Net-targeted mutant mice develop a vascular phenotype and up-regulate *egr-1*

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The ternary complex factors (TCFs) Net, Elk-1 and Sap-1 regulate immediate early genes through serum response elements (SREs) *in vitro*, but, surprisingly, their *in vivo* roles are unknown. Net is a repressor that is expressed in sites of vasculogenesis during mouse development. We have made gene-targeted mice that express a hypomorphic mutant of Net, Net δ , which lacks the Ets DNA-binding domain. Strikingly, homozygous mutant mice develop a vascular defect and up-regulate an immediate early gene implicated in vascular disease, *egr-1*. They die after birth due to respiratory failure, resulting from the accumulation of chyle in the thoracic cage (chylothorax). The mice have dilated lymphatic vessels (lymphangiectasis) as early as E16.5. Interestingly, they express more *egr-1* in heart and pulmonary arteries at E18.5. Net negatively regulates the *egr-1* promoter and binds specifically to SRE-5. *Egr-1* has been associated with pathologies involving vascular stenosis (e.g. atherosclerosis), and here *egr-1* dysfunction could possibly be associated with obstructions that ultimately affect the lymphatics. These results show that Net is involved in vascular biology and *egr-1* regulation *in vivo*.

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Introduction

The ternary complex factors (TCFs) form a subfamily of Ets-domain transcription factors. The three TCFs, Elk-1, Sap-1 and Net/Sap-2/Erp/Elk-3 (Price *et al.*, 1996; Wasylyk *et al.*, 1998), have four conserved domains, A–D. A is the Ets DNA-binding domain (DBD). The B-box interacts with the serum response factor (SRF). C is a transcriptional activation domain that is stimulated by mitogen-activated protein (MAP) kinase phosphorylation. The D-domain is a MAP kinase-binding site and a nuclear localization signal. The TCFs are nuclear mediators of

cellular responses to the activation of MAP kinase pathways. Net differs from the other TCFs in that in basal conditions, in which MAP kinases are not activated, it strongly inhibits transcription. Repression is mediated by two domains, the NID (Maira *et al.*, 1996) and the CID (Cricqui-Filipe *et al.*, 1999). The TCFs form ternary complexes with SRF on serum response elements (SREs) of immediate early gene promoters, such as *c-fos*, *egr-1* and *jun-B*. The SRE is constitutively occupied by factors, and extracellular signals are thought to lead to both phosphorylation of the complex and changes in its composition due to the exchange of TCFs.

The *in vivo* role of the TCFs is poorly understood. They may regulate the expression of immediate early genes in response to various inductive stimuli. The TCFs are expressed in many cell types and tissues (Giovane *et al.*, 1994; Lopez *et al.*, 1994; Magnaghi-Jaulin *et al.*, 1996; Nozaki *et al.*, 1996; Sgambato *et al.*, 1998), but their precise *in vivo* expression patterns are not well known. Net is expressed during mouse development at E7.5–8.5 in developing vascular primordia, including the yolk sac blood islands, allantoic vessels, heart endocardium and dorsal aortae (Ayadi *et al.*, 2001). Vascular endothelial cell expression persists throughout development, raising the possibility that Net may have functions during mouse development in vasculogenesis and angiogenesis.

In order to study the function of Net *in vivo*, we generated mice with a targeted disruption of the *net* gene. Homozygous mutant mice are born with the expected Mendelian frequency and appear normal. However, they develop chylothorax with a high penetrance within the first postnatal weeks, and die of respiratory failure. The mice have highly dilated thoracic lymphatic vessels, in which *net* is expressed. They also have elevated levels of the *egr-1* mRNA in some vascular structures in the thoracic region. Taken together, the results show that Net regulates *egr-1* and, in agreement with its expression pattern, has a role in the vasculature.

Results

Targeted disruption of the *net* gene

We deleted 1055 bp surrounding *net* exon 2, which contains the ATG and codes for the first 69 amino acids of the DBD (Figure 1A). The targeting vector had, 5' to 3', 11.5 kb of genomic sequences, the PGK-Neo^R cassette and 1.2 kb of genomic sequences. Recombinant embryonic stem (ES) cell clones were injected into C57BL/6 blastocysts and chimeras were crossed to C57BL/6 and 129/Sv females. Net^{+/mutant} mice were intercrossed to generate homozygous mutant mice, as shown by Southern blotting and PCR analysis of genomic DNA from the offspring (Figure 1B and C).

Fig. 1. Targeted mutagenesis of the *net* gene. (A) Schemes. Exon 2 contains the translation initiation codon and encodes amino acids 1–69 of *Net*. The 3' probe for Southern blots, the 13 (wild-type) and 5 (mutant) kb *Xba*I fragments and the PCR primers (uc54, uc56 and uc57) are shown. B, *Bam*HI; X, *Xba*I. (B) Southern analysis of *Xba*I-digested DNA from the progeny from a heterozygous (wild-type/mutant) inter-cross. The 3' probe reveals 13 kb wild-type (WT) and 5 kb targeted (M) alleles. (C) PCR analysis of the same progeny. The products for the wild-type (WT, 1550 bp) and the targeted (M, 1300 bp) alleles are indicated. (D) Analysis of *net* transcripts by RT-PCR. RNA from E16.5 wild-type and homozygous mutant embryos was used for RT-PCR with primers from exons 1–4. As expected, mutant RNA is not amplified with exon 2 primers due to the deletion, but is amplified with exon 3 + 4 primers. Exon 1 + 3 (ex1/ex3 set) primers produce a shorter product (90 bp) with the mutant as opposed to the wild-type (297 bp) RNA. The deduced mutant *Net* mRNA, *Net* δ , is shown. The in-frame ATG is indicated in italics. (E) Western blots of lung extracts from 2-week-old wild-type, heterozygous and homozygous mice. The #375 antibody, raised against a peptide encoded by exon 3 (Giovane *et al.*, 1994, 1997), detects a 47 000 Da protein in wild-type (+/+) and heterozygous (+/ δ) samples. The heterozygous (+/ δ) and homozygous (δ / δ) samples have a 37 000 Da band, *Net* δ , as expected from initiation at the internal ATG. (F) Basal *c-fos* SRE reporter activity in transfected MEFs generated from wild-type and homozygous mutant (*net* δ / δ) embryos. The histogram shows the average absolute luciferase activity for three experiments repeated in triplicate.

Characterization of the *Net* mutant mice

Net mRNA was examined by RT-PCR with primers that amplify across the junctions between exons 1 and 4 (Figure 1D). The expected products were obtained with wild-type and homozygous mutant mice and primers between exons 1 and 2, 2 and 3, and 3 and 4. With primers between 1 and 3, the expected 297 bp fragment was obtained from wild-type mice, and a shorter 90 bp fragment from the homozygous mutants, corresponding to splicing between exons 1 and 3, as shown by sequencing the PCR product (data not shown). The exon 1–3 splice product was not detected in wild-type mice, suggesting that it is specific for the mutant. All the *net* transcripts detected by northern blotting were found to be reduced in size, as expected from deletion of exon 2 (data not shown).

The new transcript contains an in-frame AUG in exon 3 (nucleotide 571, amino acid 93) that is expected to be a good translation initiation site since there is an A at –3 and a G at +4 (Kozak, 1991; Giovane *et al.*, 1994). The predicted 37 000 Da *Net* protein was detected in heterozygous and homozygous mice, by western blotting with two different antibodies against *Net*, PAb 375 (Figure 1E) and PAb 376 (data not shown). As expected, the wild-type 47 000 Da *Net* protein was not detected in the homozygous mutant mice. The mutant *Net* protein, *Net* δ , lacks the Ets domain (amino acids 1–89), which is encoded by sequences upstream from the new start (M93). The loss of the DBD is expected to inhibit DNA binding and thereby decrease repression, measured with a *c-fos* SRE in a reporter under basal conditions as described

Fig. 2. The phenotype of *net* δ/δ mice. (A) Genotype frequency of live-born animals from a heterozygous inter-cross. (B) Decreased survival of *net* δ/δ mice compared with +/+ and +/ δ animals. (C) Typical phenotype developed by *net* δ/δ mice. This animal had respiratory distress at 6 days and died 2 days later. The thoracic cavity is full of chyle.

previously (Giovane *et al.*, 1994). SRE activity was measured by transfection of an SRE-luciferase reporter in primary mouse embryo fibroblasts (MEFs) derived from mutant and normal mice. As expected, SRE activity was four times higher in the *net* δ/δ compared with wild-type fibroblasts under basal conditions (Figure 1F).

***Net* δ/δ mice die postnatally of chylothorax**

Cross-breeding of heterozygous *net* $^{+/\delta}$ mice (from both mixed 129Sv/C57BL/6 and pure inbred 129Sv genetic backgrounds) gave viable homozygous offspring with the expected Mendelian ratios (Figure 2A), indicating that *net* δ/δ mice develop normally. The *net* δ/δ mice began to die within a few weeks. The death rate was highest on the

129Sv background, with ~80% of the *net* δ/δ mice dying within 6 weeks (Figure 2B). The same phenotype was observed following 10 back-crosses with 129Sv mice, and 12 back-crosses with C57BL/6 mice, showing that the phenotype was not strictly dependent on the background, and was tightly linked to the mutated allele. The homozygous mice developed signs of respiratory distress, became physically inactive and died within ~2 days. *net* δ/δ animals with respiratory distress were found to have a milky liquid in the thoracic cavity (Figure 2C), which was shown to be chyle, based on its high level of triglycerides (18.4 mmol/l versus 1.5 mmol/l in the plasma), and a high proportion of lymphocytes (80% of the cells by FACS analysis; data not shown). The chylous effusion filled the pleural space and compressed the lungs, as shown by examination of paraffin sections of the thorax (Figure 3A and B). The survival of *net* δ/δ mice with respiratory distress could be extended for a few days, by draining the chylous effusion by thoracocentesis using a syringe. These results indicate that the *net* δ/δ mice die from respiratory failure due to compression of the lungs by chyle, in other words from 'congenital' chylothorax (Merrigan *et al.*, 1997; de Beer *et al.*, 2000).

Dilated lymphatic vessels in the *net* δ/δ mice

Highly dilated vessels were observed in histological sections of the thoracic wall of the *net* δ/δ mutant mice (Figure 3C and D). These appeared to be lymphatic vessels, on the basis of their extremely thin endothelial lining and the absence of luminal red blood cells. Vascular endothelial growth factor receptor 3 (VEGFR-3) knock-in mice, which specifically express LacZ in the lymphatics (Dumont *et al.*, 1998), were used to confirm the identity of the dilated vessels. The VEGF-R3 $^{+/-}$ *net* δ/δ mice, which were generated by crossing compound mutant mice (*vegfr3* $^{+/-}$ *net* $^{+/\delta}$), were phenotypically indistinguishable from the *net* δ/δ mice, indicating that the lack of one VEGFR-3 allele did not enhance the effect of the *net* mutation. The X-gal-stained blue lymphatic vessels inside the thoracic wall were very dilated in the *net* δ/δ animals with a developing chylothorax compared with their *net* $^{+/+}$ littermates (Figure 4A and B). In contrast, the lymphatic vessels of the pericardium and skin were similar (Figure 4C–F), showing that the dilation was specific for the chest wall. The increase in the diameter of the thoracic lymphatics in the *net* δ/δ mice was observed after birth (Figure 4G and H), and even at E16.5 (data not shown), well before the onset of the respiratory distress and chylothorax.

***Net* mRNA is expressed in the lymphatic endothelium**

We have found that *net* mRNA expression is highly restricted to endothelial cells during mouse development (Ayadi *et al.*, 2001). We studied whether *net* is expressed in the lymphatic vessels by *in situ* hybridization (ISH), using VEGFR-3 as a specific marker (Kaipainen *et al.*, 1995). Using serial frozen sections from E16.5 embryos, *net* and VEGFR-3 expression were found to be co-localized in the thoracic duct (Figure 5A–C), as well as in the intestinal (Figure 5D–F) and skin (data not shown) lymphatics. However, *net* expression is wider than endothelial cells, especially in the thoracic duct, where it is also detected within the muscle layer (Figure 5B; Ayadi

Fig. 3. Histology of the thorax. Haematoxylin–eosin-stained cross-sections of wild-type (A) and mutant (B) thoraxes are shown. Bars = 0.8 mm. The *net*^{δ/δ} mouse was killed at 8 days, when it developed respiratory distress symptoms, together with a *net*^{+/+} littermate control. The pleural space is expanded in the mutant, filled by the chylous effusion (asterisk), which has compressed the lungs and the heart. (C and D) Magnifications of the dashed squares in (A) and (B), respectively. The *net*^{δ/δ} thoracic wall (D) has dilated lymphatic vessels compared with the control (C). R, ribs; lv, lymphatic vessel. Bars = 0.2 mm.

et al., 2001). *net* was found to have a vascular pattern of expression on the thoracic wall by whole-mount RNA hybridization (Figure 5G and H). VEGFR-3 was expressed in a similar pattern, as shown by X-gal staining of the VEGF-R3^{+/-} mice (Figure 5I). These results show that *net* is expressed in the lymphatic vessels, which are affected in the *net*^{δ/δ} animals.

Net mutation leads to localized up-regulation of *egr-1* expression

We studied the molecular basis of the phenotype of the *net*^{δ/δ} mice by RNA ISH of E16.5 stage embryos. We initially used endothelial markers, because lymphatics have a venous origin (Kaipainen *et al.*, 1995) and *net* is expressed in endothelial cells. We chose genes with

functional Ets-binding sites in their regulatory regions, including Tie1, Tie2 and VEGFR-1 (Flt-1) (Wakiya *et al.*, 1996; Schlaeger *et al.*, 1997; Iljin *et al.*, 1999). However, no obvious differences were found (data not shown and Figure 7E and F). We also studied *c-fos* and *egr-1*, two SRE-containing genes that are regulated by the TCFs (reviewed in Treisman, 1994; Wasylyk *et al.*, 1998) and are expressed in the perichondrium of bones (Dony and Gruss, 1988; Sandberg *et al.*, 1988; McMahon *et al.*, 1990), similarly to *net* (Ayadi *et al.*, 2001). The expression levels of *c-fos* or *egr-1* in bony structures, such as in the perichondrial interface of basioccipital bone and vertebrae (Figure 6A–F) and the articular joint from the hind limb (Figure 6G–J), were similar in wild-type and *net* mutant mice. However, increased expression of *egr-1* was

Fig. 4. Dilatation of the thoracic lymphatic vessels in *net*^{Δ/Δ} mice. The LacZ-VEGFR-3 reporter strain was used to identify lymphatic vessels (Dumont *et al.*, 1998). The lymphatic vessels of *net*^{+/+}VEGFR-3^{+/+} (A, C, E and G) and *net*^{Δ/Δ}VEGFR-3^{+/+} mice (B, D, F and H) were stained with X-gal (blue). (B, D and F) Lymphatic staining of a *net*^{Δ/Δ} mouse that developed chylothorax at 10 days of age. Lymphatics of the mutant thoracic wall (B) are dilated compared with the *+/+* littermate (A). (C and D) Pericardial and (E and F) chest skin lymphatic vessels. Bars = 42 μ m. (G and H) Thorax from a 5-day-old *net*^{Δ/Δ} mouse before pleural effusion and a *net*^{+/+} littermate. R, ribs; ic, intercostal region. Bars = 90 μ m.

observed in the atrium wall of the heart of *net*^{Δ/Δ} embryos (Figure 7A–D). Surprisingly, there was no increase in adjacent tissues, such as the aorta and the perichondrium of the ribs. Although *net* was expressed in the endocardium, in the endothelial cells (compare with the VEGFR-1 marker, Figure 7E and F), the perichondrium of the ribs and the cartilaginous condensation of the trachea, its pattern of expression was similar in the wild-type and mutant mice (Figure 7G and H). At E18.5, *egr-1* up-

regulation was no longer detected in the heart, but there was a striking increase in *egr-1* expression in one or several major pulmonary arteries of the mutant embryos (Figure 8). *egr-1* up-regulation was restricted to a particular area of the lung, as shown with different sagittal sections (Figure 8C, F and I). The *egr-1* signal was homogeneous throughout the vascular wall in the mutant embryos, compared with the heterogeneous labelling in the equivalent blood vessel in the littermates (Figure 8J–L). We also studied *egr-1* expression in thoracic tissues from mutant mice after birth, but before the onset of chylothorax (Figure 9). There was a widespread increase in *egr-1* expression in the lungs of *net*^{Δ/Δ} mice (Figure 9C and D; arrowheads), which upon closer examination (Figure 9G and H) was found to be patchy compared with the more homogeneous background staining in the wild-type mice, and located in particular in blood vessels as well as other unidentified cells in the lung parenchyma (arrows). In conclusion, *net* mutation results in *egr-1* up-regulation in restricted parts of the vasculature, in the atrial wall of the heart at E16.5, in pulmonary arteries at E18.5 and in lung arteries after birth.

Net negatively regulates the *egr-1* promoter

In transfection assays, the *c-fos* SRE is negatively regulated by Net under basal conditions (Giovane *et al.*, 1994), raising the possibility that the *egr-1* promoter is also negatively regulated by Net. The *egr-1* promoter contains five SREs, of which the three upstream elements (3–5, Figure 10A) are critical for the induction of *egr-1* promoter activity (Clarkson *et al.*, 1999). We investigated whether Net negatively regulates *egr-1* promoter activity, using *net* antisense constructs to down-regulate Net (Giovane *et al.*, 1994). Mouse NIH-3T3 fibroblasts were transfected with the *egr-1200*-Luc reporter (Clarkson *et al.*, 1999) and increasing quantities of the antisense *net* plasmid. Down-regulation of Net reproducibly increased *egr-1* promoter activity (Figure 10B). The extent of up-regulation is significant, since it is quantitatively similar to the effect of co-expression of Sap-1a and Elk-1 (Clarkson *et al.*, 1999). The antisense is specific, since it down-regulates endogenous Net without affecting the levels of the closely related protein Elk-1 (Giovane *et al.*, 1994 and data not shown). Deletion of the three critical upstream SREs abolished the stimulation resulting from Net down-regulation (compare *egr-1200*- and *egr-250*-Luc, Figure 10B). These results show that the *egr-1* promoter is negatively regulated by Net.

Net is recruited to the *c-fos* SRE by SRF to form a ternary complex, but can bind alone to consensus Ets motifs (Giovane *et al.*, 1994; Maira *et al.*, 1996). SAP-1a and Elk-1 have been shown to bind autonomously to the Ets motif of *egr-1* SRE-5 and to form ternary complexes with SRF on this element (Clarkson *et al.*, 1999). We investigated whether Net has similar properties, using bandshift experiments and SRE-5 probes. We found that Net forms a complex with the wild-type probe in the absence of SRF (Figure 10C, lanes 1 and 2). Complex formation is prevented by mutation of the Ets motif (mut ets, lanes 5 and 6) but is not inhibited by mutation of the SRF motif (mut SRF, lanes 9 and 10). SRF forms a complex in an SRF motif-dependent manner (lanes 3, 7 and 11), and SRF + Net form a ternary complex when both

Fig. 5. Co-expression of *net* and VEGFR-3 RNAs in E16.5 embryos. (A–F) ISH of sagittal sections with ^{35}S -labelled riboprobes. The signal grains are white dots on a dark field. (A and D) Bright-field sections are shown for histology. (B and C) Net and the VEGFR-3 lymphatic marker are expressed in the thoracic duct (arrows). (E and F) Net is also expressed in lymphatic (arrows) and blood vessels in the gut. (G–I) Whole-mount ISH of thoracic biopsies. The specific signal has a dark blue colour. (G) Net is expressed in a plexus pattern (arrow). (H) The higher magnification shows Net staining of a lymphatic vessel (arrowhead). (I) Same magnification as (H), to show the aspect of lymphatics (arrowhead) in the thoracic wall stained with the VEGFR-3 probe. Ao, aorta; Oe, oesophagus; r, ribs; St, sternum; Td, thoracic duct; Tr, trachea; Ve, vertebrae. Bars = 50 μm (A–F), 200 μm (G), 100 μm (H and I).

motifs are intact (TC, lanes 4, 8 and 12). We compared the rate of dissociation of the ternary (TC) and binary Net complexes in the presence of excess cold SRE-5 probe (Figure 10D). The ternary complex dissociates more slowly, showing that it is more stable. These results show that Net can bind autonomously and form ternary complexes with SRF on SRE-5.

We investigated whether deletion of the Ets domain in Net δ inhibits binding and recruitment by SRF to SRE-5. Using *in vitro* translated proteins, we found that Net δ was unable to form either autonomous or ternary complexes with SRE-5 (Figure 10E, compare lanes 5 and 6 with 1–4). Using lung nuclear extracts, we detected several complexes that are sensitive to mutation of the Ets motif, B1,

B3 and B4 (Figure 10F, the part of the gel with differences is shown). B1 correspond to ternary complexes, since they are supershifted by SRF antibodies (not shown), migrate above the SRF complex (B2), have a similar migration as the ternary complex formed with *in vitro* translated proteins and are inhibited by mutation of the Ets motif. B2 is the SRF complex, since it is supershifted with SRF antibodies, inhibited by mutation of the SRF motif and co-migrates with the *in vitro* complex (data not shown). B3 is not significantly affected by Net antibodies, and does not co-migrate with *in vitro* complexes, suggesting that it is formed by other Ets proteins. B4 co-migrates with *in vitro* Net complexes and its intensity is reduced by Net antibodies, suggesting that it is composed partly of Net

Fig. 6. Unaltered expression of *c-fos* and *egr-1* RNA in bony structures of *net*^{Δ/Δ} E16.5 embryos. ISH of *net*^{+/+} (A, C, E, G and I) and *net*^{Δ/Δ} (B, D, F, H and J) embryos. (C and E), (D and F), (I) and (J) are the dark fields corresponding to the bright fields (A), (B), (G) and (H), respectively. Expression of: (i) *c-fos* (C and D) and *egr-1* (E and F) is not altered in the mutant embryo in the articular surface between the basioccipital bone and vertebrae (arrowheads); and (ii) of *egr-1* (I and J) in the articular joint space from the hind limb (arrowheads). Ar, articular space; Bo, basioccipital bone; Br, brain; Hu, humerus; OC, otic capsule; Ra, radius; Ve, vertebrae. Bars = 90 μm (A–F) and 110 μm (G–J).

complexes. The B4 complex in *net*^{Δ/Δ} extracts has a lower intensity and is not affected by Net antibodies (lanes 2, 3, 5 and 6), suggesting that it contains other Ets proteins. These results show that deletion of the Ets domain in Net^Δ inhibits binding to SRE-5 in the presence or absence of SRF.

Discussion

We produced Net mutant mice lacking exon 2, which contains the translation initiation codon and codes for most of the Ets DBD (Giovane *et al.*, 1994, 1997). The mutant mice express a mRNA isoform that lacks exon 2 due to

Fig. 7. Up-regulation of *egr-1* RNA in the heart of *net*^{δ/δ} E16.5 embryos. ISH on sagittal frozen sections of the thoracic region. (A and B) Bright fields, (C–H) corresponding dark fields for *net*^{+/+} (A, C, E and G) and *net*^{δ/δ} (B, D, F and H) mice. (C and D) *Egr-1* labelling is stronger in the atrial wall of the *net*^{δ/δ} heart (arrowheads) but not, for example, in the ribs or the aorta. (E and F) *VEGFR-1* endothelial marker. (G and H) *Net* expression. *VEGFR-1* and *net* are expressed in the atrial wall (arrowheads, E–H). Ao, aorta; At, atrium; Ht, heart; Lu, lung; Ri, ribs; Th, thymus; Tr, trachea. Bar = 110 μ m.

splicing between exons 1 and 3, and in which all the other exons are spliced normally (Figure 1D and data not shown). This RNA has an in-frame ATG translation initiation codon with an optimal Kozak sequence. It codes for a shorter protein, Net δ , which lacks the Ets DBD. The *net* gene produces several alternatively spliced mRNAs,

including *net-b* and *net-c* (Giovane *et al.*, 1997 and data not shown). We cannot exclude the possibility that the exon 1–3 splice occurs naturally, even though we did not detect it in several mouse tissues (data not shown). Net δ lacks the Ets domain and consequently does not bind to DNA *in vitro*. However, we cannot exclude the possibility

Fig. 8. Spatially restricted up-regulation of *egr-1* RNA in the lung vasculature of *net*^{δδ} E18.5 embryos. Egr-1 ISH on sagittal sections from the thoracic cage of *net*^{+/+} (B, E, H and K) and *net*^{δδ} (C, F, I and L) embryos. The bright fields (A, D, G and J) of the *net*^{+/+} sections are shown for histology. Comparable sections (1–3) are shown for *net*^{+/+} and *net*^{δδ} embryos. In section 1, *egr-1* expression is similar in the wild-type and mutant mice, whereas in the other two sections there is stronger labelling in individual pulmonary arteries in the mutant (arrowheads; F and I). There is no difference in *egr-1* labelling in other sites of expression, such as the thymus, the rib perichondrium and the walls of the vena cava as well as other vascular structures. (J, K and L) Higher magnification of sections 2, showing *egr-1* up-regulation in the wall of an artery of a *net*^{δδ} embryo (L, arrowheads), compared with the same vessel of a wild-type embryo (K, arrowheads), where *egr-1* is detected within a restricted area. Ar, artery; Ht, heart; L, vascular lumen; Li, liver; Lu, lung; Ri, ribs; Th, thymus; VC, vena cava. Bars = 200 μm (A–I) and 25 μm (J–L).

that Net is recruited to some promoters by protein–protein interactions *in vivo*, or has DNA binding-independent functions. Net^δ retains a number of functional domains, including the activation domain (C) and interaction domains with SRF (B), E47 (NID), CtBP (CID) and MAP kinases (D, JEX) (Maira *et al.*, 1996; Criqui-Filipe *et al.*, 1999; Ducret *et al.*, 2000). A brain-specific isoform of Elk-1, sELK-1, that lacks the first 54 amino acids of the DBD has been described recently. This deletion severely compromises but does not abolish ternary complex formation on the SRE *in vitro*. sELK-1 plays an opposite role to Elk-1 in nerve growth factor-driven PC12 neuronal differentiation (Vanhoutte *et al.*, 2001). As Net is a repressor under basal conditions (Giovane *et al.*, 1994), the loss of repressor function by the mutant protein should

increase the activity of cellular SRE elements. As expected, we found that *c-fos* SRE activity is enhanced in *net*^{δδ} MEFs transfected with an SRE reporter. Furthermore, Net^δ cannot bind to DNA by itself or together with SRF. In *net*^{δδ} MEFs, *egr-1* levels are higher under basal conditions and somewhat lower following induction by fibroblast growth factor-2 (FGF-2), consistent with the loss of a repressor that is converted to an activator by FGF-2-induced signalling (data not shown). These data strongly suggest that Net^δ is a hypomorphic mutant that has a weaker effect than the corresponding wild-type protein. It is unlikely that the protein has a *trans*-dominant effect, since heterozygous animals are phenotypically identical to their wild-type littermates. Net^δ is expressed in amounts similar to the wild-type protein. In

Fig. 9. Egr-1 ISH of lungs before the onset of chylothorax. Equivalent sagittal frozen sections from the thoracic region of 2-day-old *net*^{+/+} (A, C, E and G) and *net*^{δ/δ} (B, D, F and H) mice. (C and D) Lower magnification showing a specific increase in the *egr-1* signal throughout *net*^{δ/δ} lungs (arrowheads). (E–H) Higher magnifications of similar regions of the lungs. (G and H) Patchy *egr-1* up-regulation is detected in pulmonary blood vessels (arrowheads) and parenchyma (arrows). Ht, heart; Lu, lung; Th, thymus; Ri, ribs; VC, vena cava. Bars = 150 μm (A–D) and 50 μm (E–H).

addition, inhibition by a dominant-negative Net protein of the other TCFs, which are positive regulators, would decrease rather than increase the activity of the SRE (Vanhoutte *et al.*, 2001).

Homozygous *net*^{δ/δ} mutant animals develop congenital chylothorax, which has not been reported previously for mice. Chylothorax is a rare condition found in humans and other animals that results from accumulation of chyle in

the pleural space due to disruption of the thoracic duct or one of its collaterals (Merrigan *et al.*, 1997; de Beer *et al.*, 2000). Such rupture can result from identifiable causes, such as trauma, infection or neoplasia, or can arise spontaneously neonatally or during infancy, most often due to congenital defects in the circulation (van Straaten *et al.*, 1993; de Beer *et al.*, 2000). The absence of communication between the lymphatics or obstruction of

Fig. 10. Net represses the activity of and binds to the *egr-1* promoter. (A) Schemes of the mouse *egr-1* promoter–luciferase reporters. Egr-1200-Luc contains 1200 bp of the *egr-1* promoter with its five SREs (closed squares), whereas egr-250-Luc contains 250 bp upstream from the transcription start (arrows) with two proximal SREs. (B) Decreasing endogenous Net expression stimulates the *egr-1* reporter through the distal SREs. NIH-3T3 cells were transfected in triplicate with *egr-1* reporters and the p601D-anti-net plasmid that produces antisense *net* RNA, and luciferase activity was measured. An increasing amount of anti-net leads to significant and reproducible activation of the egr-1200-Luc reporter, but not egr-250-Luc. (C, D and E) Equal amounts of *in vitro* translated Net, Netδ and SRF proteins were used for gel retardation assays with wild-type or mutant SRE-5 probes (mut ets and mut SRF: mutated binding sites). Proteins and SRE-5 probes were incubated as indicated at the top and complexes were resolved by PAGE. Arrows indicate the complexes formed by Net, SRF and both proteins (TC, ternary complex), as well as a non-specific complex (NC) and the free probe (FP). (D) To compare off-rates, complexes were allowed to form for 25 min at 25°C and, after cooling on ice, a 500-fold excess of cold probe was added, the reactions were incubated for the indicated times at 0°C and then immediately run on the gel. 30'* was incubated for 30 min without competitor. (F) Nuclear extracts (Giovane *et al.*, 1997) from *net*^{+/+} and *net*^{δ/δ} lungs and *in vitro* translated Net and SRF proteins were used simultaneously for gel retardation assays with wild-type or mut ets SRE-5 probes. The Net antibodies were #375 (Giovane *et al.*, 1994).

lymphatic flow is thought to cause back pressure, dilation (lymphangiectasis) and leakage of lymph. *net*^{δ/δ} mice have extremely dilated lymphatic vessels specifically in the thoracic wall. Dilation precedes the onset of chylothorax, raising the possibility that there is a defect that alters lymphatic drainage. Net mRNA is expressed in lymphatic endothelium, including the thoracic duct and lymphatic vessels in the thoracic wall, suggesting that Net could be involved in the formation of the lymphatic network. However, we did not detect any obvious structural abnormalities in thoracic vessels or surrounding tissues by electron microscopy (data not shown), suggesting either that the primary defect is subtle or that it is located

elsewhere. Interestingly, α9 integrin 'knock-out' mice have recently been reported to develop chylothorax (Huang *et al.*, 2000). However, the phenotype of *net*^{δ/δ} differs from that of α9^{-/-} in that: (i) it is not fully penetrant and the time of onset is later; (ii) it shows no signs of oedema and inflammation surrounding the lymphatics (Figure 3D and data not shown); and (iii) it exhibits lymphangiectasis, which is not observed in α9^{-/-} mice. These differences suggest that the underlying defects are different. The causes of congenital chylothorax in humans are unknown. Our results raise the possibility that *net* gene mutation could potentially be a genetic cause of congenital chylothorax. Interestingly, the *net* gene is located on

chromosome 12q, and partial trisomy of 12q has been detected in one case of chylothorax (Houfflin *et al.*, 1993). However, additional loci may be involved, since trisomy 21 has also been associated with congenital chylothorax (Hamada *et al.*, 1992).

We have found that *net*^{δδ} mice express more *egr-1* mRNA. Transcription of *egr-1* is induced rapidly and transiently by a variety of mitogens in most cell lines without *de novo* protein synthesis, and with a pattern similar to *c-fos* (reviewed by Gashler and Sukhatme, 1995; Liu *et al.*, 1998). The similarity to *c-fos* may be due to the SRE regulatory elements (Treisman, 1992). *In vitro* studies have shown that the TCFs Net, Sap1 and Elk1 are recruited to SREs by a dimer of SRF (Treisman, 1994; Waslyk *et al.*, 1998). Net is able to down-regulate an isolated *c-fos* SRE in transient transfection assays (Giovane *et al.*, 1994; this study). However, *c-fos* expression is not altered in *net*^{δδ} animals or MEFs (data not shown). This may indicate that Net does not regulate *c-fos* expression *in vivo* (at least where studied), or that redundancy amongst the TCFs, or the nature of the Net^δ mutation, mask its contribution. The *egr-1* promoter has five SREs that mediate its response to various stimuli (McMahon and Monroe, 1995; Clarkson *et al.*, 1999; Santiago *et al.*, 1999a). Therefore, Egr-1 can be expected to be more sensitive to inactivation of TCFs (Arsenian *et al.*, 1998). Our results provide the first *in vivo* evidence for regulation of *egr-1* by a TCF.

The striking feature of *egr-1* up-regulation in *net*^{δδ} mice is its specificity. At E16.5, *egr-1* expression is increased in the atrial wall of the heart, at E18.5 in the vascular wall of some major pulmonary arteries, and after birth in the lung, in particular around blood vessels. *egr-1* expression is not altered overtly in other sites where it is expressed, including perichondrial tissues, the thymus, and MEFs in response to certain inducers (data not shown). Recently, Egr-1 protein has been shown to be expressed in a variety of cells in adult mice, including hepatocytes, neuronal cells, cardiomyocytes, and endothelial and vascular smooth muscle cells (Tsai *et al.*, 2000). The authors point out that Egr-1 expression is heterogeneous, raising the possibility that only a subset of endothelial cells or cardiomyocytes express Egr-1. These cells could be those that overexpress *egr-1* in the *net* mutant mice.

Increased *egr-1* expression could be a direct consequence of Net mutation. Net is expressed specifically in a number of cell types, including cardiomyocytes, endothelial and vascular smooth muscle cells (Ayadi *et al.*, 2001; Figure 7G and H). Furthermore, Net binds to the *egr-1* SRE and the endogenous protein represses *egr-1* promoter activity. These results are consistent with direct regulation. However, Net mutation does not increase *egr-1* expression in all the cells in which they are co-expressed. Furthermore, increased *egr-1* expression is not restricted to thoracic lymphatic vessels, the ultimate location of the phenotype, showing that the phenotype cannot be explained simply by *egr-1* up-regulation. There are various potential explanations as to why up-regulation is restricted to some cells. The TCFs regulate SRE activity in response to particular signals. The cells with increased *egr-1* expression could be responding to specific signals that are regulated negatively by Net. Alternatively, the *net*^{δδ} mutation may be hypomorphic, and only the most

sensitive pathways may be affected by this mutation. Nevertheless, this is the first *in vivo* evidence for the regulation of an immediate early gene by a TCF.

It is possible that the chylothorax phenotype and *egr-1* up-regulation are linked. There is evidence that Egr-1 is important in vascular biology, especially in pathological situations (reviewed in Khachigian and Collins, 1997; Silverman and Collins, 1999). Following vascular injury, *egr-1* expression is highly induced in endothelial and vascular smooth muscle cells at wound margins (Khachigian *et al.*, 1996; Morawietz *et al.*, 1999; Santiago *et al.*, 1999b,c). Inducible *egr-1* expression may coordinate the expression of multiple target genes involved in cell movement and replication in blood vessel walls. Ultimately, these cellular changes can result in the pathogenesis of vascular occlusive lesions such as restenosis or atherosclerosis. Egr-1 has been implicated directly in the migration and proliferation of smooth muscle cells, using antisense and DNA-targeted enzyme approaches (Santiago *et al.*, 1999b,c). *net*^{δδ} mice overexpress *egr-1* in some vessels. Increased *egr-1* expression has been associated with vascular occlusion, which could very well lead to chylothorax (Merrigan *et al.*, 1997; de Beer *et al.*, 2000). These potential links need to be studied further in more detail. In summary, the *net*^{δδ} mice show that *egr-1* is negatively regulated by the TCF Net *in vivo*, and provide an interesting model to study the role of Net in the regulation of *egr-1* and more generally in vascular biology.

Materials and methods

Generation of Net mutant mice

net gene sequences containing exon 2 were cloned from a 129Sv λ EMBL3 phage library and characterized by Southern blotting and sequencing. The targeting sequences, containing, 5' to 3', a 11.5 kb *Bam*HI–*Ava*I fragment, a 1.8 kb PGK-neo cassette and a 1.2 kb *Ava*I–*Bam*HI fragment, were excised with *Not*I and electroporated into D4 ES cells. Homologous recombinants were identified by Southern blotting and injected into C57BL/6 blastocysts to obtain chimeras. One of the positive ES clones transmitted the disrupted allele. Pure 129Sv and C57BL/6, and mixed (C57BL/6 $\times$ 129sv) genetic backgrounds were used.

Genotyping of ES cells, embryos and mice

Genomic DNA from ES cells or tail biopsies was isolated and resuspended in 100 μ l of 10 mM Tris–HCl pH 8.0, 1 mM EDTA. A 15 μ l aliquot of DNA was digested with *Xba*I and analysed by Southern blotting using a 3.8 kb 3' probe from outside the targeting construct. Routinely, mice were genotyped by PCR using the allele-specific primers: UC54, TGAAACGTGTAATCCTTGTGTCCTC; UC56, TAATTCCA-AGTTCTCGGCACGTAG; and UC57, GACCGTCTCTCGTGCTT-TACGGTA.

PCR conditions were 94°C for 2 min, 30 cycles at 94°C for 30 s, 64°C for 30 s, 72°C for 45 s and 72°C for 5 min, 0.5 μ l of DNA, 200 ng of each primer and 25 μ l of reaction containing PCR reagents (Sigma). Fragments of 1550 and 1300 bp are amplified from the wild-type and targeted alleles, respectively.

Expression analysis by RT-PCR and western blotting

RT-PCR. RNA was extracted from mouse tissues with Trizol (Gibco-BRL). A 1 μ g aliquot of total RNA was used for reverse transcription with exon-specific primers and amplified as described previously (Giovane *et al.*, 1994). The primer pairs were: EX1/EX2, CTAGAAATCTCCC-CAAGAAGACTC/GTTGTCGTCATAGTATCTCAGCGC; EX2/EX3, TGCTGGACATCGAACGATGGCGAG/ACTTGTACACAACTTCT-GCCCCG; EX3/EX4, CTGGAGCCCTGAATCTGTCATCG/TCG-AGGCCAGAAACAGTCCACTTG; and EX1/EX3, CTAGAAATC-TCCCAAGAAGACTC/ACTTGTACACAACTTCTGCCCGA.

The PCR products were electrophoresed on 5% polyacrylamide gels, stained with ethidium bromide and visualized under UV.

Western blots. Tissues were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl pH 8, 2 µg/ml aprotinin, 2 µg/ml leupeptin and 100 µg/ml phenylmethylsulfonyl fluoride) with an Ultrathorax. Proteins (200–300 µg) were fractionated by 10% SDS-PAGE, transferred to nitrocellulose membranes and revealed with purified PAb 375 (Giovane *et al.*, 1994) and the enhanced chemiluminescence detection kit (Amersham).

Histology, β -galactosidase staining and ISH

Standard conditions were used for histology, whole-mount β -galactosidase staining (Beddington *et al.*, 1989) and ISH with 35 S- and digoxigenin-labelled RNA probes (Décimo *et al.*, 1995). Probes were *net* (full-length cDNA; Giovane *et al.*, 1994), VEGF-R1 (RG 458440, Research Genetics Inc.), VEGFR-3 (Kaipainen *et al.*, 1995), *egr-1* (nucleotides 744–1400) and *c-fos* (DDBJ/EMBL/GenBank accession No. V00727). The yolk sacs of embryos were used for PCR genotyping.

Cell culture and transfections

Mefs were isolated from wild-type and homozygous mutant embryos (Robertson, 1987). Passage 3–6 Mefs were transfected in triplicate by the DEAE-dextran method (al-Moslih and Dubes, 1973) in 6-well clusters (Costar 3516) with 5 µg of DNA. After 20 h, the cells were washed twice with Dulbecco's modified Eagle's medium (DMEM), incubated in DMEM containing 0.5% fetal calf serum (FCS) for 24 h, and scraped for luciferase assays. NIH-3T3 cells in DMEM (Sigma) + 10% FCS were transfected by the BBS calcium phosphate method in 6-well clusters with 4 µg of DNA per well containing 0.5 µg of the *egr-1* reporters, 0.25–1 µg of the antisense *net* plasmid (p601D-anti-Net) and the appropriate amounts of the control vectors. After 16 h, the cells were washed, incubated for 24 h and scraped for luciferase assays.

Mobility shift assays

pSG5-based expression vectors for Net, SRF and Net δ were transcribed and translated in TNT rabbit reticulocyte lysates (Promega). Proteins (2 µl, adjusted to 4 µl with mock extracts that had been incubated with the pSG5 backbone vector) and an excess of probes in 20 µl of buffer [20 mM HEPES pH 7.8, 20% glycerol, 0.1 mM EDTA, 2.5 mM dithiothreitol (DTT), 10 µg of poly(dI-dC), 100 mM KCl] were incubated for 30 min on ice, 15 min at 37°C and 1 h at 25°C. The samples were loaded on pre-run (45 min at 8 mA) 6.6% polyacrylamide gel in 0.25× TBE and run with re-circulating buffer at 4°C.

The *egr-1* SRE-5 probes were: *egr-1* WT, 5'-GTTCGCCGACCC-GGAAACGCCATATAAGGAGCAGG-3'; *egr-1* mut ets, 5'-GTTCGC-CGACCCGCATATGCCATATAAGGAGCAGG-3'; and *egr-1* mut SRF: 5'-GTTCGCCGACCCGGAAACGCCATATGAAGAGCAGG-3'.

One strand of the double-stranded blunt-end probes is shown. The Ets and SRF motifs are underlined. Oligonucleotides were 5'-end-labelled with T4 polynucleotide kinase and purified on native 10% polyacrylamide gels.

Plasmids

Plasmids were constructed by standard methods: pTL2-Net, pTL2-SRF, p601D and p601D-anti-Net (Giovane *et al.*, 1994); pKOZ1-Net δ , the net fragment from GAL-N1 in pKOZ1 (Maira *et al.*, 1996); pGL2-*egr*-1200 and pGL2-*egr*-250 (Clarkson *et al.*, 1999), kindly provided by Michael J. Waters.

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