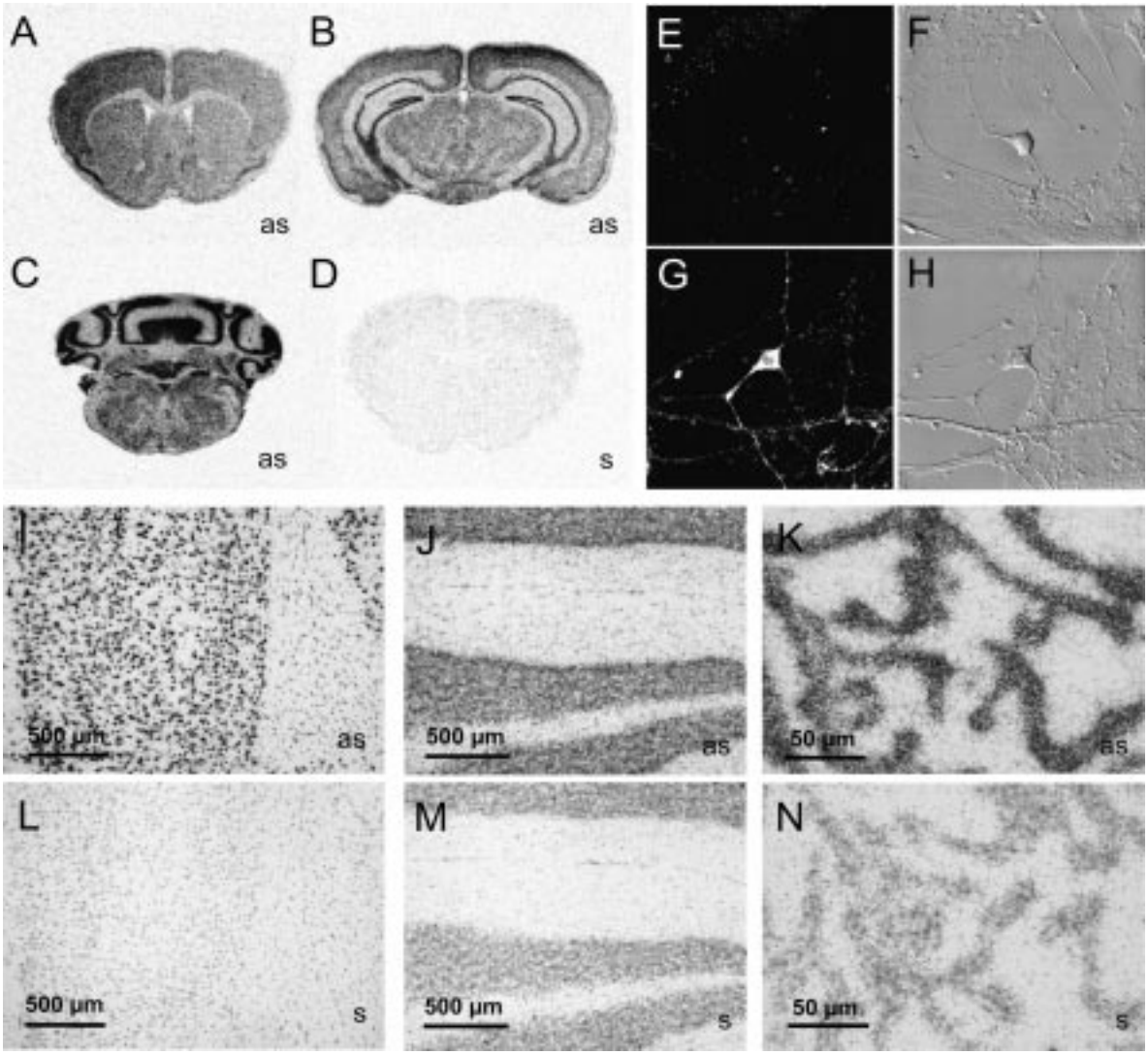

Fig. 12: *In situ* hybridization analysis of brain sections and immunofluorescence labelling of hippocampal neurons. ^{35}S labelled TN-N specific antisense (as - A, B, C, I, J, K) and sense (s – D, L, M, N) riboprobes were hybridized to frontal cryosections of 3-month-old mice. Note that the autoradiographic detection (A, B, C) revealed staining in layers of the brain which predominantly consist of neurons, e.g. pyriform cortex, neocortex, thalamus, and granule cell layer, whereas white matter structures of corpus callosum, hippocampus and cerebral peduncles are spared. These observations are confirmed by higher magnification of the neocortex and corpus callosum (I), the cereballar

cortex including Purkinje cells and granule layer (J), and the choroid plexus (K). Sections used for micrographs at higher magnifications were counterstained with hemalum to reveal the distribution of neuronal and glial perikarya (I to N). Immunofluorescence labelling of dissociated hippocampal neurons were incubated with preimmune serum (E) or TN-N antibody (G) and demonstrate specific staining of neurons and their processes. The micrographs in F and H represent the phase contrast images corresponding to E and G, respectively.

1.9 Production of recombinant TN-N domains

To investigate the functional roles of TN-N and to distinguish possible functional differences between the two TN-N isoforms, recombinant protein fragments of TN-N were expressed in *E.coli.* Four different protein fragments of the FN III region were produced which surrounded the spliced domain of TN-N, including the FN III domains 2 to 4, domains 2 + 4 (lacking the spliced domain 3), domain 2 only, and, as a control, domains 5-7 (Fig. 13A). The protein fragments carried an amino-terminal 6 x His tag and could thus be purified in a single step as soluble proteins (Fig. 13B). The purity of the recombinant proteins as judged from densitometric analysis of Coomassie-stained gels (Fig. 13B) was at least 97%. The apparent molecular masses visible in Fig. 13 were in accordance with the molecular masses calculated from the protein fragment sequences. The purified fragments were extensively dialyzed against PBS and concentrated prior to application to cell culture experiments. The yield of purified proteins after elution from Ni^{2+}-ion coupled agarose, dialysis, and concentration was between 5 and 10 mg/l of bacterial culture. The TN-N antibody was used to detect the recombinant protein fragments by Western blot analysis (Fig. 13C). The fragments corresponding to FN III domains 2 to 4 and 2 + 4 of TN-N showed strong bands of expected sizes. In contrast, when the recombinant protein corresponding to FN III 5 to 7 was loaded, no signal was obtained (Fig. 13C). Since the antibody was directed against a peptide located within FN III domain 2, these experiments demonstrate the specificity of the antibody. Furthermore, no degradation products were detected, confirming the integrity of the expressed proteins (Fig. 13C).

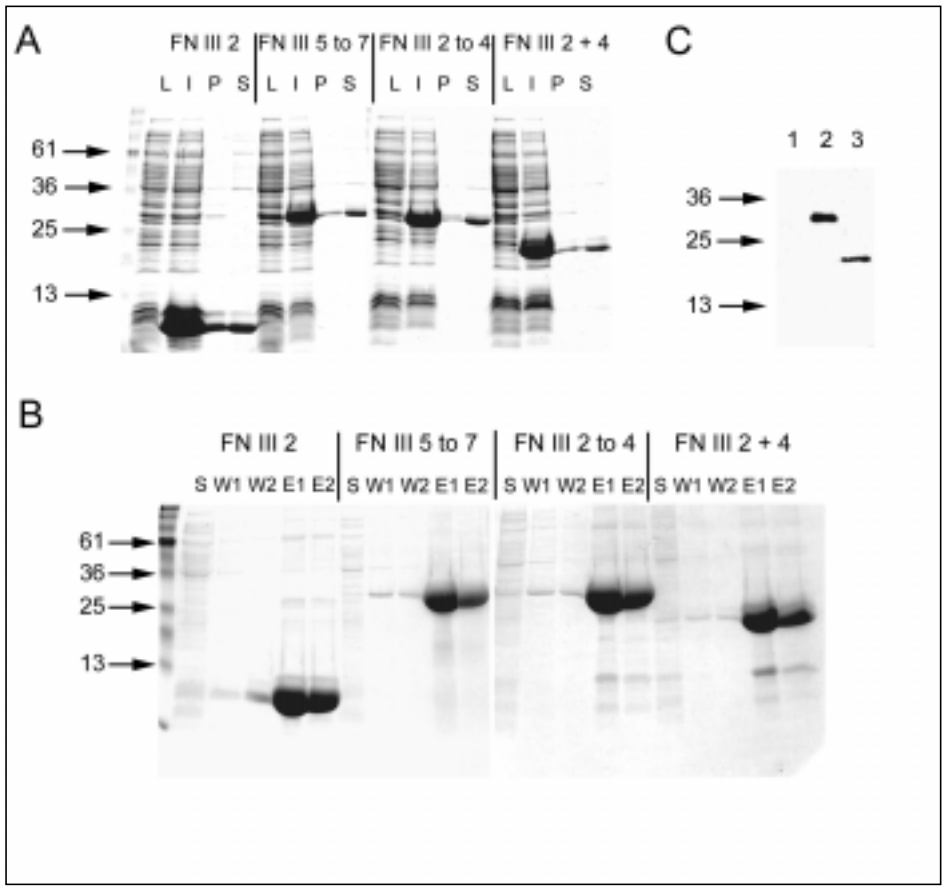

Fig. 13: SDS-PAGE and immunoblot analysis of recombinant TN-N fragments. Four TN-N fragments comprising distinct FN III domains were expressed in bacteria: the single FN III domain 2 (FN III 2), FN III domains 5, 6 and 7 (FN III 5 to 7), FN III domains 2, 3 and 4 (FN III 2 to 4), and FN III domains 2 and 4 (FN III 2 + 4). (A) Coomassie stained gels loaded with bacterial lysates harvested before (L) and 4 h after IPTG induction (I) of protein expression, and with fractions of the induced lysates (insoluble pellet (P); soluble supernatant (S)). (B) Purification of 6xHis-tagged fragments from the supernatant fractions. Aliquots were taken from the supernatant after incubation with Ni^{2+}-agarose beads (S), from two washing steps (W1, W2), and two elution steps (E1, E2), subjected to SDS-PAGE, and stained with Coomassie blue. (C) Western blot analysis of the purified FN III domain 5 to 7 (1), FN III domain 2 to 4 (2), and FN III domain 2 + 4 (3). Marker sizes are shown in kD at the left margin.

1.10 Inhibition of neurite outgrowth and cell migration at a TN-N substrate border

The following assays aimed to elucidate whether the two splice variants of TN-N have different functions. Therefore, outgrowing neurites and migrating cells from hippocampal

CA3 region explants were exposed to substrate borders of the recombinant protein fragments of TN-N (Fig. 13). The CA3 subregion of the hippocampus was prepared from newborn mice and oriented around a substrate spot that had been coated with the purified TN-N fragments. As a positive control, the amino-terminal fragment of tenascin-C (the cysteine-rich segment and all EGF repeats) was used, which had previously been shown to repel outgrowing neurites in choice situations (Dorries et al., 1996; Gotz et al., 1996). The substrate border was made visible by fluorescence of rhodamine-coupled BSA, which was coated in a mixture with the recombinant TN-N fragment (Fig. 14D). When substrates contained BSA only, neurites readily crossed the border (Fig. 14B). In contrast, nearly all neurites were repelled at the boundary of the region where the amino-terminal fragment of TN-C had been coated (Fig. 14A). The shorter splice variant of TN-N showed similar effects as TNC and the vast majority of neurites were not able to penetrate into the coated area (Fig. 14C).

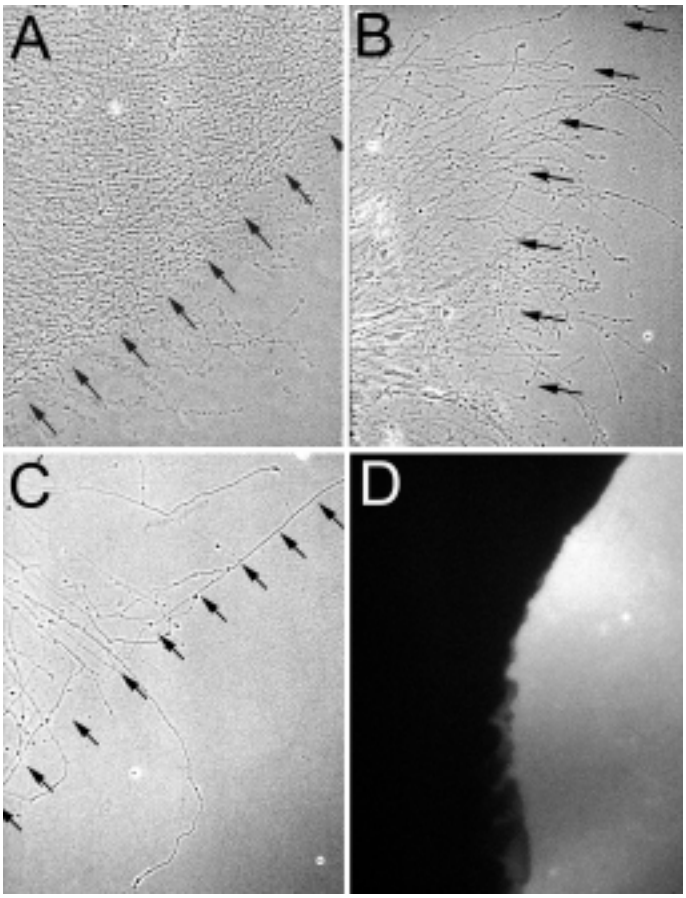

Fig. 14: Outgrowing neurites are respelled by the recombinant TN-N fragments corresponding to FN III 2 + 4. Explants from the hippocampal CA3 region were

positioned close to an area coated with FN III 2 + 4 of TN-N (C) or control proteins. Arrows point towards the uncoated regions of the substrate. (A) A recombinant fragment comprising the EGF-L domain of TN-C served as a positive control (Dorries et al., 1996). (B) Rhodamin-coupled BSA was used as a negative control. The fluorescence image of the protein border corresponding to B is shown in D. Outgrowing neurites do not enter the area coated with TN-N FN III 2 + 4 (C) or EGF-L of TN-C (A).

To analyze whether the observed inhibition of neurite outgrowth is specific for the shorter splice isoform of TN-N, we analyzed possible inhibitory effects of other FN III subfragments of the molecule. In Fig. 15A the CA3 explant was positioned directly adjacent to the FN III 2 + 4 substrate border and neurites were able to grow in any direction from the explant with exception of the area coated with the shorter splice variant fragment. In contrast, a substrate containing the FN III domains 2 to 4, including the spliced domain 3 (Fig. 15D), showed no repellent effect on neurites, nor did FN III 2 (Fig. 10C) and FN III 5 to 7 (Fig. 15B) that were tested as controls.

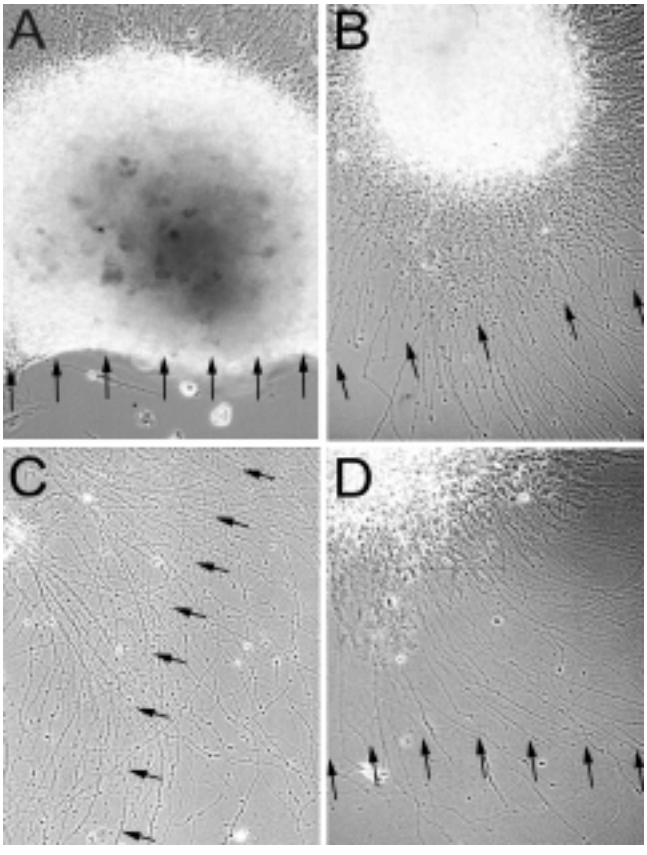

Fig. 15: Repulsion of neurite outgrowth is specific for the smaller isoform of TN-N. Neurites growing from explants of the hippocampal CA3 region do not pass the substrate border when the TN-N fragment FN III 2 + 4 was coated (A), whereas they entered areas coated with fragments FN III 5 to 7 (B), FN III 2 (C), or FN III 2 to 4 (D).

We then asked whether the fragment lacking the FN III domain 3 of TN-N was repellent not only for neurites, but also for migrating cells. The behavior of cells migrating from the CA3 explants towards an area coated with the respective protein fragment was therefore studied. Cells migrated readily into areas coated with FN III 5 to 7 (Fig. 16A) or FN III 2 to 4 (Fig. 16B), whereas they hardly entered a substrate containing the fragment corresponding to the spliced isoform of TN-N (Fig. 16C). This indicates that the TN-N fragment lacking the third FN III domain is a repellent substrate not only for outgrowing neurites but also for migrating cells.

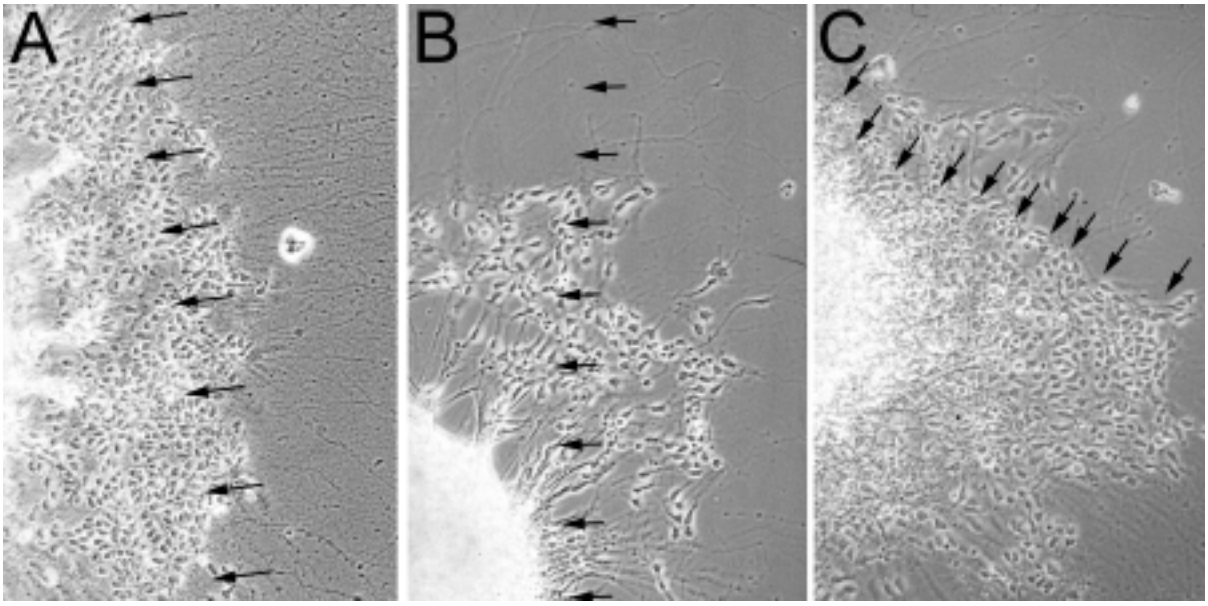

Fig. 16: Effects of recombinant FN III fragments of TN-N on migrating cell bodies. Explants from the hippocampal CA3 region were positioned close to an area coated with recombinant TN-N fragments. Cells migrate into areas coated with FN III 5 to 7 (A) and FN III 2 to 4 (B), but stop at the border of areas coated with FN III 2 + 4 (C).

2 Conditional gene targeting of the tenascin-R gene

Gene targeting has become a powerful and indispensable tool to study gene function *in vivo* and to develop mouse models for biomedical research. The combination with site-specific recombination systems such as the Cre/loxP system of the bacteriophage P1 or the yeast derived FLP/FRT system in conditional gene targeting has further extended the power of this technique.

2.1 A versatile Neo resistance selection cassette for gene targeting experiments

The key step of gene targeting is the modification of the gene of interest via homologous recombination in embryonic stem (ES) cells with a gene targeting vector including a positive selection cassette to enrich for the rare stably transfected ES cell clones. In conditional gene targeting experiments, this selection marker is removed after identifying correctly recombined ES cell clones to avoid interference with the endogenous gene function. To improve the often poor efficiency of the removal and to reduce the risk of unwanted ES cell differentiation, we have designed a Neo resistance selection cassette with a tandem arrangement of both the loxP and the FRT recognition sequences at both ends. This allows excision of the selection marker by expression of either the Cre or the FLP recombinase and helps to overcome the problems arising with only one recombination system. Furthermore, a frequently observed problem, when using only the Cre/loxP system, is the very low rate of type II recombination products leading to floxed alleles after transient expression of Cre recombinase *in vitro*. Therefore it was decided to construct the improved Neo resistance cassette in order to allow the versatile and alternative use of either both or one of the Cre/loxP or FLP/FRT recombination system depending on the experimental demand. In addition, the cassette was flanked with suitable restriction sites that allow the insertion of the long and short arm or the transfer of the

cassette to another vector. The vector was termed pJN1 and was applied for conditional modification of the tenascin-R (TN-R) gene

2.1.1 Construction of the versatile Neo resistance cassette

To construct the plasmid named pJN1, the Neo resistance cassette from the vector pKO SelectNeo (Lexicon Genetics) consisting of the neomycin phosphotransferase gene driven by the pgk promoter and terminated by the bgh polyA sequence was excised via the flanking AscI sites and cloned into the unique AscI site of the plasmid pKO Scrambler-V901 (Lexicon Genetics). For further steps, a clone with the desired orientation was chosen and its sequence integrity was confirmed by sequencing.

A fragment carrying the tandemly arranged loxP and FRT sequence was generated by a standard PCR reaction with a pair of partially overlapping primers that were extended to the double stranded DNA (Fig. 17). The two primers were: 1) primer "loxP" which contained the loxP sequence (bold), a BglII restriction site (italic) and the overlap with primer "FRT" (underlined): (5'-ttt*AGATCT***ATAACTTCGTATAATGTATGCTATA<u>CGAAGTTAT</u>**<u>GAAGTTCCTATT</u>-3'); 2) primer "FRT" that contained the FRT sequence (bold), SalI and SacII restriction sites (italic) and the overlap with primer "loxP" (underlined): (5'-aaa*GTCGACCCGCGG***GAAGTTCCTATACTTTCTAGAG<u>AATA</u>**<u>**GGAACTTC**</u>ATAACTTCG-3').

The PCR product (Fig. 17) was purified in a gel after digestion with BglII and SalI to be cloned in a defined direction in the BglII and XhoI sites 5' of the Neo resistance cassette, and was cloned as a BglII-SacII fragment into the BamHI and SacII sites at the 3' site respectively (Fig. 18). In the final construct the correct, unidirectional orientation and the sequence integrity of the loxP and FRT sites and the central Neo resistance cassette was confirmed by sequence analysis.

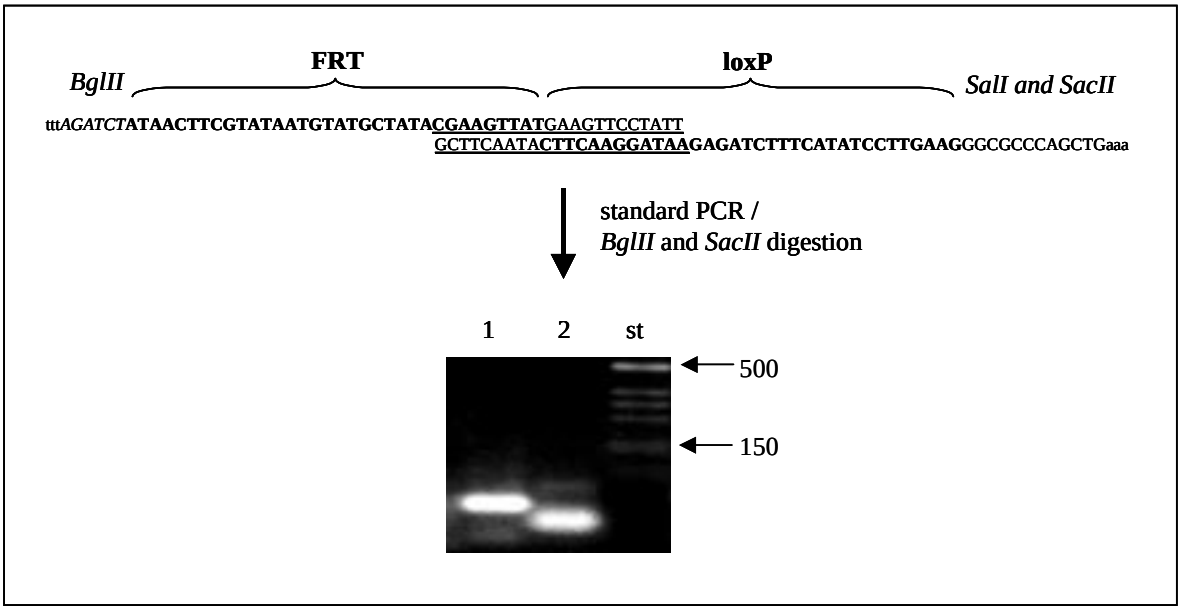

Fig. 17: Generation of the loxP/FRT site by PCR. Two overlapping primers were amplified to double stranded DNA (1) and digested with BglII and SalI (2). The digested DNA fragments were used to construct the plasmid pJN1. Marker sizes are given in bp.

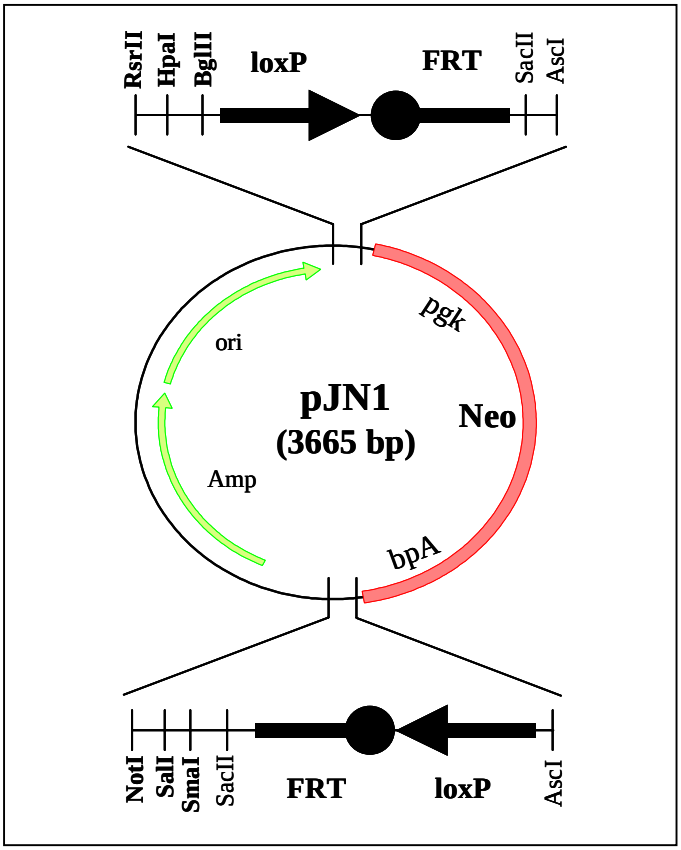

Fig. 18: Construction of plasmid pJN1 with a Neo resistance cassette suitable for both Cre mediated and FLP mediated excision. The structure of plasmid pJN1: two tandemly arranged loxP/FRT sites are flanking the Neo resistance cassette consisting of the neomycin phosphotransferase gene (Neo) driven by the pgk promoter (pgk) and terminated by the bgh polyA sequence (bpA). The loxP sequence is symbolized by an arrow pointing with its head to the 3' site, the FRT sequence by a circle with a tail pointing towards the 3' site. Only relevant restriction sites are shown, single cutters are in bold.

2.2 Construction of the targeting vector to generate a conditional knockout of tenascin-R

The key step in a gene targeting experiment is the design and cloning of a targeting vector that is used to generate recombinant ES cell clones carrying the desired gene modification. The application of site specific recombination systems is increasing the complexity of this task and requires extensive handling and manipulation of the ES cells.

In the first place, a detailed knowledge of the organization of the tenascin-R gene was needed to select the sequences used for the targeting vector backbone. Therefore, a 18 kb genomic clone was analyzed by systematic restriction endonuclease digestion to identify the position and cutting frequency of restriction sites (Fig. 19). The results of this analysis were used to plan the targeting vector and equip it with all desired features, like position of the Neo resistance gene, orientation of loxP/FRT sites and identification of homologous and Cre mediated recombined clones by their restriction fragment length in Southern blot analysis.

The targeting vector backbone was generated by successive ligations of two endonuclease digestion fragments from the total genomic clone into a pBlue-KS cloning vector. The genomic sequence was first digested with EcoRV, the 5 kb fragment was extracted from the gel, and ligated to pBlue-KS. In the next step, a 2.5 kb EcoRV and XhoI fragment isolated from a digestion of the genomic clone was ligated to the 3’ end of this 5 kb EcoRV fragment (Fig. 19). The cloned part of the TN-R genomic sequence was used as the targeting vector backbone.

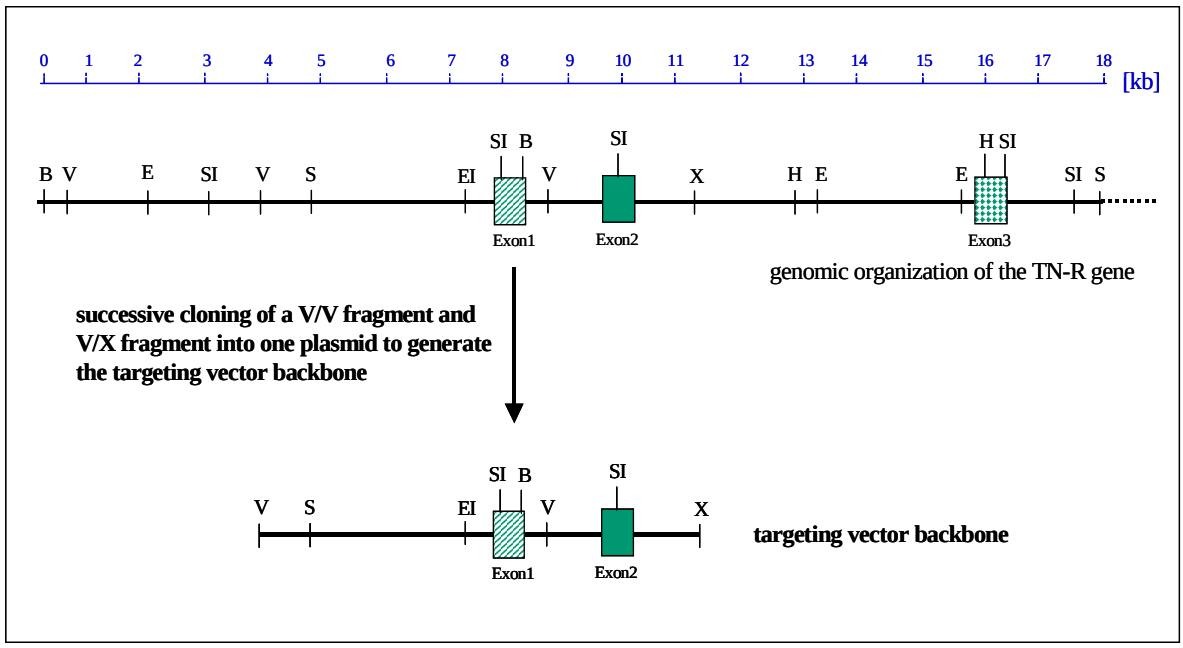

Fig. 19: Genomic organization of the 5' end of the TN-R gene and generation of the targeting vector backbone. A 18 kb genomic ES cell clone containing exons 1 to 3 of TN-R was analyzed by systematic restriction endonuclease digestion to identify the position and frequency of restriction sites on this clone. The 5 kb EcoRV and the 2.5 kb EcoRV/XhoI fragments were cloned into pBlue-KS to generate the targeting vector backbone. Abbreviations are: B: BamHI, E: EcoRI, EI: EcoNI, H: HindIII, S: SpeI, SI: SacI, V: EcoRV, X: XhoI.

The targeting vector backbone (Fig. 19) and the plasmid pJN1 (Fig. 18) were used to construct the final targeting vector for conditional ablation of the mouse TN-R gene. Therefore, the loxP/FRT Neo resistance cassette of the plasmid was excised by HpaI and SmaI restriction as a blunt end fragment and inserted into a EcoRV site located within intron 1 of the mouse TN-R gene (Fig. 20). A third loxP site was generated by PCR using again partially overlapping primers and was equipped with an additional 5' EcoRI site. This additional restriction site facilitated the detection in subsequent Southern blot analyze. The PCR fragment was flanked by two EcoNI restriction sites for cloning and the EcoNI digested fragment was ligated into a single EcoNI site upstream of exon 1 of TN-R (Fig. 20). Clones with the correct orientation of loxP were identified by sequence analysis, thereby confirming the sequence identity of the newly introduced loxP site.

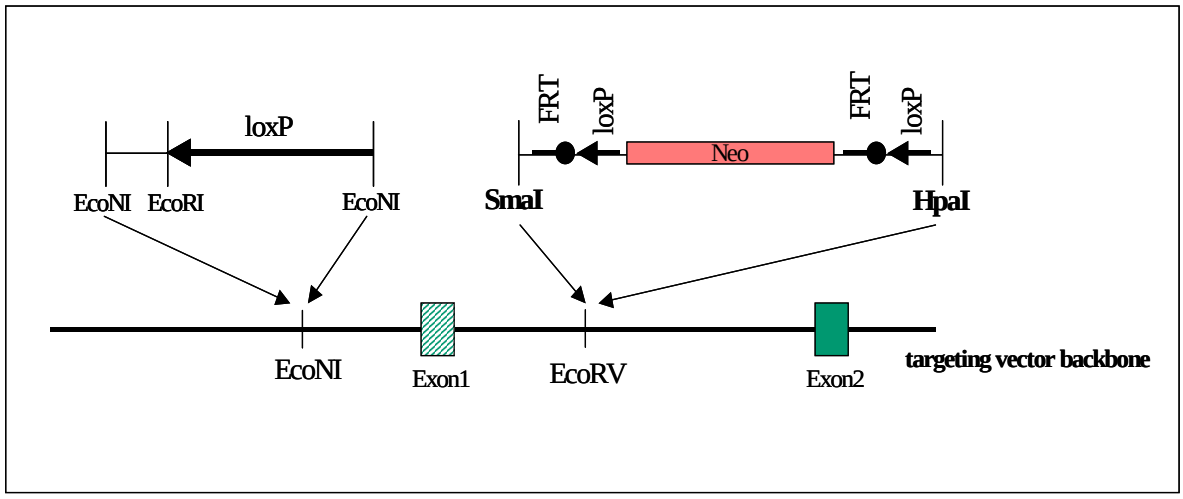

Fig. 20: Cloning steps during the generation of the final targeting vector. A PCR fragment containing a single loxP site with an additional EcoRI was ligated 5' of exon 1. Note that the additional EcoRI site is needed to distinguish between recombination events by Southern blot analysis. The blunt end fragment obtained by digestion of the pJN1 plasmid with SmaI and HpaI was ligated to intron1 of the targeting vector backbone. This cloning step equipped the targeting vector with a Neo resistance gene flanked by tandemly arranged loxP and FRT sites which are suitable for manipulation after homologous recombination in ES cells.

Before the final targeting vector (Fig. 20) was used to mutate ES cells by homologous recombination, the functionality of both the loxP/Cre and the FRT/FLP site specific recombination was confirmed by Cre or FLP expressing *E. coli* strains. The strains are capable to recombine loxP and FRT sites on exogenous plasmids. The strains 294-Cre and 294-FLP were kindly provided by F. Steward (Buchholz et al., 1996). The targeting vector was transformed into both bacterial strains and 10 clones each were selected for plasmid preparation and restriction enzyme analysis. All ten clones showed the site specific recombination event, namely the excision of the FRT flanked Neo resistance cassette in 294-FLP and the deletion of exon 1 plus Neo resistance cassette between the first and the third loxP site in 294-Cre (type I deletion). Intermediate excision products between the first and second loxP site (type III deletion) or the second and third loxP site (type II) were not observed demonstrating that these intermediates are not stable at higher Cre expression levels.

2.3 Identification of homologously recombined ES cell clones

The ES cell line R1 (Nagy et al., 1993) was cultured on a feeder layer of primary mouse embryonic fibroblast cells, which were treated with mitomycin C to block their proliferation. ES cells were transfected by electroporation with the linearized targeting vector and selected with G418 for the presence of the Neo resistance selection marker. Genomic DNA from individual ES cell clones was prepared. For Southern blot analysis 5 - 10 µg of genomic DNA were digested with EcoRI, electrophoresed and transferred onto a nylon membrane. A 500 bp probe was labelled with ^{32}P to detect homologous recombination events. In this case, the targeting vector replaced one allele of the TN-R gene and the digestion by EcoRI generated two fragments of different sizes. The digestion of the wildtype allele generates a fragment that runs at a size of 11 kb, whereas the band of the mutated allele has a size of 7.3 kb in Southern blot analysis (Fig. 21 A and B). The 500 bp probe was derived from intron 2 of the mouse tenascin-R gene located outside of the targeting vector at the 3' site (Fig. 21A).

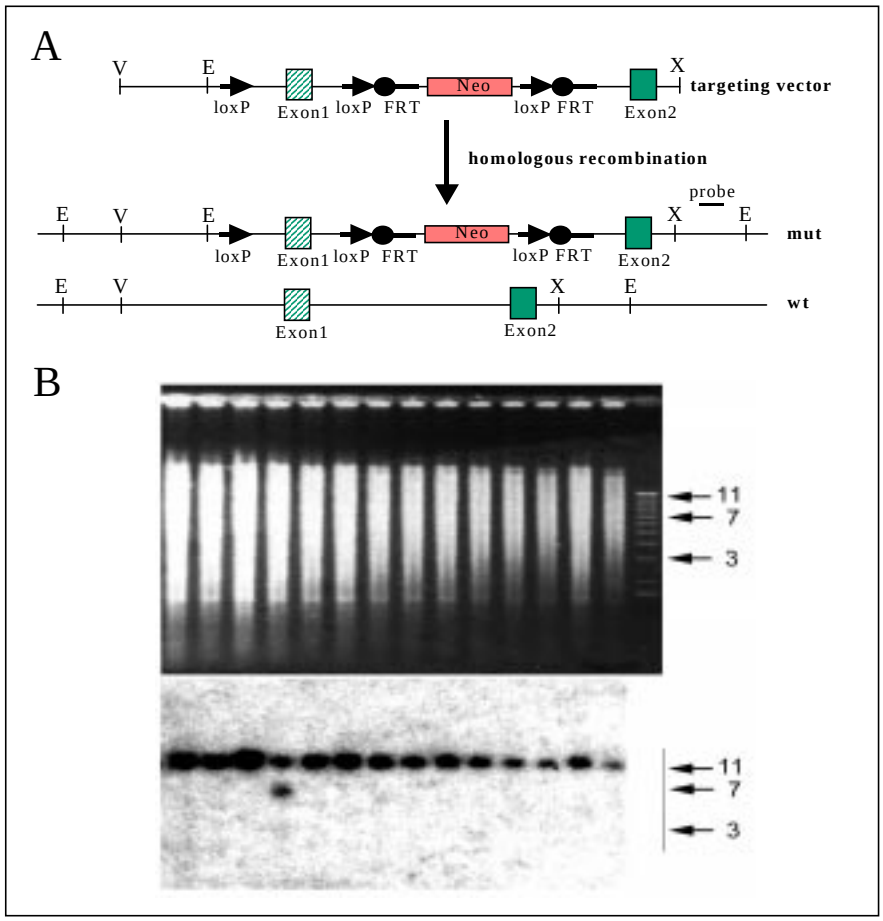

Fig. 21: Homologous recombination of the targeting vector with the TN-R gene and detection by Southern blot analysis. (A) The targeting vector sequence replaces one wildtype allele of the TN-R gene by homologous recombination. Thus, one allele is mutated to contain a Neo resistance gene (Neo), loxP and FRT recombination sites (loxP, FRT), and an additional EcoRI restriction site. The additional EcoRI site allows to distinguish between the homologously recombined allele (mut) and the wildtype allele (wt) in southern blot analysis. (B) Southern blot analysis was performed to identify ES cell clones which had undergone homologous recombination. The upper panel shows an ethidiumbromide staining of the gel electrophoresis of digested genomic ES cell DNA. In the lower panel the wildtype band runs at 11 kb, whereas the mutated band is detectable in the fourth lane at 7.3 kb.

In total, 1100 clones were analyzed from two independent transfections of ES cells. From these, three different ES cell clones were identified that had undergone homologous recombination. Thus, without applying any negative selection markers to counterselect for random integration, the targeting frequency was approximately 0.25 %. As estimated from the ratio of the band intensity of the mutated vs. the wildtype allele, the three clones were 90 %, 85% and 60 % pure for homologously recombined cells.

2.4 Cre and FLP mediated recombination in ES cells

The Neo selection marker was removed from homologously recombined ES cell clones by Cre and FLP mediated recombination. Two homologously recombined ES cell clones were subjected to electroporation with plasmid pOG-FLPe (Buchholz et al., 1998) and plasmid pPGKcrebpA (Torres and Kühn, 1997) for transient transfection and induction of the corresponding site specific recombination system. Transient expression of FLP was used to delete the FRT flanked Neo resistance cassette in order to create a floxed allele of the tenascin-R gene at exon 1. Clones with the FLP recombined allele were identified by their characteristic 5.5 kb EcoRI signal. In parallel experiments, Cre mediated recombination in ES cells resulted in type I deletion or type II deletions (Fig. 22 A and B). As previously observed, transient expression of Cre recombinase mainly resulted in clones with a type I deletion detectable by its characteristic 4.5 kb EcoRI fragment in Southern analysis (Fig. 22A and B). Such a clone with a deletion of exon 1 could be used to

generate mice with a constitutive inactivation of the tenascin-R gene. Only after extensive modulation of transfection conditions and plasmid concentrations, conditions were found under which a significant portion of clones exhibited type II Cre recombination with a floxed allele identical to the FLP recombination product (Fig. 22). Cre recombinations of type III have not been observed. Type II recombinants were used to create a mouse model with conditional ablation of TN-R.

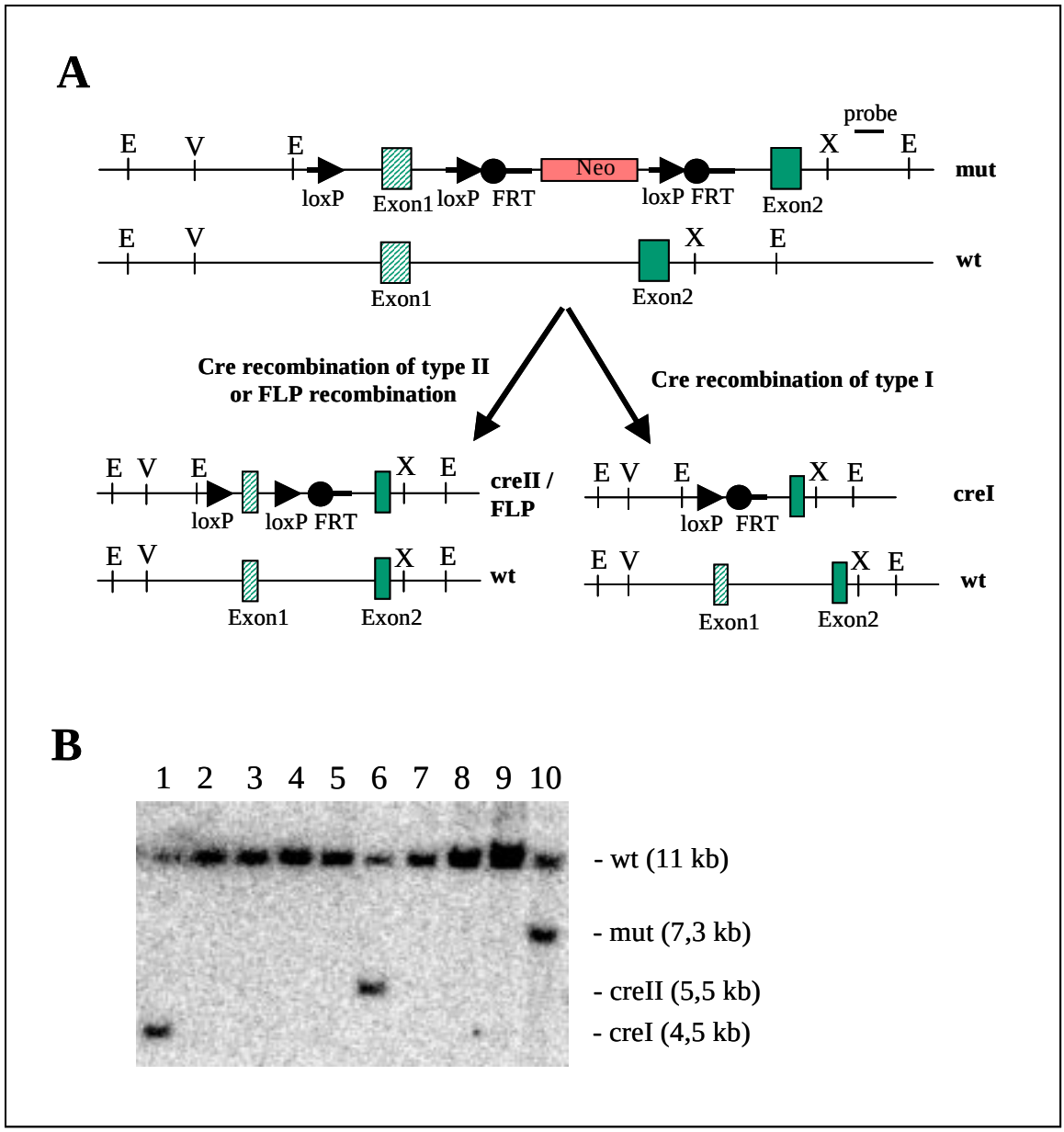

Fig. 22: Cre and FLP mediated recombination in ES cells. (A) Homologous recombination of the tenascin-R gene in ES cells. Presented are the mutated allele (mut) after homologous recombination and the wildtype allele (wt). These are compared to the

recombination products after transient expression of Cre or FLP in ES cells. Either Cre recombination of type II or FLP recombination (cre II/FLP) selectively excises the Neo resistance cassette leaving the floxed exon 1 and one FRT site in intron 1. Cre recombination of type I (cre I) deletes the segment between the first and the third loxP site. This removes also exon 1 and creates a constitutive knockout allele. Abbreviations are: E: EcoRI, V: EcoRV, X: XhoI. Note, that the figures are not drawn to scale. (B) Southern blot detection of site specific recombination events after transient expression of the Cre recombinase. Genomic DNA from individual ES clones was restricted with EcoRI and hybridized with the radioactively labelled probe. The correctly targeted clone obtained by homologous recombination is presented in lane 10 and shows a mutated 7.3 kb band in addition to 11 kb wildtype signal. The wildtype fragment is shortened to 7.3 kb in the mutated allele by introduction of the additional *Eco*RI site associated with the 5' loxP site. By excision of the Neo resistance cassette, the mutated fragment is shortened to 5.5 kb (cre II / FLP: lane 6) or is converted to 4.5 kb in case of Cre mediated type I deletion (cre I).

2.5 Generation and breeding of chimeric mice to obtain homozygously floxed animals

Two ES cell clones with a floxed TN-R allele were selected for injection into pre-implantation blastocysts. Ten chimeric mice were born from the transplantation of these blastocysts to 3 host mothers. Five-week-old chimeric males were crossed with wildtype females to generate heterozygously floxed offspring. Two of six chimeras gave no germline transmission. Heterozygously floxed mice were crossbred to generate homozygous mice. The genotypes of these mice were characterized by PCR. Therefore, primers were designed to bind to the 5' region of TN-R and to differentiate between wildtype, heterozygous, and homozygous genotypes (Fig. 23)

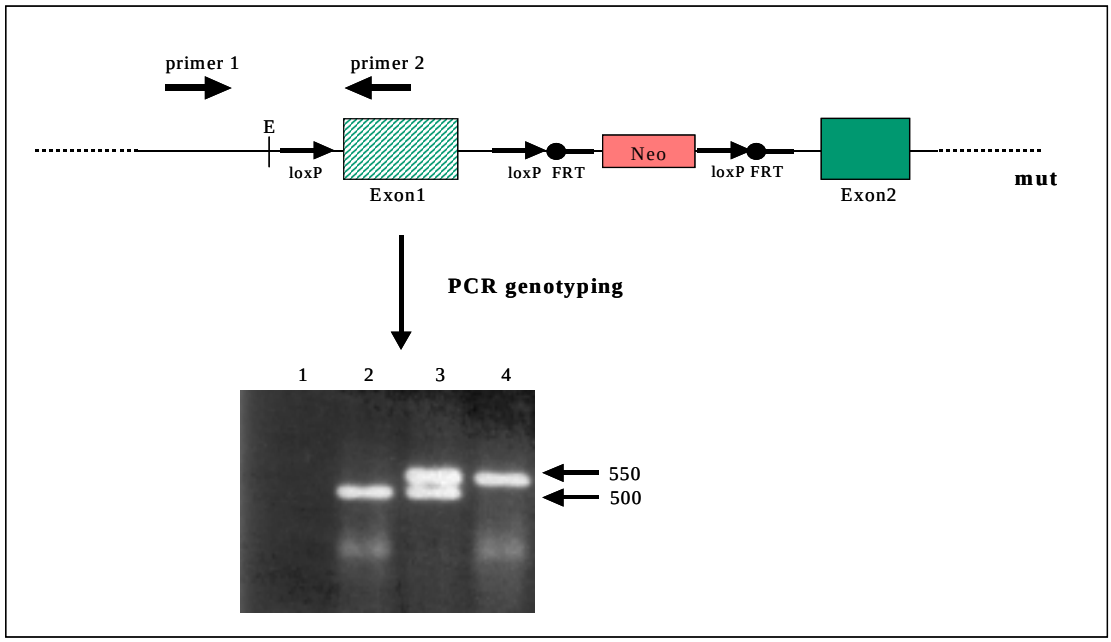

Fig. 23: Genotyping of targeted mice. Two primers were deduced from the genomic sequence of TN-R to amplify the 5' part of the gene. The PCR results in a 500 bp band for the wildtype animals (2), a double band pattern for heterozygously floxed mice (3), and a single 550 bp band for homozygously floxed mice (4). The negative control is shown in (1).

2.6 Conditional gene inactivation of TN-R

TN-R is expressed predominantly by oligodendrocytes of the CNS, but is also produced by subpopulations of neurons. It was aimed to generate a mouse model in which TN-R is conditionally ablated only from oligodendrocytes of the CNS, thus creating a tissue specific and time controlled knockout.

TN-R was conditionally removed by three different Cre recombinase expressing mice, which were crossed with homozygously floxed TN-R animals. The different mouse lines were bred until both TN-R alleles were floxed and the Cre transgene was heterozygous ($TNR^{FL/FL;Cre}$) and were compared to homozygously floxed littermates which lacked the Cre transgene ($TNR^{FL/FL;WT}$).

The transgenic mouse lines that were tested, expressed the Cre recombinase under the control of different oligodendrocyte specific promoters: 1) the myelin/oligodendrocyte

glycoprotein (MOG) promoter, 2) the proteolipid protein (PLP) promoter, and 3) the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) promoter. The activity of these three promoters is regulated by the developmental status of the oligodendrocytes. The MOG and PLP promoters are predominantly active in mature oligodendrocytes, whereas the CNP promoter is also active in oligodendrocyte precursor cells (Nave and Lemke, 1991; Jaquet et al., 1999; Berndt et al., 2001; Scherer et al., 1994; Gravel et al., 2000).

2.6.1 MOG-Cre mediated recombination in floxed TN-R mice

The Cre mediated recombination controlled by the MOG promoter in floxed TN-R mice was determined by PCR. Three different primer combinations were used to amplify the 5' part of TN-R, such that the resulting PCR products differ for the floxed allele and the recombined allele (Fig. 24). Genomic DNA was prepared from 8 optic nerves taken from adult $TNR^{FL/FL;MOG\text{-}Cre}$ and $TNR^{FL/FL;WT}$ animals and subjected to PCR. The recombination occurred specifically in floxed mice which contained the MOG-Cre transgene, whereas in floxed littermates without the Cre transgene, no recombination was detectable by PCR. This result was confirmed by three different primer combinations and all showed that the floxed TN-R gene had been deleted by the Cre recombinase under the control of the MOG promoter (Fig. 24A).

When Cre recombination occurs at the floxed TN-R locus, the RNA transcribed by the mutated TN-R gene is lacking the region encoding the ribosomal binding site, the translation initiation codon, the signal sequence, the EGF domains, and the first FN III domain (Weber et al., 1999). Thus, the TN-R protein cannot be produced properly by oligodendrocytes after the recombination is completed.

Western blot analysis was performed to determine whether the rather late MOG promoter controlled recombination resulted in reduced protein levels of TN-R. Equal protein amounts were loaded onto the gel and TN-R was detected by the monoclonal 596 antibody directed against the FN III domains 3 to 5 of TN-R. The protein concentration, as determined by densitometric measurements of the band intensities, was only slightly reduced in the optic nerve and protein preparations of total brain (Fig. 24A and B). A maximum of 12 % reduction was measured by this method.

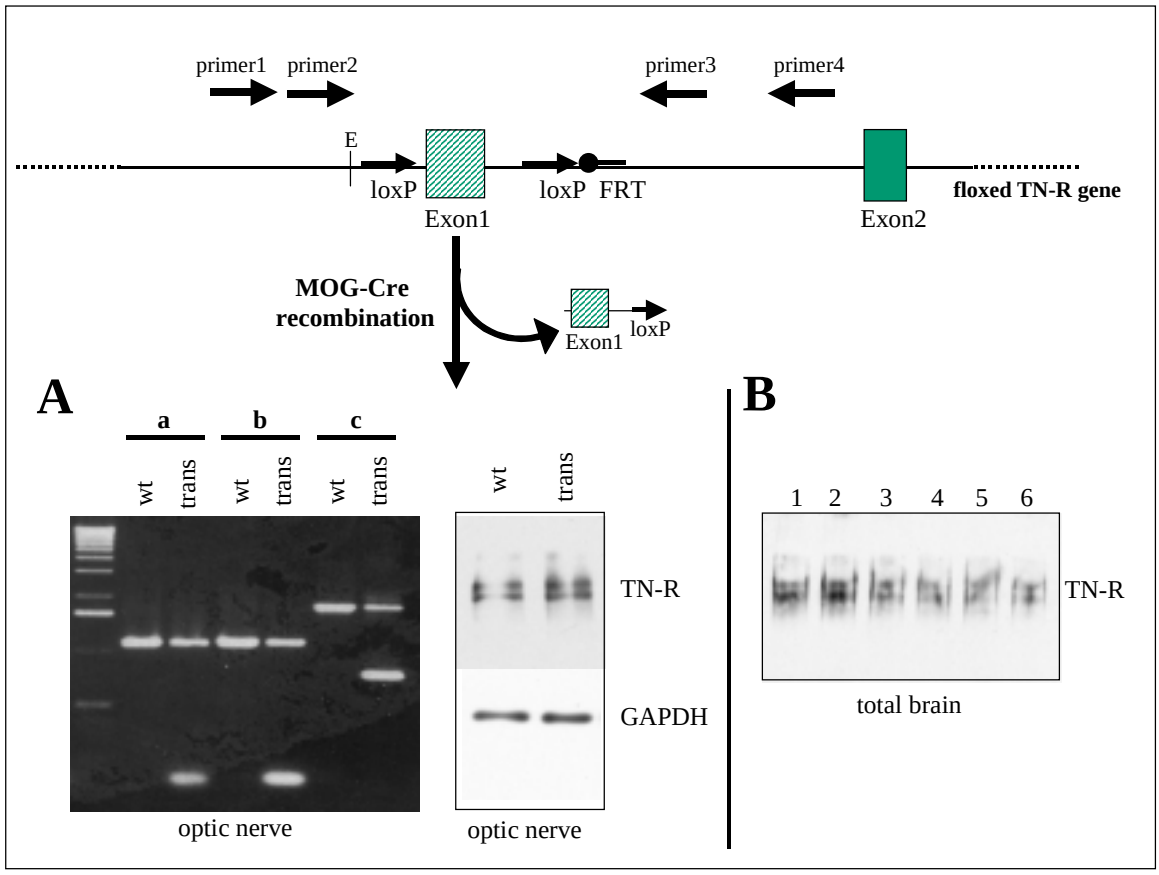

Fig. 24: MOG-Cre mediated recombination in floxed TN-R mice. The localization of primers used to determine the recombination event is presented in the schematic drawing (primer 1 to 4). (A) Optic nerves were analyzed. Three different primer combinations (a, b, c) amplified either a single band specific for not recombined alleles of $TNR^{FL/FL;WT}$ (wt) animals or an additional smaller band specific for recombination in $TNR^{FL/FL;MOG\text{-}Cre}$ (trans). In spite of the recombination of the TN-R gene in $TNR^{FL/FL;MOG\text{-}Cre}$ mice, the Western blot analysis (left panel) detected no reduction of the protein. Protein amounts loaded to the gel were controlled by GAPDH. (B) Protein extracts from adult total brain showed no reduction in TN-R levels: 1 = $TNR^{WT/FL;WT}$; 2 = $TNR^{FL/FL;WT}$; 3 = $TNR^{WT/FL;WT}$; 4 = $TNR^{WT/FL;MOG\text{-}Cre}$; 5 = $TNR^{FL/FL;WT}$; 6 = $TNR^{FL/FL;MOG\text{-}Cre}$. Note that the MOG promoter is active predominately in the mature oligodendrocyte.

2.6.2 PLP-Cre and CNP-Cre mediated recombination in floxed TN-R mice

The recombination of the floxed TN-R gene mediated by the MOG-Cre transgenic mouse line did not result into significant reduction of TN-R protein levels (Fig. 24). Since the MOG promoter is predominantly active in mature oligodendrocytes, it was of special

interest to analyze other promoters which were active earlier in oligodendrocyte development and test them for their ability to reduce the TN-R protein levels. Therefore, a PLP-Cre and a CNP-Cre transgenic mouse line were crossed with floxed TN-R mice.

The conditional ablation of the TN-R gene was tested by PCR with specific primer combinations (Fig. 25). The result demonstrated the specific recombination in $TNR^{FL/FL;PLP\text{-}Cre}$ and $TNR^{FL/FL;CNP\text{-}Cre}$, whereas in wildtype littermates no recombination was detectable.

Protein levels were tested by Western blot analysis using the monoclonal 596 antibody, which is directed against the FN III domains 3 to 5 of TN-R. Protein extracts were obtained from optic nerves prepared from the respective genotypes. The TN-R protein level was not reduced in $TNR^{FL/FL;PLP\text{-}Cre}$ compared to $TNR^{FL/FL;WT}$, but showed a strong reduction of 70 % in the $TNR^{FL/FL;CNP\text{-}Cre}$ mice (Fig. 25).

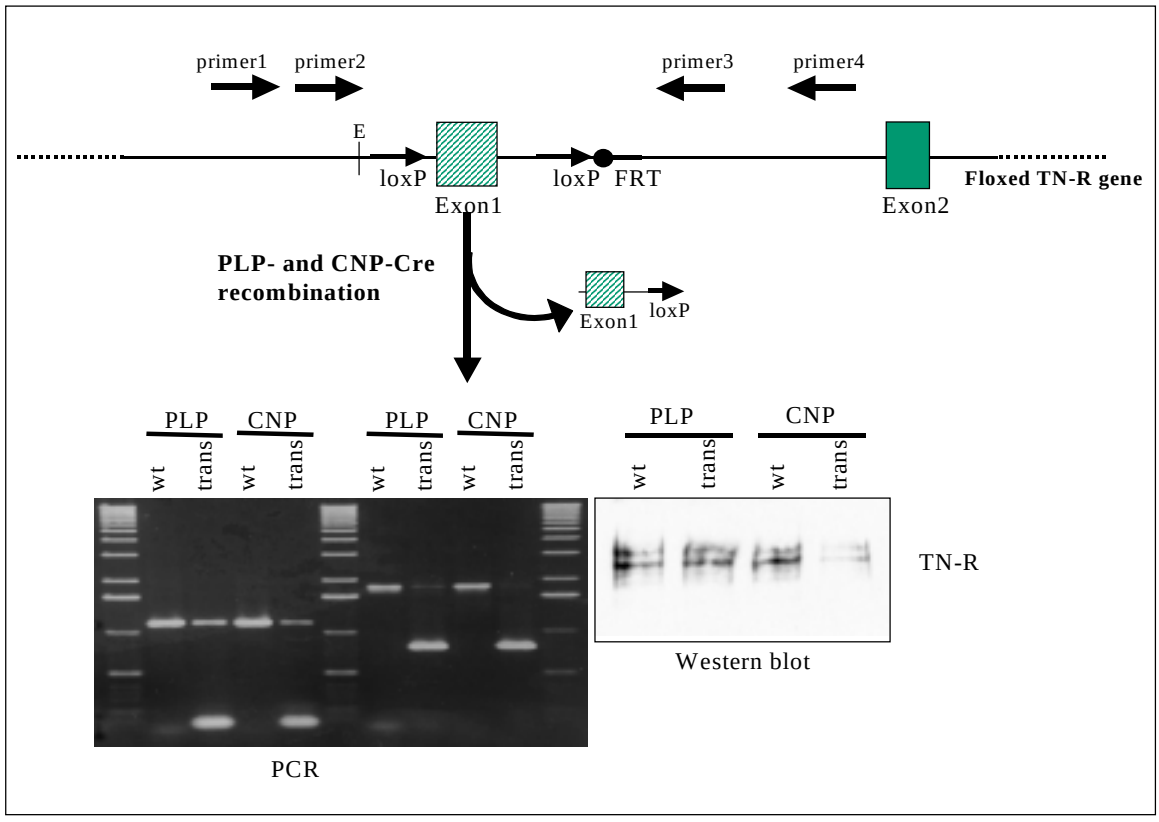

Fig. 25: PLP-Cre and CNP-Cre recombination in optic nerves of floxed TN-R mice. The recombination of the floxed TN-R gene leads to a deletion of exon 1 and was mediated by two different Cre expressing mouse lines (schematic drawing). The genomic recombination occurred specifically in both $TNR^{FL/FL;PLP\text{-}Cre}$ and $TNR^{WT/FL;CNP\text{-}Cre}$ mice as shown by PCR: two primer combinations amplified either a single band specific for not

recombined alleles from $TNR^{FL/FL;WT}$ (wt) animals or an additional smaller band specific for recombination in $TNR^{FL/FL;PLP\text{-}Cre}$ or $TNR^{FL/FL;CNP\text{-}Cre}$ (trans). Protein levels were tested in Western blot analysis and revealed a strong reduction of TN-R in optic nerves of $TNR^{FL/FL;CNP\text{-}Cre}$ mice (CNP, trans), whereas in $TNR^{FL/FL;PLP\text{-}Cre}$ animals (PLP, trans) the protein is not reduced when compared to wildtype littermates.

Discussion

1 Tenascin-N: characterization of a novel tenascin family member

A new member of the tenascin family in mouse (*Mus musculus*), designated tenascin-N (TN-N), was cloned and characterized. The sequence data have been deposited in the GenBank/EMBL/DDBJ under the accession number AF455756. At the molecular level, TN-N is clearly different from the other tenascins described in mammals, chicken and fish. It is composed of a cysteine-rich segment, 3.5 EGF repeats, 12 FN III domains as well as a domain homologous to fibrinogen. The second FN III domain contains an arginine-glycine-aspartate (RGD) motif. Two isoforms of the molecule generated by alternative RNA splicing were also identified. Thus, TN-N is a typical member of the tenascin family.

An interesting feature of the molecule is that two of its FN III repeats are identical, suggesting a recent duplication event during evolution. Phylogenetic considerations place TN-N of the mouse closest to the structure of TN-W from zebrafish. This makes these two proteins less related to TN-C and TN-R which are more closely conserved among each other. TN-X and TN-Y are the least related members of the tenascin family with regard to TN-N and TN-W on the one hand and TN-C and TN-R on the other. TN-N and TN-W do not appear to be species orthologues, since their overall structure is significantly different from each other in that TN-W contains only 5 FN III repeats, whereas TN-N contains 12 of these. Furthermore, the expression profiles of TN-N and TN-W are strikingly different from each other and the comparison of subdomain homologies of TN-N with the family members confirms that TN-N represents a so far unknown tenascin family member. In databases an *in silico* predicted molecule from human with a similar domain structure as TN-N and up to 89 % homology in the FBG domain can be found (accession number: AL049689). This demonstrates that orthologues to the mouse TN-N occur in other species which have not been characterized yet. Interestingly, the cDNA of this putative human

orthologue of TN-N is predicted to contain only 9 FN III domains instead of 12 FN III domains as found in the mouse TN-N. The missing FN III domains are probably due to differences between the two species. This conclusion is drawn since the analysis of the genomic structure of the human TN-N predicted exons coding only for 9 FN III domains. Since the analysis was done *in silico*, it is also possible that additional FN III domains will be found when the complete cDNA of the human TN-N is characterized.

While among the lymphoid organs TN-C expression is maximal in the thymus of developing and adult mice (Matsumoto et al., 1994), highest levels of TN-N transcription has been detected in the spleen of young adult animals, as shown in this work. TN-N expression in the white pulp of the adult spleen exhibits a distinct pattern associated with the functionally mature lymphoid tissue. Expression of TN-N mRNA occurs predominantly in the periarterial lymphatic sheath (PALS, the T cell zone of the white pulp) where the distribution pattern of silver grain accumulations makes TN-N production by lymphocytes unlikely, since in this case a diffuse, more homogeneous picture of the radioactive label should be expected in the PALS. Rather, the dotted pattern of TN-N expression is compatible with the anatomical distribution of resident macrophages in the PALS. The splenic localization of TN-N transcripts is clearly different from that of TN-C which is associated throughout the white and red pulp of human and murine spleens with the extracellular matrix (ECM) of the reticular meshwork formed by developing and mature fibroblasts (Liakka and Autio-Harmainen, 1992; Ocklind et al., 1993). It is remarkable that the multitude of red pulp macrophages and the specific macrophages of the B cell follicles of the white pulp are not labelled by the TN-N probe. The restriction of TN-N expression to a subpopulation of mononuclear phagocytes is in line with the well established functional and molecular heterogeneity of splenic macrophages (Gordon, 1998) contributing to the highly compartmentalized organization of this hemolymphoid organ and suggests a specific functional role of TN-N producing macrophages in cellular immunity. It remains to be investigated whether TN-N is laid down by PALS macrophages onto the ECM of the reticular framework of the PALS and thus contributes to its specific local microenvironment.

As in the spleen, strong TN-N mRNA expression in the kidneys becomes evident not before morphological and functional maturity during the first 14 postnatal days. TN-N expression is confined to the medullary region of the kidneys where transcriptional activity can be assigned to epithelial tubular and collecting duct cells. More exactly, TN-N mRNA is found in medullary areas where descending and ascending limbs of long loops

of Henle and collecting ducts are in close contact with specialized blood vessels. Here, the ECM of the interstitial space forms the anatomical basis for the essential water and ion exchanges which take place in this part of the nephron during diuresis and antidiuresis. It is tempting to speculate that TN-N is deposited together with the TN-C into the interstitial ECM of the renal medulla, thus helping to establish a barrier and specific transport systems essential for the regulation of water and salt turnover. It is intriguing in this context that high TN-N expression, as seen in the epithelial structures of the renal medulla, also occurs in the epithelium of the choroid plexuses of the cerebral ventricles. The choroid epithelium, although of different histogenetic origin, shares many morphological and functional characteristics with the renal tubular epithelium. Similar to that of the tubular epithelium for diuresis, its main function is the production of the cerebrospinal fluid by selectively excreting water, ions and some other substances into the ventricular cavity and simultaneously sustaining a barrier between the perivascular and ventricular spaces. It is tempting to speculate that TN-N might interact with ion channels as suggested for TN-R and TN-C (Srinivasan et al., 1998). The decrease of conduction velocity along the optic nerve in TN-R deficient mice (Weber et al., 1999) and the functional modulation of sodium channel beta subunit by TN-R *in vitro* (Xiao et al., 1999) are in agreement with this notion.

In contrast to TN-N, TN-C is predominantly expressed by mesenchymal cells during early embryonic stages of kidney development, e.g. during the initial epithelial/mesenchymal interaction in the metanephros anlage and during the fetal stage. In this stage, further differentiation of the renal tissues occurs until the formation of nephrons is completed in the young adult age. During development TN-C is strongly expressed in both the cortical and medullary parts of the kidneys, but in the mature, adult kidneys of mice TN-C like TN-N is exclusively expressed in the medulla (Aufderheide et al., 1987; Truong et al., 1996). TN-C, however, is synthesized by interstitial connective tissue cells while TN-N is produced by medullary epithelial cells. At this anatomical site, as in others, the two members of the tenascin family may have complementary roles: “mesenchymal” TN-C on the one hand acting as an oncofetal ECM glycoprotein with the capacity of being reactivated during tumorigenesis, inflammation, and tissue repair, while “epithelial” TN-N, on the other hand, represents an ECM constituent of functionally mature tissues.

Since TN-C expression as well as that of other tenascin molecules is more widespread during prenatal development than after birth, the studies of the TN-N expression have been extended to representative stages of late embryogenesis. In summary, at E16

widespread and strong TN-N-specific *in situ* hybridization signals were seen in the axial, appendicular and cranial skeleton. A less intense presence of TN-N mRNA was also observed in the walls of the digestive tract and in large blood vessels. At P0 a strong *in situ* hybridization signal of distinct anatomical sites such as the epithelial connective tissues areas of the lips, the dorsal specialized mucosa of the tongue, the epidermis of the skin of the back, and the apical regions of the maturing intestinal villi was seen.

Skeletogenesis is a complex multi phase process regulated over time and TN-C appears to be one of the prominent components of the associated skeletal ECM. At an early stage of skeletal organogenesis TN-C is involved in the formation of mesenchymal condensations, which are regarded as the first structural units of later bones (Hall and Miyake, 2000). Thereafter its expression is associated with the differentiation of various cartilaginous, osseous and tendinous tissues to be finally down regulated with the exception of a few restricted sites when growth and maturation of the organism is completed (Mackie and Murphy, 1998). As in the case of spleen and kidneys TN-N transcripts, in contrast to those of TN-C, are not detectable during early stages of skeletal development but can readily be visualized when the relatively late ossification event of bone development sets in. Similar to TN-C, TN-N expression is then predominantly found in the mesenchymal primordia of certain cranial bones as well as in the perichondral (to become periostal later) tissue of the axial and appendicular skeleton to expand subsequently to regions of bones where typical enchondral ossification occurs. Coincident with the progression and extension of ossification, an increase in TN-N transcription can be observed irrespective of enchondral or membraneous modes of ossification. Importantly and in contrast to reports on TN-C, cartilaginous structures do not express TN-N. Summarizing, TN-N and TN-C are expressed in the developing skeletal system with unique but partly overlapping spatio-temporal expression patterns. Whether TN-N disappears from the mature bone matrix as does TN-C which only persists in periostal and endostal surfaces (Mackie et al., 1987) or more likely is persisting there lifelong remains to be investigated.

Likewise, systemic TN-N expression in tissue layers of the developing digestive and vascular system corresponds to the associated developing smooth musculature. Interestingly, TN-C was also reported to appear in the smooth musculature of the oesophagus of E15.5 murine embryos but not more caudally in the stomach and the intestines where TN-C together with TN-X is localized in the loose connective tissue of the lamina propria (Aufderheide and Ekblom, 1988; Matsumoto et al., 1994). Colocalization of TN-C with forming smooth muscle of the developing small intestine

was also reported for humans (Beaulieu et al., 1993). Recently, it has been shown that in the adult rat TN-C colocalizes with proliferating smooth muscle cells of the aorta and pulmonary artery in various experimental settings of vessel wall modelling and remodelling (Jones and Rabinovitch, 1996; LaFleur et al., 1997). At present it remains an open question whether TN-N represents a permanent component of the differentiated vascular smooth musculature or is a temporal and inducible molecule in this tissue, as TN-C. It is tempting to speculate that prominent occurrence of TN-N in e.g. the developing lips, the epithelium of the dorsal tongue surface, or the apical parts of the intestinal villi reflects the extensive epithelial-mesenchymal interactions. These are necessary to form and sustain such specialized epithelial-connective tissue structures as the different forms of glottal papillae or functionally active villi of the small intestine. These morphogenetic processes are highly dependent on the elaboration of a specifically adapted ECM.

Also in the central nervous system, TN-N appears to be spatially and temporally regulated and its expression is complementary to TN-C or TN-R. TN-C and TN-R are expressed by astrocytes and oligodendrocytes, respectively, whereas TN-N is predominantly expressed by neurons. However, TN-R and TN-C are also expressed by certain subpopulations of neurons such as horizontal and amacrine cells of the retina, respectively (Bartsch, 1996). In the hippocampus, TN-R is expressed by inhibitory interneurons that carry the molecule in a particular configuration, the so-called perineuronal nets (Weber et al., 1999 Bruckner et al., 2000), whereas TN-C is expressed by granule cells in the adult hippocampus after stimulation (Nakic et al., 1998). Thus, some overlap in the expression of TN-N, TN-C, and TN-R by neurons is seen, but TN-N is more widely expressed by neurons than TN-R and TN-C. Another difference between TN-C and TN-N expression is evident during development: Whereas TN-C expression is more predominant at early developmental stages, TN-N is more strongly expressed in the adult.

The question is now which functions TN-N is conveying. First steps towards this analysis are presented here, by using an established cell culture system in which TN-C and TN-R have been investigated with regard to their ability to promote or inhibit neurite outgrowth (Xiao et al., 1996; Dorries et al., 1996; Gotz et al., 1996; Becker et al., 2000). Furthermore, the influence of TN-N on neuronal migration was studied. It was of particular interest to investigate the functions of the two isoforms of TN-N isolated so far. Neurites and migrating neurons of the CA3 region of early postnatal hippocampus were confronted with a sharp molecular boundary containing different fragments of TN-N on a continuous substrate of a neurite outgrowth and neuronal migration promoting molecule,

such as laminin. It could be shown that FN III 3 to 5 and alternatively spliced domains A1 to A4 of TN-C are repellent for neurite outgrowth in choice situations. It should be mentioned as well that the alternatively spliced domains B and D of TN-C enhance neurite outgrowth when coated as a homogenous substrate (Gotz et al., 1996). As TN-C and TN-R, TN-N is repellent for neurites mediated by the FN III domains. Interestingly, the FN III domains of TN-N show inhibition of neurite outgrowth and cell migration when the third FN III domain is missing in the shorter isoform of the molecule. To our knowledge, this is the first time that a splice variant of a tenascin is directly compared to its non-spliced counterpart in neurite repulsion and cell migration assays. Differences were found between splice variants of TN-C in cell adhesion assays and suggested different and/or additional cell binding sites located on the isoforms of TN-C (Prieto et al., 1992). Therefore, a possible interpretation of the findings on the different functions of the TN-N isoforms in neurite outgrowth and cell migration assays could be that the RGD sequence motif in the second FN III domain of TN-N and thus the binding to integrins may be masked by the absence of the third FN III homologous repeat. The interaction of TN-C and TN-R with integrins via the RGD motif has been well established (for review see: Jones and Jones, 2000) and the inhibition of neurite outgrowth by a RGD dependent mechanism has been proposed for TN-C (Probstmeier and Pesheva, 1999). Another possible model explaining the observed effect is that the contiguity of the second and fourth FN III homologous repeat could form a structural motif that is repellent for neurites. This motif would not be present if the second and fourth FN III repeats were separated by the third FN III repeat, supporting the idea that several receptors or interaction partners are responsible for the observed differences of the spliced isoforms of TN-N, as described for other tenascins (Joester and Faissner, 2001; Jones and Jones, 2000; Prieto et al., 1992). Whether F3/contactin is a receptor for TN-N, as shown for TN-R and TN-C, remains to be elucidated. It is noteworthy that in the nervous system TN-N is synthesized predominantly by neurons and could thus exert an autocrine function on neurite outgrowth. This autocrine function in the adult nervous system could lead to an inhibitory environment in a situation where the molecule is present in an inhomogeneous extracellular matrix environment, preventing neurite outgrowth under stable conditions. Whether TN-N is differentially regulated under conditions of lesion and enhances synaptic efficiency, as with TN-C and TN-R, remains a topic for further investigations.

2 Conditional gene ablation of tenascin-R

The construction of a floxed TN-R mouse and the conditional gene inactivation *in vivo* is described.

An important step during the generation of a conditional gene deficient mouse is the construction of the targeting vector. To simplify this often complicated procedure, a plasmid was constructed with a novel arrangement of a Neo resistance cassette flanked by tandemly orientated loxP and FRT sequences. The resulting final plasmid was named pJN1 and was additionally equipped with suitable restriction sites for further cloning steps. The plasmid pJN1 was tested in a specific targeting project with the purpose of generating a conditional knockout mouse model of the tenascin-R gene and its functionality was demonstrated. The cassette can be applied to the construction of all kind of replacement type targeting vectors and thus facilitates the generation of knockout mice. Generally, it is desired to remove the Neo resistance cassette from the genome of ES cells after homologous recombination. A frequently observed problem of the Cre mediated recombination is that type I deletions between the outermost of three accessible loxP sites dominate over the desired type II deletion leading to a floxed allele. Moreover, time consuming optimization steps are required to find the correct conditions for Cre mediated type II deletions. The pJN1 plasmid offers an alternative to the Cre recombination, since the removal of the Neo cassette can be obtained more easily by FLP recombination. In the meantime, the restriction of FLP recombination to 30 °C has been overcome by mutagenesis and the use of the improved FLP recombinase is a true alternative to the application of the Cre recombinase (Buchholz et al., 1998). Depending on the genetic context, the presence of the loxP/FRT-Neo-resistance cassette may create a hypomorphic allele (Meyers et al., 1998) that can be efficiently converted to the wildtype allele by crossing with a FLP expressing deleter mouse strain (Rodriguez et al., 2000), leaving back a floxed allele that subsequently can be subjected to restricted gene inactivation by crossing with a suitable Cre expressing mouse strain. Moreover, when a new gene is targeted, the versatility of this new selection cassette in plasmid pJN1 can be used to generate a constitutive and conditional mouse model with only a single targeting vector: The same ES cell clone can be treated either with the Cre recombinase to generate a constitutive knockout situation or with the FLP recombinase for selective generation of the floxed allele needed for conditional applications.

The targeting vector constructed and used in this work was a replacement type vector including the positive selection marker gene Neo. After homologous recombination and removal of the Neo resistance cassette, exon 1 of TN-R was flanked by loxP sites. It was chosen to flox exon 1, because it contains the 5' untranslated region and the 5' coding region of TN-R. Thus, the deletion of exon 1 removes the ribosomal binding site, the translation initiation codon, the signal peptide responsible for excretion of TN-R, and a large portion of the molecule required for its proper function. Furthermore, possible translation initiation codons of exon 2 and 3 are located in wrong reading frames of the molecule. Thus, unexpected splicing events in the mutated TN-R gene would lead to nonsense proteins. In conclusion, it is highly probable that the deletion of exon 1 leads to a complete abolishment of the TN-R expression.

Working with ES cells requires optimal culturing conditions to prevent the ES cell from differentiation. The pluripotent ES cell will efficiently contribute to the developing embryo, whereas pre-determined cells only show a low rate of germline transmissions (Torres and Kühn, 1997). R1 ES cells were chosen in this work, because it was previously reported that they have been successfully applied to other targeting projects (Nagy et al., 1993). However, an unusually high number of ES cell clones (approximately 1100) had to be analyzed to find candidates which had incorporated the targeting vector by homologous recombination. In a comparable targeting project in our group in which the same targeting, recombination, and culturing conditions have been used, only a few hundred cells had be screened to find homologous recombinants. This indicates that the accessibility of the targeted gene locus is of importance for the rate of homologous recombination (Haines and Brodeur, 1998). The incorporation of the herpes simplex thymidine kinase as a negative selection marker together with gancyclovir treatment of the ES cells might have improved the selection for homologous recombination (Chen and Bradley, 2000). During the selection for clones with a Neo resistance cassette many cells showed a flat morphology and started to spread. These ES cells were considered as predetermined or differentiated (Torres and Kühn, 1997) and were not picked as single colonies and tested for homologous recombination. According to Torres and Kühn (1997), it is likely that this strict selection for clones with a round and sharp-bordered morphology contributed to the high rate of germline transmission that was observed in this study.

A tissue specific and time controlled knockout of TN-R was generated by three different transgenic mice which were crossed with the floxed TN-R mouse line. The homozygously floxed and heterozygously transgenic mice differed from each other by the promoter

which regulates the Cre expression. In the first experiments, it was of special interest to analyze if the desired recombination event at the TN-R locus occurred. It was shown by PCR that in all conditional knockouts the floxed genomic locus of TN-R was recombined by the Cre. This recombination deletes exon 1 of TN-R from the genome and leads to an abolishment of the protein expression. The reduction in TN-R levels can be detected by the monoclonal antibody 596 (Weber et al., 1999). However, only one mouse line, namely the $TNR^{WT/FL;CNP\text{-}Cre}$, showed a reduction of the protein by 70 % in adult animals, whereas the MOG promoter and PLP promoter controlled Cre transgenic mouse lines were unable to reduce the TN-R protein significantly. This observation can be explained by the time-controlled activity of the three different promoters. MOG and PLP are predominantly active during the late phase of oligodendrocyte differentiation (Nave and Lemke, 1991; Jaquet et al., 1999; Berndt et al., 2001), whereas the CNP promoter also shows activity in oligodendrocyte progenitor cells (Scherer et al., 1994; Gravel et al., 2000). This leads to the conclusion that TN-R is predominantly expressed by pre-mature oligodendrocytes and is down regulated in finally differentiated cells. The peak in TN-R expression at day 14 of mouse development which coincides with the phase of active myelination in the CNS and the down regulation to a constant level in the adult are in agreement with this notion (Bartsch et al., 1993).

TN-R is not only expressed by oligodendrocytes, but also by subpopulations of neurons (Fuss et al., 1993; Wintergerst et al., 1993). By crossing the MOG, PLP, and CNP transgenic mice with the floxed TN-R mouse line, it was aimed to delete TN-R in oligodendrocytes only. Thus, it is likely that the remaining TN-R expression in the conditional knockout is due to the neuronal protein production. It is a matter of further investigations to exclude a mosaic expression profile of the CNP promoter driven Cre which could also lead to an incomplete reduction of the TN-R protein.

The constitutive knockout of TN-R and TN-C showed only mild phenotypes (Weber et al., 1999; Settles et al., 1997; Forsberg et al., 1996; Mitrovic and Schachner, 1995; Saga et al., 1992) and did not come up to expectations indicated by many other studies. For example, investigations demonstrated anti-adhesive properties of TN-R and TN-C for cell bodies and repellent functions on neurite outgrowth (Dorries et al., 1996; Xiao et al., 1996). Even functionally specialized domains have been identified and different types of receptors interfere with tenascins (Joester and Faissner, 2001). The discrepancy between the constitutive knockout phenotype and results from other investigations could be explained by regulatory mechanisms which compensate for the lacking molecule (Erickson, 1993;

Faissner et al., 1994; Faissner, 1997). This problem might be overcome by conditional mutagenesis, allowing normal development of the organism until the spatially restricted and time controlled knockout sets in.

Summary

Tenascin-N, a novel member of the tenascin family, was identified by cDNA cloning. The total mRNA is 5773 bp in length and was completed by 5' and 3' RACE experiments. Northern blot analysis confirmed the size of the transcript. Tenascin-N was shown to encode in an open reading frame of 4683 bp the characteristic structural motifs of a cysteine-rich stretch, 3.5 epidermal growth factor-like repeats, 12 fibronectin type III homologous domains, and a fibrinogen-like domain. Phylogenetic analysis revealed that tenascin-N is an independent homologue of the family and not an orthologue of a known tenascin. In the central nervous system, tenascin-N was found to be synthesized predominantly in neurons, but it was most highly expressed in specific cell types of kidney, spleen, and bones. The expression pattern suggests a predominant localization of tenascin-N to late developmental stages of different tissues, since it is produced by a subpopulation of macrophages in the adult spleen, by the medullary region of the mature kidney, and by the perichondrial region of later stages of the bone development. An alternatively spliced isoform lacking the third fibronectin type III homologous domain was identified by RT-PCR analysis. The shorter splice variant repelled neurites and migrating neurons from hippocampal CA3 region explant cultures, while the longer splice form did not inhibit neurite outgrowth or cell migration. The combined observations show that tenascin-N shares functional features with other members of the tenascin family. However, although tenascin-N and tenascin-C are expressed in the same tissues, the spatial and temporal expression appears to be more complementary than overlapping.

A conditionally targeted mouse line containing a floxed tenascin-R gene was generated by homologous recombination in ES cells. To facilitate the construction of the targeting vector, a new plasmid that improves the often poor efficiency of the removal of the Neo resistance cassette and reduces the risk of unwanted ES cell differentiation was developed and functionally tested. For conditional ablation of the tenascin-R gene, three different transgenic mouse lines were crossed with floxed tenascin-R mice, in which the transgenic Cre expression was controlled by the oligodendrocyte specific MOG, PLP, or CNP promoters. Although the recombination at the tenascin-R locus was catalyzed by all transgenic mouse lines, the tenascin-R protein was only reduced in the CNP promoter controlled Cre mice. The MOG and PLP promoters are predominantly active in mature oligodendrocytes, whereas the CNP protein is also produced in oligodendrocyte progenitor cells. This leads to the conclusion that tenascin-R is predominantly expressed by oligodendrocyte precursor cells.

Zusammenfassung

Tenascin-N, ein bisher unbekanntes Mitglied der Molekülfamilie der Tenascine wurde identifiziert und charakterisiert. Die vollständige cDNA enthält 5773 bp und wurde durch 5' und 3' RACE Experimente komplettiert, wobei Northern-Blot Analysen die Länge des Transkripts bestätigten. Der offene Leserahmen der mRNA beträgt 4683 bp und codiert für typische Strukturmotive der Tenascine: N-Terminal befindet sich ein cysteinreicher Bereich, gefolgt von 3.5 epidermalen, wachstumsfaktorähnlichen Domänen, 12 fibronektinähnlichen Domänen und am C-Terminus findet man eine Domäne mit Homologien zu Fibrinogen. Phylogenetische Analysen zeigten, daß Tenascin-N ein eigenständiges Homolog der Familie darstellt und keine orthologen Verwandtschaften zu bekannten Familienmitgliedern bestehen. Im zentralen Nervensystem wird das neue Tenascin vorwiegend von Neuronen gebildet, wobei es noch stärker in der Niere, Milz und in Knochen synthetisiert wird. Es tritt besonders in späteren Entwicklungsstadien der Gewebe auf. So findet sich Tenascin-N in der Medulla der funktionell reifen Niere, in einer Subpopulation der Makrophagen der adulten Milz und in der späten Phase der Verknöcherung des Skelettsystems. Eine alternative Splißform von Tenascin-N wurde durch RT-PCR kloniert. Ihr fehlt die 3. fibrobnektinähnliche Domäne des vollständigen Moleküls. In Explantatstudien zeigte sich, daß die kürzere Splißvariante das Neuritenwachstum und die Zellwanderung von hippocampalen Neuronen aus der CA3 Region inhibiert, wobei sich das vollständige Molekül neutral verhält. Obwohl Tenascin-C und Tenascin-N in denselben Organen exprimiert werden, zeigen sie ein zeitlich und räumlich unterschiedliches Expressionsmuster.

Im zweiten Teil dieser Arbeit wird die Etablierung einer Mauslinie vorgestellt, in der das Gen für Tenascin-R konditional entfernt wurde. Um die Klonierung des Mutationsvektors zu erleichtern, wurde ein neuartiges Plasmid konstruiert, in dem das Neo-Resistenzgen von gruppierten LoxP- und FRT-Elementen flankiert wird. Diese Neo-Resistenz-Kassette wurde zur Konstruktion der konditionalen Tenascin-R Knockout Mauslinie eingesetzt. In diesen Mäusen wurde das Gen für Tenascin-R zelltypspezifisch aus Oligodendrozyten entfernt, indem drei unterschiedliche transgene Mauslinien verkreuzt wurden. Diese Linien exprimierten die Cre Rekombinase entweder unter der Kontrolle des MOG-, PLP- oder CNP-Promotors. Die Rekombination fand in jeder Linie statt, jedoch wurde nur in der Verkreuzung mit der CNP-Cre Maus das Tenascin-R Protein reduziert. Da die MOG- und PLP-Promotoren erst in der späten Phase der Oligodendrozytendifferenzierung aktiv sind, wogegen das CNP-Protein bereits in Oligodendrozytenvorläufern vorkommt, führt dies zu der Schlußfolgerung, daß Tenascin-R überwiegend in der frühen Entwicklungsphase der Oligodendrozyten gebildet wird.

Keywords

extracellular matrix
tenascin family
conditional knockout

Schlagwörter

Extrazelluläre Matrix
Tenascin
konditionale Knockout

Abbreviation list

aa	amino acid
bp	base pair
cDNA	complementary DNA
CNP	2’,3’-cyclic nucleotide 3’-phosphodiesterase
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor-like
ES	embryonic stem
EST	expressed sequence tags
FBG	fibrinogen-like
FN III	fibronectin type III
kD	kilo dalton
MOG	myelin/oligodendrocyte glycoprotein
mRNA	messenger RNA
Neo	aminoglycoside phophotransferase
P	postnatal day
PALS	periarterial lymphatic sheath
PCR	polymerase chain reaction
PLP	proteolipid protein
RACE	rapid amplification of cDNA ends
RGD	arginine-glycine-aspartate
rpm	round per minute
RT-PCR	reverse transcriptase PCR
TN	tenascin
$TNR^{FL/FL;Cre}$	homozygously floxed TN-R mice + heterozygous Cre transgene
u	unit
UTR	untranslated region
wt	wildtype

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Appendix

1 The complete cDNA and deduced protein sequence of the mouse tenascin-N

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1    agtaccaggaccagctcacaggaggcaggggattggaagtggtgcccagcagcctcccaggcctcctgg

71   agtgtctgatccctttctccaagaATGGGTCTCTGGGGGATGCTCGCCTTCCCCCTGGGATTTCTGCTTG
                             M  G  L  W  G  M  L  A  F  P  L  G  F  L  L
141  CTTCTGTGCTCCTGGTGGCTTCGGCCCCAGCCACTCCAGAGTCTCCCGGCTGCAGCAACAAAGAGCAACA
     A  S  V  L  L  V  A  S  A  P  A  T  P  E  S  P  G  C  S  N  K  E  Q  Q
211  GGTCACTGTTAGCCACACCTACAAGATTGACGTGCCCAAGTCTGCTCTGGTTCAAGTAGAGACCGACCCA
       V  T  V  S  H  T  Y  K  I  D  V  P  K  S  A  L  V  Q  V  E  T  D  P
281  CAGTCACTCAGCGATGATGGGACATCACTCTTGGCTCCCGGGGAGGATGGGGAGGAGCAGAACATTATCT
      Q  S  L  S  D  D  G  T  S  L  L  A  P  G  E  D  G  E  E  Q  N  I  I
351  TCAGGCACAACATCCGTCTTCAGACACCGCAGAAGAATTGCGACCTGGCAGACAGTGTCCAGGACCTGCT
     F  R  H  N  I  R  L  Q  T  P  Q  K  N  C  D  L  A  D  S  V  Q  D  L  L
421  AGCCCGGATGAAAAAGCTGGAGGAAGAGATGGCAGAGCTGAAGGAGCAGTGCAATACCAACCGCTGCTGC
       A  R  M  K  K  L  E  E  E  M  A  E  L  K  E  Q  C  N  T  N  R  C  C
491  CAGGGGGCTGCTGATCTGAGCCGTCACTGCAGTGGCCACGGGACCTTCCTCCCTGAGACCTGCAGCTGCC
      Q  G  A  A  D  L  S  R  H  C  S  G  H  G  T  F  L  P  E  T  C  S  C
561  ACTGTGACCAGGGCTGGGAGGGCGCAGACTGTGATCAGCCCACCTGTCCTGGGGCTTGCAACGGCCACGG
     H  C  D  Q  G  W  E  G  A  D  C  D  Q  P  T  C  P  G  A  C  N  G  H  G
631  GCGCTGTGTGGATGGGCAGTGCGTGTGTGACGCGCCCTATGTGGGGGTCGACTGCGCCTACGCCGCCTGT
       R  C  V  D  G  Q  C  V  C  D  A  P  Y  V  G  V  D  C  A  Y  A  A  C
701  CCCCAGGACTGCAGTGGGCATGGCGTGTGCGTGCAGGGTGTCTGCCAGTGCCACGAGGACTTCACAGCAG
      P  Q  D  C  S  G  H  G  V  C  V  Q  G  V  C  Q  C  H  E  D  F  T  A
771  AGGACTGCAGCGAGCAGCGCTGTCCTGGCGACTGTAGTGGCAATGGTTTCTGTGACACTGGCGAGTGTTA
     E  D  C  S  E  Q  R  C  P  G  D  C  S  G  N  G  F  C  D  T  G  E  C  Y
841  CTGTGAGATGGGCTTTACTGGCCCCGACTGTTCCCAGGTGGTGGCTCCTCAGGGCCTGCAGTTGCTCAAG
       C  E  M  G  F  T  G  P  D  C  S  Q  V  V  A  P  Q  G  L  Q  L  L  K
911  AGCACGGAGAACTCTCTGCTGGTGAGTTGGGAGCCCTCCAGTGAGGTAGACTACTACCTGCTCAGCTACT
      S  T  E  N  S  L  L  V  S  W  E  P  S  S  E  V  D  Y  Y  L  L  S  Y
981  ACCCCCTGGGGAAGGAGCAAGCTACAAAACAGGTCCGGGTACCCAAGGAGCAGCACACCTATGACATCAC
     Y  P  L  G  K  E  Q  A  T  K  Q  V  R  V  P  K  E  Q  H  T  Y  D  I  T
1051 CGGCTTGCTGCCTGGAACCAAGTACATAGTCACCCTGCGCAACGTGAAGAAAGACATTTCCAGCAGCCCT
       G  L  L  P  G  T  K  Y  I  V  T  L  R  N  V  K  K  D  I  S  S  S  P
1121 CAGCATCTACTTGCCACCACAGATCTTGCTGTGGTGGGCACAGCCTGGGTAAATGAAGAGACTGAGACAT
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1261  AGGGCAGGAGGTGACAGAGGTCACTGTGCCCAAGAGCCGTGATCCCAAGAGCAGATATGACATCACTGGT
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1331  CTGCAGCCTGGAACGGAATATAAAATCACAGTTGTGCCCATCCGAGGTGATCTGGAGGGAAAGCCGATTC
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1401  TCCTGAATGGCAGGACAGAAATTGATGGACCAACCAATGTGGTCACAAATCAGGTGACAGAAGACACAGC
      L  L  N  G  R  T  E  I  D  G  P  T  N  V  V  T  N  Q  V  T  E  D  T  A
1471  ATCTGTTTCCTGGGATCCAGTGAGGGCTGACATAGACAAGTATGTGGTGCGCTATATCGCCCCTGATGGG
        S  V  S  W  D  P  V  R  A  D  I  D  K  Y  V  V  R  Y  I  A  P  D  G
1541  GAGACCAAGGAGAAGGCAGTACCAAAGGACCAGAGCAGCACCGTTCTCACAGGCCTGAAGCCAGGAGAGG
      E  T  K  E  K  A  V  P  K  D  Q  S  S  T  V  L  T  G  L  K  P  G  E
1611  CCTACAAAGTCTTTGTGTGGGCTGAGAGGGGCAACCAAGGCAGCAAGAAAGCAGACACCAAGGCCCTCAC
      A  Y  K  V  F  V  W  A  E  R  G  N  Q  G  S  K  K  A  D  T  K  A  L  T
1681  AGAAATTGACAGTCCAGAAAACCTGGTGACTGACCGGGTGACAGAGAACAGCCTCTCTGTCTCGTGGGAC
        E  I  D  S  P  E  N  L  V  T  D  R  V  T  E  N  S  L  S  V  S  W  D
1751  CCAGTGGAGGCTGACATCGACAGGTACGTGGTAAGCTACACTTCCGTGGATGGAGAGACGAAGCAGGTTC
      P  V  E  A  D  I  D  R  Y  V  V  S  Y  T  S  V  D  G  E  T  K  Q  V
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      P  V  K  K  D  Q  R  S  T  V  L  T  G  L  S  P  G  V  E  Y  K  V  Y  V
1891  GTGGGCAGAGAAAGGCGATCGGGAGAGCAAGAAGGCCAACACCAAGGCTCCCACAGACATCGACAGCCCC
        W  A  E  K  G  D  R  E  S  K  K  A  N  T  K  A  P  T  D  I  D  S  P
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      K  N  L  V  T  D  Q  V  T  E  N  T  L  S  V  S  W  D  P  V  Q  A  N
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      I  D  R  Y  M  V  S  Y  T  S  A  D  G  E  T  R  E  V  P  V  P  K  E  K
2101  GAGCAGTACCGTCCTGACTGGCCTGAGGCCAGGTGTGGAGTACAAGGTCCATGTGTGGGCCCAGAAGGGG
        S  S  T  V  L  T  G  L  R  P  G  V  E  Y  K  V  H  V  W  A  Q  K  G
2171  ACCCAGGAGAGCAGAAAGGCCAACACCAAGGCCCCCACAGATATTGATGGCCCCAAAAACCTGGTGACTG
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2241  ACCAGGTGACCGAGACCACTCTTAGTGTCTCCTGGGACCCAGTGGAGGCTGACATTGATAGGTACATGGT
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      D  G  E  T  R  E  F  L  V  G  K  D  Q  T  S  T  V  L  T  G  M  R  P
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2731  CCCAACAGATATTGATGGCCCCAAAAACCTGGTGACTGACCAGGTGACCGAGACCACTCTTAGTGTCTCC
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      K  A  L  T  E  I  D  P  P  R  N  L  R  P  F  G  V  T  H  S  G  G  V
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      D  A  R  F  P  H  P  S  D  C  S  Q  V  Q  Q  N  T  N  A  A  S  G  L
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     Y  T  I  Y  L  N  G  D  A  S  R  P  M  Q  V  Y  C  D  M  D  T  D  G  G
4201 CGGCTGGATTGTCTTCCAGAGACGGAACACTGGGCAGCTGGATTTCTTCAAGCGTTGGCGGAGTTATGTA
      G  W  I  V  F  Q  R  R  N  T  G  Q  L  D  F  F  K  R  W  R  S  Y  V
4271 GAAGGTTTTGGGGACCCCATGAAGGAGTTCTGGCTTGGACTTGATAAACTACATAATCTCACCACTGGCA
      E  G  F  G  D  P  M  K  E  F  W  L  G  L  D  K  L  H  N  L  T  T  G
4341 CCACCACTCGGTATGAGGTGAGGGCAGACTTACAGACTTTCAATGAATCTGCCTACGCTGTATATGATTT
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     T  T  T  R  Y  E  V  R  A  D  L  Q  T  F  N  E  S  A  Y  A  V  Y  D  F
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       F  Q  V  A  S  S  K  E  R  Y  K  L  S  V  G  K  Y  R  G  T  A  G  D
4481 GCTCTCACCTACCACAATGGATGGAAGTTCACGACTTTTGACAGAGACAATGATATTGCCCTCAGCAACT
      A  L  T  Y  H  N  G  W  K  F  T  T  F  D  R  D  N  D  I  A  L  S  N
4551 GTGCACTGACGCATCATGGTGGCTGGTGGTATAAGAACTGCCATTTGGCCAACCCGAATGGCAAATATGG
     C  A  L  T  H  H  G  G  W  W  Y  K  N  C  H  L  A  N  P  N  G  K  Y  G
4621 GGAGACCAAGCACAGCGAGGGGGTGAACTGGGAGCCATGGAAGGGACATGAGTTCTCCATTCCTTATGTG
       E  T  K  H  S  E  G  V  N  W  E  P  W  K  G  H  E  F  S  I  P  Y  V
4691 GAGCTGAAAATCCGCCCGTTTGGTTACAGCAGAGACCGTTTCTCTGGCAGAAAGAAGCGCTCCATAGGAA
      E  L  K  I  R  P  F  G  Y  S  R  D  R  F  S  G  R  K  K  R  S  I  G
4761 AAGCAAGGATGTTCTGAaggcttgtctgagctgtcttcacaggagacaagagaaacttggggttggcgggg
     K  A  R  M  F  *
4831 gtagatggtgcctaggaagctgaagtttgagggatgctcatagcctccgacttcctagattgctgggtac

4901 acagtgctaatgacgtcatcacactttgacctttagaggttccttccatgtcatctgcaagtttgactgt

4971 ctctactgagggcttcattgttggctttcaggagccaacaatgttgtaactgaacagtacatatggatag

5041 aacacgtgttggaatcacaaggtttttcagcccatccttaatgacagatgtattgaattagggcaaaaa

5111 ggtgaaccatcctgtagaccatcaattggaaatctttagaaattggtggagatctctgtatccatctgat

5181 aatatagatctttgtagaaaaaaaaagacaaagtgaggttctgatcccctgggtgtcagagctgaggcga

5251 cctgctacttcttgtctctcctggacctcctggtgtttgaaggaaatgtttatatcttactacttttaaa

5321 cttcctgtgtgatgaagaatgaagagactacaaaggggccacgatgcagtcatagttaagttctgactag

5391 tctctagcattctagagaccaaagctcccattttattgctatttttaactgccccttttccagacatttg

5461 cataagttctttcatagatctgcatatgttgtaaataaatttgcagtcatttaaactttaaataacccat

5531 ctctttaaataactggtcccctcagctccccccccaccacttcactcacctccatgcctctcccctccccc

5601 gggtccatcagccctcagactccctccttccctgctccccacactggaagcttcctcacatcatctttct

5671 tgcgtgtttggtgtcattttggaaagttttctgtaaccaagtcagtgacctttgaagtaacttaaattcc

5741 tactcaagaaaacacaaataaactgtttgattt
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2 Publications and poster presentations

Neidhardt, J., S. Fehr, M. Kutsche, J. Löhler, and M. Schachner. 2001. Tenascin-N: characterization of a novel member of the tenascin family. *J. Cell. Biol.*, submitted

Neidhardt, J., and M. Bösl. 2001. A versatile Neo resistance selection cassette for gene targeting experiments. *Biotechniques*, submitted

Neidhardt, J., and M. Schachner. 2001. Tenascin-N: Cloning and characterization of a novel tenascin family member. Poster presentation, Herbsttagung der Gesellschaft für Biochemie und Molekularbiologie (GBM), Bochum, 9th-12th September 2001.

3 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Fremde Hilfe ist, soweit sie in Anspruch genommen wurde, vermerkt.

Diese Arbeit ist zuvor noch keiner Prüfungsbehörde in gleicher oder ähnlicher Form vorgelegt worden.

Hamburg, den 14.12.2001

(John Neidhardt)

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5 Curriculum vitae

John Per Neidhardt
Geboren am 7. Februar 1973 in Hamburg

Schulbildung:

1979-1983	Grundschule Strenge, Hamburg
1983-1989	Gymnasium Albert Schweizer, Hamburg
1990-1992	Gymnasium Karl von Ossietzky, Hamburg
1992	Abitur mit der Durchschnittsnote 1,3

Hochschulbildung:

1992-1997	Studium der Biochemie, Universität Hannover
1996	Praktikum am Zentrum für Medizin und Biowissenschaften (Borstel) mit dem Thema: „Enzymkatalysierte Synthese des Endotoxinbestandteils 2-Keto-3-Desoxyoctonsäure.“
1996	Praktikum im Institut für Peptidforschung (Hannover) mit dem Thema: „Etablierung von Reporterassays zur Analyse von Promotorsequenzen.“
1997	Auslandspraktikum am Department of Plant Biology, University Berkeley (USA) mit dem Thema: „Photosysthem-II repair and chloroplast recovery from irradience stress.”
1997-1998	Diplomarbeit am Max-Planck-Institut für Experimentelle Endokrinologie (Hannover) mit dem Thema: „Zur Phylogenie des TRH abbauenden Ektoenzyms.“
1998	Diplom der Biochemie in Hannover mit der Note „sehr gut“.
1999-2001	Aufbaustudium der Molekularbiologie, Universität Hamburg
2001	Abschluß des Aufbaustudiums mit der Note „sehr gut“.

Berufliche Weiterbildung:

1995	Fortbildung an der Medizinischen Hochschule Hannover zum Beauftragten für Strahlenschutz und Biologische Sicherheit.
1995	Fortbildung an der Medizinischen Hochschule Hannover zum Thema: "Tierexperimentelle Methoden"
1998	Fortbildung an der Universität Hannover zum Thema: "Patentrecht und Patentrecherche"

Veröffentlichungen:

Neidhardt J, Benemann JR, Zhang L and Melis A, "Photosysthem-II repair and chloroplast recovery from irradience stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in *Dunaliella salina* (green algae)", 1998, *Photosynthesis Research*, 56:175-184

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