FEBS Letters 523 (2002) 12–16 FEBS 26212

Temporal and spatial expression of two isoforms of the *Dutt1/Robo1* gene in mouse development

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Received 11 February 2002; revised 13 May 2002; accepted 14 May 2002

First published online 20 June 2002

Edited by Ned Mantei

Abstract The mammalian homologue of the *Drosophila* axonal guidance receptor *roundabout* is expressed in a wide range of tissues. Here we show that alternative splicing of the *Dutt1/Robo1* gene results in two mRNA transcripts with different signal peptides, which are differentially expressed throughout mouse embryogenesis. Since mice with a targeted deletion in the *Dutt1/Robo1* gene have abnormal lung pathology, immunohistochemistry was used to identify the cellular expression pattern of Dutt1/Robo1 during lung development. Dutt1/Robo1 expression was widespread and diffuse in the lung at embryonic day 17.5 but became increasingly localised to the bronchial epithelium in newborn and adult mice.

Key words: *Roundabout*; *DUTT1*; *ROBO1*; Alternate splicing; Bronchial epithelium; Signal peptide

1. Introduction

The *roundabout* (*robo*) gene was identified in a large scale *Drosophila* mutant screen for the genes which control midline crossing of axons. In *robo* mutants axons aberrantly cross and re-cross the midline creating characteristic whirls. The gene was found to encode a transmembrane receptor and identified as a member of the immunoglobulin superfamily [1]. Three *robo* genes have since been described which have distinct, but overlapping expression patterns that define pathways for neuronal migration [2]. Biochemical purification and genetic linkage analysis identified *slit*, a large extracellular matrix protein, as the ligand for *robo* [3,4]. *slit* is expressed by midline cells in *Drosophila* and loss-of-function mutations are unable repel commissural neurones away from the midline [4]. Together the *slit* and *robo* genes define the neuronal patterning of commissural neurones crossing the midline in *Drosophila* [2].

The expression patterns of the vertebrate homologues of *robo* suggested that the neuronal guidance function had been evolutionally conserved. Robo1 and Robo2 have been detected in cells in mammalian brain [5] and spinal cord [6], which would be expected to contribute to midline development. In addition, Slit2 was biochemically isolated as a regulator of sensory axon elongation and branching [3] and Slit2 has been shown to be important in controlling the divergence of retinal ganglion cell axons in vertebrates [7,8]. However, analysis of retinal axon guidance in *Slit1/2*-deficient mice [9] and in *astray/robo2*-deficient zebrafish [10] indicates that Slit/Robo signalling does not regulate midline crossing in vertebrates. Instead, these loss-of-function mutations identify a role for Slit/Robo signalling in establishing the correct placement of retinal axons and in mice, the position of the optic chasm. Recently, a more general role in cell migration has been suggested by the observation that Slit2, signalling through Dutt1/Robo1, inhibits leukocyte migration induced by chemokines [11].

The human homologue of the *robo* gene was independently cloned as a potential tumour suppressor gene from within overlapping homozygous deletions in breast and small cell lung cancer cell lines and designated *DUTT1* [12]. This gene was identified as a member of the NCAM family with an unusually broad pattern of expression and was hypothesised to have additional functions to those performed in neuronal cells. Interestingly, mice with a targeted deletion in the *Dutt1/Robo1* gene show a delay in lung development at birth and bronchial epithelial abnormalities in adults [13]. These observations suggest that the Slit/Robo signalling pathway may have an additional function in lung development and adult lung physiology. The spatial and temporal expression pattern of Dutt1/Robo1 in mammalian lung is poorly understood. Here we describe the expression pattern of Dutt1/Robo1 during mouse lung development. In addition, we show that the *Dutt1/Robo1* gene is alternatively spliced and that the transcripts are differentially expressed during mouse development.

2. Materials and methods

2.1. Gene nomenclature

A gene cloned from within overlapping homozygous deletions was mapped to chromosome 3p and designated *DUTT1* (accession no. Z95705; [12]). Subsequently this gene was independently isolated as the human homologue of the *Drosophila robo* gene and submitted to GenBank as *H-robo1* (accession no. AF040990; [1]). Alignment of the two sequences and analysis of the draft human genome indicates that *DUTT1* results from alternate splicing of *H-robo1* gene [1]. The gene name suggested by HUGO nomenclature committee is *ROBO1*. We use the name *DUTT1* when a transcript [or polymerase chain reaction (PCR) product] includes the sequence corresponding to the signal peptide which is coded for in the cDNA we described as *DUTT1*. When the transcript (or PCR product) includes the sequence corresponding to the signal peptide which is coded for in the cDNA isolated by Kidd et al. [1]we refer to this as *ROBO1*, otherwise we refer to the gene as *DUTT1/ROBO1* (Fig. 3A).

2.2. cDNA clones, transfection and cell culture

The human *DUTT1* sequence was assembled from overlapping cDNA clones from HeLa, foetal brain and heart cDNA libraries as

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0014-5793/02/$22.00
PII: S0014-5793(02)02904-6

described previously [12]. Full-length *DUTT1* and *ROBO1* cDNAs were assembled using standard restriction digestion and ligation protocols and were verified by sequencing across ligation junctions. The clones were ligated into the mammalian expression vector pcDNA3.1 (Clonetech). Linearised expression constructs or control empty vector were introduced into the *DUTT1*/*ROBO1* null cell line U2020 [14] using effectene transfection reagent (Qiagen). Stable cell lines were selected by growth in G418-containing medium (500 μg/ml) for three weeks. Individual clones were isolated and tested for DUTT1 or ROBO1 expression by Western blotting and reverse transcription (RT)-PCR experiments as described below.

2.3. Polyclonal antibody production and Western blot analysis

A polyclonal antiserum was raised in rabbits against the C-terminal peptide of DUTT1/ROBO1 (CYERGEDNNEELEETES) by Zeneca (Cambridge Research Biochemicals, UK). The antiserum specifically recognised a band at approximately 190 kDa in cells transfected with either a *DUTT1* or *ROBO1* expression vector. Staining was blocked by pre-incubation of the immunising peptide with the antiserum. Preparation of cell lysates and Western blotting was performed as previously described [13].

2.4. Immunohistochemistry

Tissues or whole embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline for at least 24 h, paraffin embedded and processed to give 4 μm sections. Sections were deparaffinised and endogenous peroxide quenched. After blocking, Dutt1/Robo1 was detected using the polyclonal antiserum described above followed by donkey biotin-conjugated anti-rabbit secondary antibody (Santa Cruz, USA) and amplification with the Vectastain ABC kit (Vector Laboratories, USA) as per manufacturer's protocol. Positive staining was detected using nickel-enhanced diaminobenzidine tetrahydrochloride (DAB) and counterstained with Fast Red. Slides were mounted with Vectashield-mounting medium. Staining was blocked by pre-incubation of the immunising peptide with the antiserum. Adjacent sections were stained with haematoxylin and eosin (H and E).

2.5. RT-PCR of mouse tissues

Adult, newborn pups (P0) or timed pregnant C57Black/6 mice were killed at the gestational day indicated (based on appearance of vaginal plug = day 0) and total RNA was prepared from various tissues with Trizol reagent (Gibco BRL). Reverse-transcription reactions were performed on total RNA (5 μg) in 20 μl as previously described [13]. The primers used were: *Robo1*-specific exon 1 forward 5′-CTGGCCCAGCTTATCCA-3′, *Dutt1*-specific 1 forward 5′-AGGGATTGACAAGCCTCCGG-3′ and *Robo1*/*Dutt1*-common exon 2 reverse 5′-AGCTACCTCCAGCGATGCGT-3′. The *Dutt1*-specific product is 571 bp and the *Robo1*-specific product is 428 bp. Products were separated on a gel containing gelstar (BioWhitaker) and the images obtained by scanning using a Typhoon multi-load imager.

3. Results

3.1. Analysis of Dutt1/Robo1 protein expression by Western blotting

To analyse the protein expression pattern of Dutt1/Robo1 an antibody was generated against a C-terminal peptide (CYERGEDNNEELEETES). The specificity of the antibody was confirmed by the detection of a band at approximately 190 kDa in Western blots of cells transfected with an expression vector for *DUTT1* (Fig. 1A, lane 3) or *ROBO1* (Fig. 1A, lane 4) but not control cells transfected with vector alone (Fig. 1A, lane 2). A band of the same size was detected in mouse tissue and human cells by this antibody but not by the pre-immune serum (Fig. 1A). Pre-incubation of the antibody with the antigenic peptide blocked the detection of the 190 kDa band in transfected cells and mouse tissues (Fig. 1A). The expression pattern of Dutt1/Robo1 in mouse tissues was examined by Western blotting (Fig. 1B). In all tissues examined Dutt1/Robo1 expression was detected and the expression level decreased with developmental stage. The strongest expression

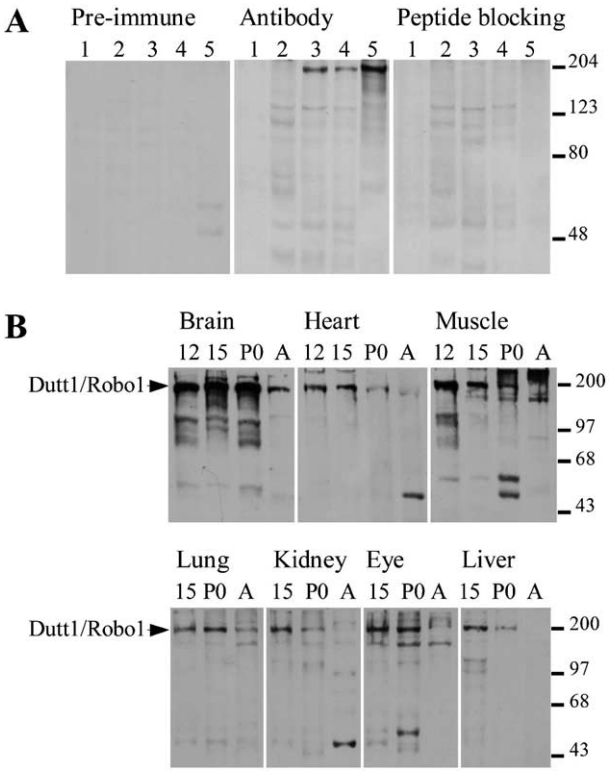

Fig. 1. Expression of Dutt1/Robo1 in transfected cell lines and a range of mouse tissues. Total protein extracts were prepared from tissue culture cells or mouse tissues and analysed by Western blotting on a 10% polyacrylamide gel using an antibody raised against the C-terminal of DUTT1/ROBO1. Panel A shows total protein extracts from U2020 cells (lane 1) or cell lines selected following transfection with either empty vector (lane 2), *DUTT1* expression vector (lane 3), *ROBO1* expression vector (lane 4) or newborn mouse brain (lane 5). The blots were probed with the pre-immune serum, C-terminal antibody or with antibody pre-incubated with the antigenic peptide as indicated. In panel B, extracts were prepared from C57black/6 mouse organs at the indicated developmental stage (12, embryonic day 12.5; 15, embryonic day 15.5; P0, newborn and A, adult). The arrowhead indicates the migration of DUTT1ROBO1 at ~190 kDa. The migration of pre-stained molecular weight markers is indicated.

was seen in the brain. Shorter fragments were observed in the heart, kidney and eye in adult tissues and in the newborn muscle, which may represent tissue-specific proteolysis. The molecular weight of the fragment observed in adult heart was estimated to be approximately 50 kDa, which would generate a cytosolic protein fragment. Interestingly, proteolytic fragments of hemaglutinin-tagged Robo1 were detected when an expression construct was transfected into human embryonic kidney cells [15] but not in the clones derived from U2020 cells shown in panel A.

3.2. Detection of Dutt1/Robo1 in mouse lung by immunohistochemistry

We have previously reported the expression of Dutt1/Robo1 in the bronchial epithelial cells of newborn mice [13]. Because of the expression of Dutt1/Robo1 in adult mouse lung tissue shown above and our demonstration of abnormal lung phenotypes in adult mice with a targeted mutation in the *Dutt1*/*Robo1* gene [13], we chose the developing mouse lung to investigate the cellular distribution of Dutt1/Robo1 during development.

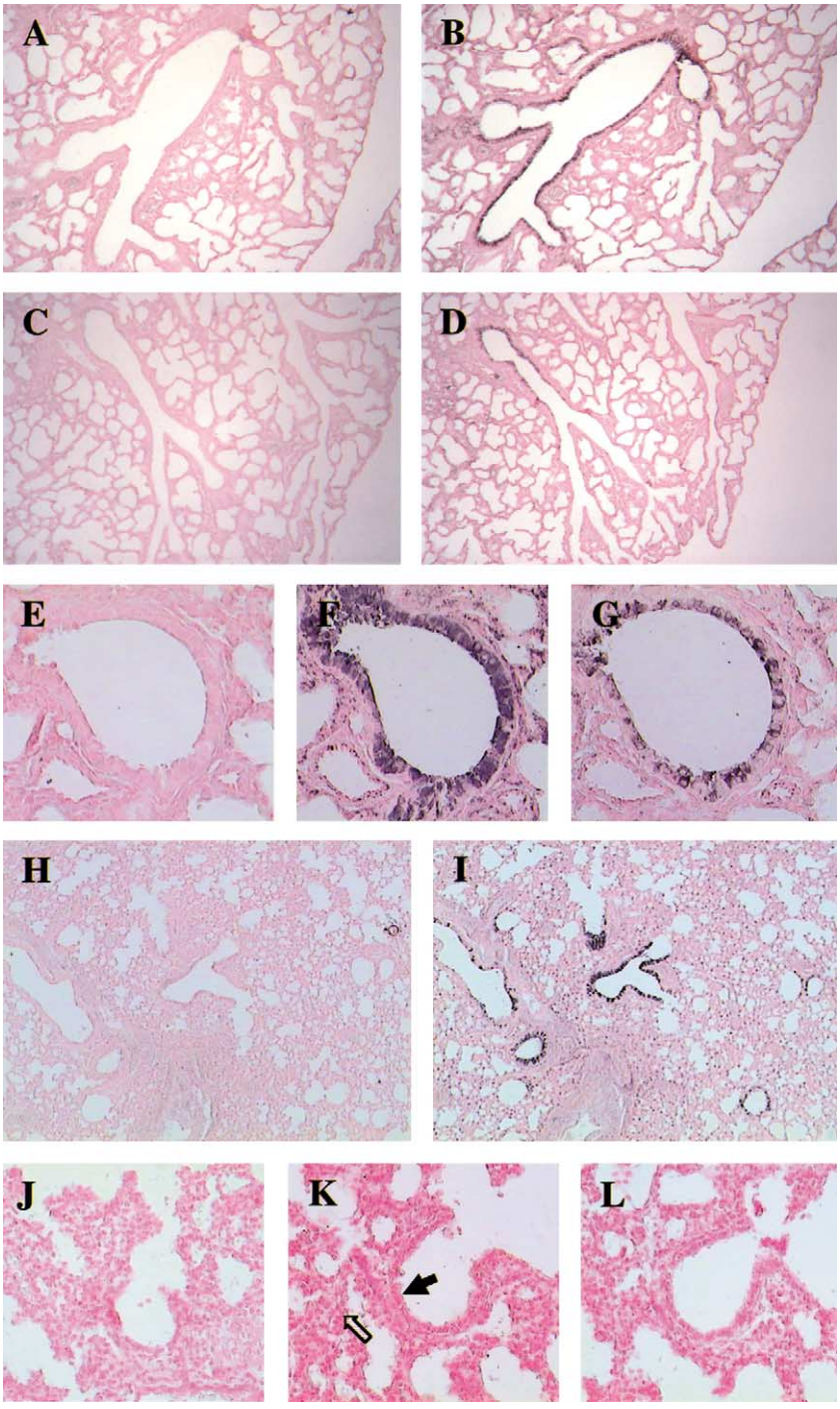

Fig. 2. Bronchial epithelial expression of Dutt1/Robo1 in mouse development. Whole embryos or dissected lungs were fixed in paraformaldehyde and processed to paraffin. Serial sections of lung (4 μm) were incubated with antibody as indicated below. Positive staining was detected using the biotin–avidin amplification system (Vectastain ABC kit) with DAB colour detection. The sections were counterstained with Fast Red. Lung tissue from embryonic day 17.5 (panels J–L), newborn (panels A–G) or adult mice (panels H and I) is shown. The sections were stained with either a control pre-immune serum (panels A, E, H and J) or with the C-terminal DUTT1/ROBO1 antibody (panels B, D, F, I and K), or a CC10 antibody (panel G). In panels C and L a control section where the staining has been blocked by pre-incubation of the antibody with the immunising peptide is shown. The original magnification was either 100× (panels A–D, H and I) or 200× (panels E–G and J–L).

Lung tissue or whole embryos were fixed and processed for analysis by immunohistochemistry using the C-terminal antibody described above. Initial experiments to characterise the antibody showed staining of the bronchial epithelium of newborn mice (Fig. 2B). The staining pattern was most intense in large proximal airways but not detectable in the smaller distal airways (Fig. 2D). Control experiments with the pre-immune serum from the same rabbit (Fig. 2A) and pre-incubation of the anti-serum with the immunising peptide (Fig. 2C) showed no detectable staining. Staining of adjacent sections of newborn mouse lung with the bronchial epithelial marker CC10 (Fig. 2G) co-localises with the cells positive for Dutt1/Robo1

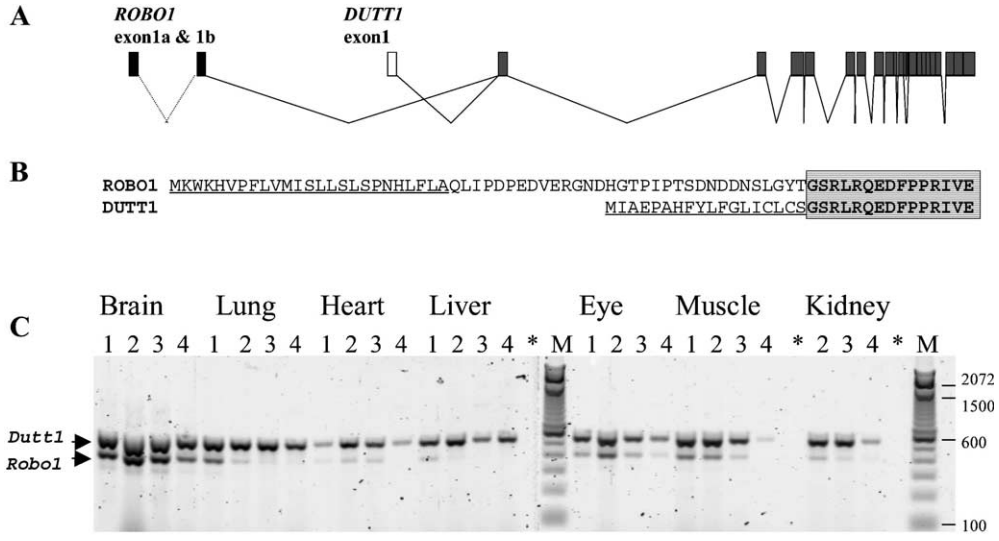

Fig. 3. Alternate splicing of the Dutt1/Robo1 gene generates two differentially expressed transcripts. A: Genomic organisation of the human *DUTT1*/*ROBO1* gene (adapted from the NCBI map). The dashed lines between the first two *ROBO1* exons indicate that the size of the intron is unknown. B: The translated products with the predicted signal peptides are underlined. C: Total RNA was extracted from C57black/6 mouse organs indicated at the embryonic day 12.5 (1), at the embryonic day 15.5 (2), at birth (3) and at three months (4) using Trizol reagent as per manufacturer's instructions. cDNA was generated using a *ROBO-1* exon 3 specific primer which is present in both *DUTT-1* and *ROBO-1* isoforms. *DUTT-1*-specific exon 1 forward primers to exon 2 reverse primers generate a 571 bp PCR fragment. *ROBO-1*-specific exon 1 forward primers to exon 2 reverse primers generate a 428 bp PCR fragment. Samples were loaded on a gel containing gelstar and the images obtained by scanning using a Typhoon multi-load imager. Lane 1: embryonic day 12.5; lane 2: embryonic day 15.5; lane 3: neonate; lane 4: adult. Lanes marked M contain a 100 bp ladder (GibcO BRL; where the darkest bands are 600, 1500 and 2072 bp as indicated on the left hand side of the panel) and lanes marked * indicate the water controls.

expression (Fig. 2F). In adult mouse lung, Dutt1/Robo1 expression is seen in the bronchial epithelial cells (Fig. 2I). A broader mesenchymal expression pattern is seen in day 17.5 embryonic lung (Fig. 2K, indicated by the open arrow), with some staining of the columnar cells of the developing bronchi (Fig. 2K, indicated by the closed arrow).

3.3. *DUTT1 and ROBO1 are splice variants of the DUTT1/ROBO1gene*

Genome structure analysis (results not shown) and alignment with the draft sequence of the human genome using the Blast program [16] indicate that there are at least two exons of the *DUTT1*/*ROBO1* gene corresponding to the *ROBO1*-specific signal peptide. We have called these *ROBO1a* and *ROBO1b* (Fig. 3A). The first 85 residues of this *ROBO1*-specific region called *ROBO1a* in Fig. 3A are not identified by Blast analysis of the Aug 2001 freeze of the draft genome. The line connecting *ROBO1a* and *ROBO1b* is dashed to indicate that the distance between these exons is unknown. A single exon encodes the *DUTT1*-specific signal peptide and 5′ untranslated region (Fig. 3A). The sequences of *DUTT1* and *H-robo1* cDNAs confirm that they are the same gene and show that, besides minor differences [1], the cDNAs differ only in sequences corresponding to their signal peptides and 5′ untranslated regions. Analysis of the transcripts using SignalP [17] predicts that the first exon of *DUTT1* encodes a signal peptide that would be cleaved in the mature protein (Fig. 3B). The *ROBO1* transcript is longer and is predicted, following cleavage of the signal peptide, to encode 32 unique amino acids at the N-terminus of the mature protein (Fig. 3B). Although the mouse gene structure has not been determined, analysis of mouse cDNAs indicates a very similar structure (results not shown).

3.4. *Differential expression of Dutt1/Robo1 alternate splicing transcripts during mouse development*

Our studies on the protein expression pattern of Dutt1/Robo1 revealed protein expression in adult tissues. We hypothesised that the adult expression may be due to translation of the Dutt1 transcript, which has a widespread expression in tissues and cell lines [12]. To test this hypothesis we performed RT-PCR experiments to determine the spatial and temporal expression pattern of the *Dutt1* and *Robo1* variants during mouse development. Primers pairs were designed to amplify the *Dutt1* and *Robo1*-specific signal peptide regions and were used to amplify RNA extracted from various mouse tissues at several stages of development. The *Dutt1* exon1 splice variant was more widely expressed in development and was frequently the only transcript detectable in adult tissues, whereas *Robo1* expression was detected in all tissues examined at embryonic day 12.5 and 15.5 but only in the brain, kidney and eye in adult tissue (Fig. 3C). The detection of RNA by RT-PCR did not always correlate with the level of protein expression. In the adult liver and eye for example, RNA was detected but little or no protein, which may reflect the sensitivity of the RT-PCR experiments or indicate additional controls on protein expression or half-life.

4. Discussion

We isolated the human homologue of the *Drosophila* axonal guidance receptor *robo* as a candidate tumour suppressor gene involved in lung cancer development [12,14] and as part of that study demonstrated the wide expression of the *DUTT1*/*ROBO1* gene outside the nervous system. Here we extend those observations and show that the level of expression is developmentally regulated. In lung, we have shown a marked

expression of Dutt1/Robo1 in the bronchial epithelial cells of the large proximal airways.

Two independent sequence analyses were performed on cDNAs of the *DUTT1/ROBO1* gene [1,12]. Comparison of the sequences indicated that there were two signal peptides available to this gene [1]. In view of the widespread expression of this gene as indicated by Northern blotting we speculated that it might be involved in functions other than axonal guidance [14] and that this might be achieved by tissue-specific differential transcription of the two isoforms. RT-PCR reactions, which distinguished the two splice variants, showed that there was not mutually exclusive expression of the isoforms at a tissue level. The *Dutt1* form was always expressed, whereas the *Robo1* form was only detectable during embryogenesis except in brain, kidney and eye where expression persists into adult life. Almost all of the human cell lines we have assessed express only the *DUTT1* form (unpublished observations) but this is not surprising since most cell lines are isolated from adult epithelial (tumour) cells. This issue could be resolved by in situ hybridisation to tissue sections of probes corresponding to each signal peptide.

There are two distinct signal peptides for the *DUTT1/ROBO1* gene and thus two distinct proteins are synthesised. Use of signalP to predict the signal peptide suggests that the mature ROBO1 isoform should be longer than the DUTT1 isoform by 32 amino acids. However, an anti-peptide antibody raised against these amino acids (IPDPEDVERGNDHGT) does not detect a protein of the expected size in COS cells transfected with the ROBO1 isoform (data not shown). N-terminal sequencing of purified protein(s) should resolve this uncertainty. Even if the mature proteins are the same, their different signal peptides imply some functional differences perhaps by altering the mRNA half-lives or their cellular location. DUTT1/ROBO1 is a transmembrane protein and is expected to be located on the cell surface for interaction with extracellular SLIT. However, Slit3 has recently been detected in mitochondria of rat kidney epithelial cells [19]. Although its function there and interactions are unknown, the discovery of Slit in vertebrate mitochondria suggests further degrees of complexity in Dutt1/Robo1–Slit interactions compared to *Drosophila*. The observation of two distinct signal peptides in mammalian Dutt1/Robo1 may contribute to this complexity.

Acknowledgements: The authors are grateful to Professor James Greenberg and Dr. Jian Xian for helpful discussion and technical advice. We also acknowledge the excellent technical services of Mrs Beverly Haynes from the Histopathology Department at Addenbrooke's Hospital and the animal technologists in the MRC-LMB. This work was supported by Grant G9703123 from the Medical Research Council awarded to P.H.R.

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