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BIOCHEMICAL AND CELL BIOLOGICAL STUDIES OF TRIM37 DEFECTIVE IN MULIBREY NANISM

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ACADEMIC DISSERTATION

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"Olen tullut luoksesi, koska muistin,
että omenapuun varjo on hyvin kaunis
puutarhassasi syyskuussa, kun istumme portailla."

"Niin, olet matkustanut viisisataa kilometriä
nähdäksesi omenapuun valkean ja sinisen varjon.
Tloitsen siitä - siis istukaamme portaille."

Helvi Hämäläinen

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List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-IV. In addition, some unpublished data are presented.

I Kallijärvi J, Avela K, Lipsanen-Nyman M, Ulmanen I, Lehesjoki A-E. (2002) The *TRIM37* gene encodes a peroxisomal RING-B-Box-Coiled-Coil protein: Classification of Mulibrey nanism as a new peroxisomal disorder. Am J Hum Genet 70:1215-1228

II Hämäläinen R, Avela K, Lambert J, Kallijärvi J, Eyaid W, Gronau J, Ignaszewski AP, McFadden D, Sorge G, Lipsanen-Nyman M, Lehesjoki A-E. (2004) Novel mutations in the *TRIM37* gene in mulibrey nanism. Hum Mutat 23:522

III Kallijärvi J, Lahtinen U, Hämäläinen R, Lipsanen-Nyman M, Palvimo J, Lehesjoki A-E. (2005) TRIM37 defective in mulibrey nanism is a novel RING finger ubiquitin E3 ligase. Exp Cell Res 308:146-55

IV Kallijärvi J, Hämäläinen R, Karlberg N, Sainio K, Lehesjoki A-E. Tissue expression of the mulibrey nanism-associated Trim37 protein in embryonic and adult mouse tissues. *Submitted*

Abbreviations

ACTH	adrenocorticotropic hormone
ALDP	adrenoleukodystrophy protein
ASC	apoptotic speck protein
BHK	Baby hamster kidney cells
BLAST	basic local alignment search tool
bp	base pair
cDNA	complementary deoxyribonucleic acid
CHF	congestive heart failure
COS-1	African green monkey kidney cells
DNA	deoxyribonucleic acid
E	embryonic day
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
FMF	familial Mediterranean fever
GST	glutathione-S-transferase
HA	hemagglutinin
HECT	homologous to E6-AP carboxyl terminus
HGP	Human Genome Project
kb	kilobase
kDa	kiloDalton
Mb	megabase
mRNA	messenger ribonucleic acid
NLS	nuclear localization signal
OMIM	Online Mendelian Inheritance in Man
ORF	open reading frame
P	postnatal day
PCR	polymerase chain reaction
PHD	plant homeodomain
PP2A	protein phosphatase 2A
PTS1	peroxisomal targeting signal 1
PTS2	peroxisomal targeting signal 2
RING	really interesting new gene
SDS	standard deviation score
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SRS	Silver-Russell syndrome
SULT	sulphotransferase
TNF	tumour necrosis factor
TRAF	tumour necrosis factor receptor-associated factor
TRIM	tripartite motif
UCH	ubiquitin C-terminal hydrolase
USP	ubiquitin-specific protease
UTR	untranslated region
wt	wild-type
WT	Wilms tumour

Summary

Mulibrey nanism (muscle-liver-brain-eye nanism) is a rare growth disorder first described in Finland in the early 1970s. Typical clinical features of mulibrey nanism include prenatal-onset growth failure, progressive cardiopathy, dysmorphic facial features, failure of sexual maturation, and metabolic syndrome. Prior to this study, the *TRIM37* gene at chromosome 17q23-25 was identified as the causative gene for mulibrey nanism. The gene encodes a 964-amino acid TRIM (tripartite motif, RING-B-box-coiled-coil) family protein of unknown function. Four disease-associated frameshift mutations predicting truncated proteins were initially identified in the gene. In this study, we set out to 1) explore the subcellular localization of wild-type and mutated TRIM37 proteins, 2) investigate the biochemical function of the protein and the effect of the patient mutations on the function, 3) identify proteins interacting with TRIM37, and 4) analyze the distribution of Trim37 in embryonic and adult mouse tissues. Moreover, novel *TRIM37* mutations were sought in patient DNA samples in order to confirm the diagnosis of suspected mulibrey nanism in patients outside of Finland, and to identify the second mutation in a Finnish patient heterozygous for the major Finnish mutation. Five frameshift mutations and two missense mutations (p.Gly322Val, p.Leu76Pro) were identified in the screening. The novel missense mutations were employed in subsequent functional studies.

Computer-based amino acid sequence analysis predicts that TRIM37 is a soluble (non-transmembrane) protein containing a putative nuclear localization signal close to its carboxyl terminus. We found that transiently expressed TRIM37 was not targeted to the nucleus but localized to cytoplasmic granular structures in cultured cells. The TRIM37-positive granules partly co-localized with peroxisomal marker proteins. The correct subcellular localization was lost in a mutant protein truncated at the coiled-coil region (Fin_{major} patient mutation) and in the p.Gly322Val mutant (representing a patient missense mutation), but retained in a protein truncated close to the carboxyl terminus (Fin_{minor} patient mutation). Endogenous TRIM37 immunoreactivity was detected in several cell lines and it localized to peroxisomes, as assessed by immunofluorescence analysis. High-level expression of TRIM37 in various cell lines induced the formation of poorly soluble inclusion bodies, or aggresomes, and

consequent loss of staining for organellar markers. An artificial mutant protein with disrupted RING finger (p.Cys35Ser;Cys36Ser) and the Gly322Val mutant protein did not form aggresomes and were markedly more soluble that the wild-type protein, suggesting that aggresome formation reflects a physiological function of the wild-type protein.

Several RING finger proteins, including members of the TRIM subfamily, act as ubiquitin E3 ligases. This prompted us to investigate whether TRIM37 is an E3 ligase. We first transiently expressed TRIM37 or its mutant forms in cell culture and studied their ubiquitination by means of immunoprecipitation and immunoblot analysis. The results showed that both full-length TRIM37 and its TRIM fragment are highly polyubiquitinated under such conditions. The polyubiquitination was diminished in the RING finger mutant (p.Cys35Ser;Cys36Ser), and in the p.Leu76Pro mutant (representing a novel missense mutation identified in a mulibrey nanism patient), implying that the modification is RING-dependent autoubiquitination. The results were corroborated by analyzing the ubiquitination of a bacterially-produced TRIM fragment in a reticulocyte lysate-based assay, and indicated that TRIM37 possesses ubiquitin E3 ligase activity. This finding raises the possibility that defective proteasomal degradation or, alternatively, defective regulatory ubiquitination of an as-yet-unidentified target protein underlies mulibrey nanism.

Finally, by means of immunohistochemical staining, we analyzed the distribution of Trim37 protein in various mouse tissues during ontogenesis. During embryonic development Trim37 immunoreactivity was detected in many epithelial tissues, including epithelium of developing lung and gut, in the developing pancreas and in ganglia. In adult tissues, Trim37 staining was detected in the central and peripheral nervous system, adenohypophysis, adrenal medulla, and in distinct cell types in the digestive system. Moreover, Trim37 was very highly expressed in maturing spermatids and oocytes.

Introduction

Initiated in the mid 1980s, the Human Genome Project (HGP) reached its first goal in 2001 when two draft sequences of the 3-billion-base pair human genome were published simultaneously by the HGP and by Celera, a private company (Lander et al. 2001; Venter et al. 2001). One of the most unexpected results of the HGP was that the estimated number of protein-encoding genes in humans was reduced from as high as 100,000 to about 25,000. This finding has, in part, lead to the realization that much of the diversity of gene products in higher vertebrates has to be generated by other means, for example alternative splicing, multifunctional proteins and post-translational modification (Levine and Tjian 2003; Pennisi 2005). However, the actual gene number in humans remains uncertain because new classes of gene-like elements, micro-RNAs and other non-protein-encoding transcripts have been discovered in mammals (Bartel 2004; Claverie 2005).

The sequencing of the human genome has enormously aided in the identification of disease-associated genes. The Online Mendelian Inheritance in Man (OMIM) database lists more than 2000 diseases and other phenotypes the underlying genes for which are currently known. Positional cloning has proved a particularly efficient method in identifying disease genes because it does not require any prior knowledge about the gene. It is based on DNA samples from disease families and on polymorphic DNA markers that are utilized to localize the disease locus by linkage analysis (Collins 1992). The Finnish disease heritage refers to rare genetic disorders that are much more common in Finland than elsewhere in the world (Norio 2003a), the result of our unusual history with small, isolated founder populations (Norio 2003b). At the turn of the millennium, 36 diseases were classified as belonging to the Finnish disease heritage. Research on the molecular genetics of the Finnish disease heritage has been tremendously successful, as thus far the underlying genes for all but a few of the diseases have been identified (www.findis.org).

Mulibrey nanism, a peculiar growth disorder with multiorgan manifestations, is a typical example of the Finnish disease heritage. It is autosomal recessive, 90% of known patients are Finnish, and it is concentrated in late-settlement areas in Finland (Perheentupa et al. 1973; Norio 2003c). The underlying gene for mulibrey nanism was identified by positional cloning

(Avela et al. 2000). The gene, *TRIM37*, encodes a previously uncharacterized protein of unknown function. What is the normal function of this protein? How do mutations in the *TRIM37* gene perturb the function of the protein product and lead to an unusual combination of both developmental and adult manifestations as is seen in mulibrey nanism? Functional studies, including biochemical characterization, analysis of subcellular localization and investigation of the tissue distribution of the defective protein aim at answering these questions, and are the subject of this study.

Review of the literature

1. Mulibrey nanism

1.1 Background

Mulibrey nanism (OMIM 253250), then a new disorder to the Finnish disease heritage, was first described in the early 1970s (Perheentupa et al. 1970; Perheentupa et al. 1973). The acronym mulibrey, for muscle-liver-brain-eye, is composed on the basis of some of the clinical findings, i.e. muscle hypotonia, hepatomegaly, enlarged brain ventricles, and abnormal retinal pigmentation. Mulibrey nanism cases are typically clustered in the late-settlement regions in Savo and northern Carelia (Lipsanen-Nyman 1986, Norio 2003c). The Finnish mulibrey nanism-associated founder mutation most likely arose on the shores of Lake Pielinen in the early 1600s (Lipsanen-Nyman 1986; Kristiina Avela, unpublished data). Molecular genetic studies of mulibrey nanism were initiated in the mid 1990s and resulted in the identification of the underlying gene in the year 2000 (Avela et al. 2000). At present, a total of 90 Finnish mulibrey nanism patients and nine patients from outside of Finland with a diagnosis confirmed by mutation analysis are known.

Reports on various clinical aspects of mulibrey nanism, such as craniofacial dysmorphism, the occurrence of Wilms tumour, and ophthalmologic findings were published by Finnish authors during the 1970s and early 1980s (Myllärniemi et al. 1978; Tarkkanen et al. 1982). The first suspected mulibrey nanism patients from outside of Finland were described in 1976 (Cumming et al. 1976; Voorhess et al. 1976). In the early 1980s, a study was initiated to investigate the genealogy, incidence and clinical features of the disease, based on all known Finnish patients. This work resulted in a monograph thesis that confirmed the autosomal recessive inheritance, established areas of enrichment of the disease gene, reported novel clinical findings in the patients, and summarized prevailing clinical data (Lipsanen-Nyman 1986). The clinical features and diagnostic criteria have been summarized and revised recently (Karlberg et al. 2004a). The revised diagnostic criteria are based on a study of 85 Finnish mulibrey nanism patients whose clinical data were systematically analysed from birth to the time of diagnosis. The diagnostic signs and their prevalence are presented in Table 1.

MAJOR SIGNS		FREQUENCY (%)
growth failure (A or B or C)	A) small for gestational age lacking catch-up growth	95
	B) height in children 2.5 SDS below population mean for age	94
	C) height in adult 3.0 SDS below population mean	90
characteristic radiological findings (A or B)	A) slender long bones with thick cortex and narrow medullar channels	93
	B) low and shallow (J-shaped) sella turcica	89
characteristic craniofacial features	scaphocephaly, triangular face, high and broad forehead, low nasal bridge and telecanthus	90
characteristic ocular findings	yellowish dots in retinal mid peripheral region	79
other	mulibrey nanism in a sibling	17
MINOR SIGNS	peculiar high-pitched voice	96
	hepatomegaly	70
	cutaneous naevi flammei	65
	fibrous dysplasia of long bone	25

Table 1. Diagnostic features of mulibrey nanism. For the diagnosis, three major signs with one minor sign or two major signs with three minor signs are required. SDS denotes standard deviation score. The table is modified from Karlberg et al. (2004a).

Silver-Russell syndrome (SRS, OMIM 180860) is another dysmorphic growth disorder characterised by prenatal-onset progressive growth failure (Hitchins et al. 2001). Patients with mulibrey nanism and SRS are both gracile with similar facial dysmorphism, which makes differential diagnosis important. These two conditions, however, have several distinct characteristics. Clinodactyly (curving of the fifth finger), small face with marked triangularity, micrognatia (small jaws), downturned mouth corners, and skeletal asymmetry with hemihypertrophy are characteristics of SRS. On the other hand, hepatomegaly,

pericardial constriction or cardiomyopathy, yellow dots in ocular fundi, and fibrous dysplasia of long bones typical of mulibrey nanism do not occur in SRS (Hannula et al. 2001; Karlberg et al. 2004a).

1.2 Clinical features

Pregnancies of mothers carrying a fetus with mulibrey nanism, and deliveries following such pregnancies, are in most cases normal. The prenatal-onset growth failure in mulibrey nanism is clearly apparent at birth, with mean birth length SDS -3.1 and mean birth weight SDS -2.8 in newborns (Karlberg et al. 2004a). Head circumference SDS is on average -0.5, indicating macrocephaly relative to the small stature of the patients (Karlberg et al. 2004a). The growth failure progresses in later infancy, and average length SDS drops to -4.4 at two years of age, a typical time of diagnosis. Nearly all (96%) mulibrey nanism patients are markedly growth-retarded and gracile at this point. Failure to thrive and feeding difficulties are the most common clinical problems during infancy. In addition, the patients have a high frequency of upper respiratory infections and middle ear infections in infancy, and pneumonias are diagnosed in nearly half of the infants by the age of two years (Karlberg et al. 2004a). Antibody deficiency and diminished antibody response *in vivo* has been reported in a Dutch patient with suspected mulibrey nanism (Haraldsson et al. 1993).

Typical craniofacial features (see Table 1), including triangular face, high forehead and low nasal bridge are present in over 90% of mulibrey nanism patients at diagnosis (Karlberg et al. 2004a). Other features present in over half of the patients are a high-pitch voice, yellow dots and abnormal pigment dispersion in ocular fundi, and naevi flammei mainly in the skin of the lower limbs. Another common finding is hepatomegaly (enlarged liver), which develops in 45% of the patients. Psychomotor development is within normal limits in the majority of mulibrey nanism patients, although one third of the patients show a mild delay in motor and speech development (Karlberg et al. 2004a).

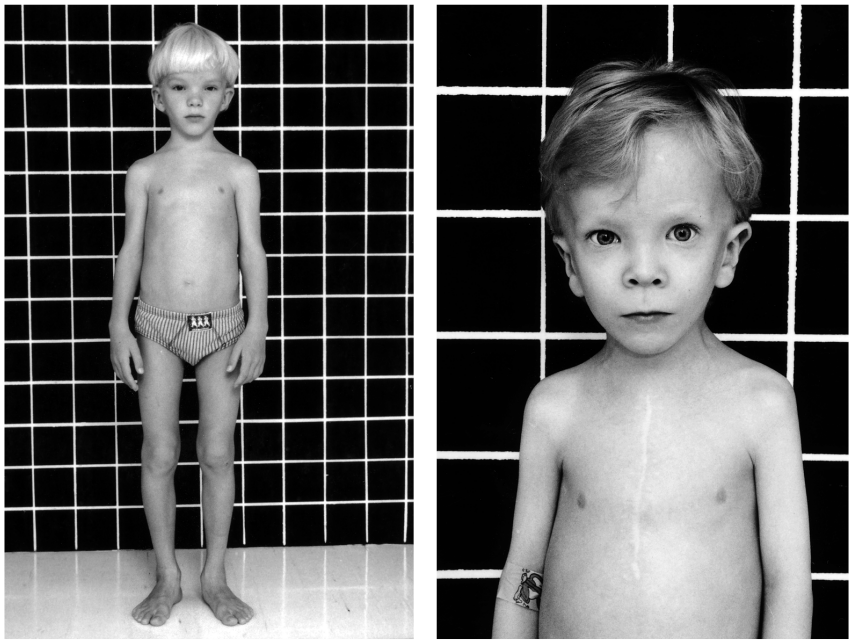

Figure 1. Two children with mulibrey nanism showing the typical external features, i.e. gracile body, triangular face and low nasal bridge. The vertical scar running the length of the chest of the patient on the right is due to cardiac surgery performed to relieve pericardial constriction.

Congestive heart failure (CHF) is the most serious feature of mulibrey nanism and the main factor affecting the life span of patients. Of 49 Finnish patients followed for up to 25 years, 51% developed CHF, 39% underwent pericardiectomy and 22% died of cardiac causes (Lipsanen-Nyman et al. 2003). Two thirds (12/19) of patients who undergo cardiac surgery gain lasting clinical benefit from the operation, while one third (6/19) eventually die of unrelieved or recurrent CHF. Histopathological analysis of autopsy samples shows fibrotic thickening of the pericardial leaves, myocardial hypertrophy, and variable but mostly mild myocardial fibrosis (Lipsanen-Nyman et al. 2003).

1.3 Endocrinological and metabolic findings in mulibrey nanism

Mulibrey nanism patients present defects in a number of functions that are regulated by the endocrine system. For example, the patients develop hypergonadotrophic hypogonadism, i.e. failure of sexual maturation with high gonadotropin levels (Lipsanen-Nyman 1986). The female patients present spontaneus pubertal development but develop signs of ovarian failure approximately two years after menarche. At pelvic ultrasonography, the females have small ovaries with only a few or no follicles (Karlberg et al. 2004b). In males, testicular hypoplasia and a reduced number of sperm has been observed (Lipsanen-Nyman 1986). Another finding related to endocrine function is elevated levels of plasma adrenocorticotropic hormone (ACTH) in some mulibrey nanism patients (Lipsanen-Nyman 1986). However, adrenal steroid hormone levels are normal or only mildly subnormal (Lipsanen-Nyman 1986), and the adrenocortical function appears to be normal in the patients (Karlberg N, unpublished data).

Mulibrey nanism shares some features with peroxisomal disorders, in particular with peroxisomal biogenesis disorders. These include growth failure, facial dysmorphism, retinal pigmentary changes, muscular hypotonicity, hepatomegaly, and, in Refsum's disease, cardiomyopathy (Leys et al. 1989; Sacksteder and Gould 2000). Because of the overlap of clinical features, the peroxisomal function in mulibrey nanism patients has been studied previously (Schutgens et al. 1994). In a small study involving two Finnish mulibrey nanism patients, the plasma concentrations of phytanic acid, pristanic acid, tri- and dihydroxycholestanic acid and cerotic acid were found to be within reference values. Moreover, the profile of very-long-chain fatty acids and *de novo* plasmalogen biosynthesis in fibroblasts derived from the patients were normal. In conclusion, no biochemical evidence of gross peroxisomal dysfunction in mulibrey nanism was found in this study (Schutgens et al. 1994).

Recently, early-onset insulin resistance in mulibrey nanism patients was reported (Karlberg et al. 2005b). In this study, insulin resistance was detectable already in some slim prepubertal children. Clinically, abdominal obesity started to appear after puberty so that 42% of the adults were overweight (Karlberg et al. 2005). Serum leptin, uric acid, total cholesterol, and triacylglycerols increased with age in the patients studied. Fatty liver and hypertension were frequent findings among the adolescent and adult patients. Of the adults, half had type-2

diabetes, 42% had impaired glucose tolerance and 70% fulfilled the National Cholesterol Education Program treatment panel III (NCEP) criterion for metabolic syndrome.

1.4 Mulibrey nanism-associated tumours

Mulibrey nanism patients have an increased risk for both benign and malignant tumours (Seemanova and Bartsch 1999; Karlberg et al. 2004a). Wilms tumour (WT, OMIM 194070) is among the most common solid tumours of childhood, occurring in 1 in 10,000 children. The incidence of WT is increased in several congenital malformation syndromes and trisomies although the vast majority of cases are sporadic (Dome and Coppes 2002). In mulibrey nanism, the incidence of WT is elevated (Lipsanen-Nyman 1986). One patient has been reported from Finland (Similä et al. 1980) and another outside of Finland (Seemanova and Bartsch 1999). WT is believed to arise from embryonic cells, referred to as nephrogenic rests, which retain embryonic differentiation potential after birth (Beckwith 1998). Germline mutations in the *WT1* gene and somatic mutations in the *BRCA2* (a breast cancer susceptibility gene) and *GLYPICAN 3* (encoding a heparan sulphate proteoglycan) genes have been identified in Wilms tumours (Haber et al. 1990; White et al. 2002; Reid et al. 2005). *WT1* encodes a zinc finger transcription factor that is critical to normal kidney and gonad development. Germline mutations in a single allele of *WT1* cause either Wilms tumour-aniridia-genotourinary malformations-mental retardation syndrome or Denys-Drash syndrome, depending on the location of the mutation (Dome and Coppes 2002). Several additional chromosomal loci, including 17q12-q21, have been associated with WT (Dome and Coppes 2002).

Mulibrey nanism patients have a 55% risk of developing fibrothecomas, benign ovarian tumours of stromal cell origin (Karlberg et al. 2004b). The stroma of the ovary consists of undifferentiated mesenchymal cells and their endocrine derivatives. Ovarian fibromas that show differentiation towards theca cells are referred to as fibrothecomas. In general, the tumours become malignant in less than 1% of cases (Zhang et al. 1982). Benign liver tumours have been also been associated with mulibrey nanism. Histopathologically, the tumours resemble hamartomas with fibrosis and lipid degradation (Karlberg et al 2004).

2. *TRIM37* - the mulibrey nanism gene

2.1 Positional cloning of the gene

The search for the mulibrey nanism gene was initiated with a genome-wide scan of six multiplex families with 240 polymorphic markers (Avela et al. 1997). A marker at 17q was found to give evidence of suggestive linkage. Genotyping of additional markers and the inclusion of new families with only one affected person defined a 7-cM region at 17q as the critical mulibrey nanism gene region (Avela et al. 1997). Linkage disequilibrium and haplotype analyzes were subsequently used to narrow the critical region down to 800 kb at 17q22-23. In order to proceed to candidate gene analysis, a physical map of the chromosomal region was constructed (Paavola et al. 1999). Several apparently relevant candidate genes are located in the region, including the growth hormone gene, *HOXB* cluster, *TBX2* and *PNUTL2*, but they were all eventually excluded as causative genes. A disease-associated mutation was identified in a candidate gene corresponding to a previously uncharacterized KIAA0898 cDNA clone (Nagase et al. 1998). The KIAA0898 cDNA includes an open reading frame of 2892 bp, coding for TRIM37, a predicted 964-amino acid protein. The major Finnish mutation underlying mulibrey nanism was found to be a c.493-2A>G transition in the 3' splice site of exon 7 resulting in aberrant splicing at the next AG site. The mutation predicts a frameshift and truncation after 10 frameshifted amino acids in the coiled-coil region of the protein. The minor Finnish mutation, c.2212delG, has so far been detected in only two Finnish patients who are compound heterozygous for this and the major mutation. Two additional homozygous mutations, c.838delACTTT and c.11346insA, were found in patients from outside of Finland. All mutations identified predict a frameshift and truncated protein (Avela et al., 2000). An additional *TRIM37* frameshift mutation predicting a protein truncation has been identified in a Turkish patient (Jagiello et al. 2003).

2.2 Expression of *TRIM37* mRNA

On the basis of Northern blot analysis, a *TRIM37* transcript of approximately 4.5 kb is present in most human tissues studied (Avela et al. 2000; Hämäläinen et al. 2005). An additional transcript of 3.6 kb, generated by use of an alternative 3' untranslated region, and encoding a protein identical to that encoded by the 4.5-kb transcript, is seen in the testis. Quantitative

real-time PCR analysis of various fetal and adult human tissues shows the highest mRNA expression level is the testis and brain (Hämäläinen et al. 2005). By primer extension analysis, several transcription initiation sites have been localized to a region between nucleotides -246 and -373 upstream of the ATG codon. Basal promoter activity, determined by means of luciferase reporter assays, resides within 600 nucleotides upstream from the ATG codon of *TRIM37* (Hämäläinen et al. 2005).

The expression of *TRIM37* during mouse and human embryogenesis has been studied by means of RNA *in situ* hybridization (Lehesjoki et al. 2001). In mice, weak diffuse expression is first detected at embryonic day (E) 9.5. At E11.5, expression appears in the epithelial lining of the oesophagus and bronchi. Between E12.5 and E14.5, *TRIM37* is highly expressed in epithelia of ectodermal or endodermal origin, but not in neuroepithelium. Tissues showing high expression include epithelium of the gut and stomach, epithelium of the developing nephron, ductal cells of the pancreas, lens epithelium and medulla of the adrenal glands. Intensive expression is also found in the trigeminal, symphatetic and dorsal root ganglia, and in the liver. The expression pattern in 4- to 10 week-old human embryos is very similar to that of mouse embryos (Lehesjoki et al. 2001). Interestingly, *TRIM37* is highly expressed at many sites of mesenchymal-epithelial interaction.

3. TRIM family proteins

3.1 General features

TRIM37 is a member of the TRIM (tripartite motif or RING-B-box-coiled-Coil) subfamily of zinc finger proteins. At present (GenBank release 150.0, October 8, 2005), the cDNAs for 68 TRIM family proteins have been annotated in GenBank. The classification of the TRIM family was presented in a paper describing a large-scale analysis of 40 TRIM proteins (Reymond et al. 2001). The order of the domains from the N- to C-termini is strictly conserved (RING, B-box 1, B-box 2, coiled-coil) within the TRIM family and throughout evolution, suggesting that the TRIM forms an integral functional unit (Reymond et al. 2001).

The RING domain (finger) is cysteine-rich, zinc-binding domain found in a large number of eukaryotic proteins (Saurin et al., 1996). RING proteins are involved in diverse cellular processes such as oncogenesis, apoptosis, viral replication, organelle transport, cell-cycle

control and peroxisomal biogenesis. In many cases RING domains mediate protein-protein interactions and the formation of macromolecular complexes. The RING domain contains eight zinc-coordinating amino acid residues, six or seven of which are cysteines and one or two of which are histidines (Borden 2000; Jackson et al. 2000; Joazeiro and Weissman 2000).
The RING domain, present in most but not all TRIM family members, is followed by an approximately 40-amino acid zinc-binding domain, the B-box, of which there can be one or two (B1 and B2). The B-box domain appears to be a critical element of the TRIM because it is almost exclusively found in members of this protein family (Reymond et al. 2001). Four amino acid residues are used for zinc coordination in the B-box and they bind one zinc atom in a Cys2-His2 tetrahedral arrangement. The nuclear magnetic resonance structure of the B-box of the *Xenopus* Xnf7 protein has been determined. It comprises two β-strands, two helical turns and three extended loop regions that are different from any other zinc binding motif (Borden et al. 1995).
The third element of the TRIM, the coiled-coil region, is thought to mediate homo-oligomerization of TRIM proteins (Reymond et al. 2001). Roughly 5% of all coding sequences in a eukaryotic genome code for coiled-coil domains, implying that the domain is involved in numerous cellular processes (Gillingham and Munro 2003). Coiled-coils consist of at least two α-helices that wrap around each other and form a slight left-handed superhelical twist. The amino acid sequence which forms this structure consists of a heptad (seven-amino acid) repeat, in which positions 1 and 4 of the sequence are usually hydrophobic (Gillingham and Munro 2003). The coiled-coil region in TRIM family proteins is followed by a variable C-terminal sequence that may contain known domains, such as B30.2, NHL (NCL-1, HT2A and LIN-41) or ARF (ADP-ribosylation factor) domains.

3.2 The TRIM37 protein

TRIM37 possesses a typical N-terminal tripartite motif comprising a RING domain, a single B-box domain, and a coiled-coil region. A schematic structure of TRIM37 is shown in Figure 2. The TRIM unit is followed by a tumour necrosis factor (TNF)-receptor-associated factor (TRAF) domain (Zapata et al., 2001). TRAF proteins act as signal transducers for the TNF receptor superfamily and the interleukin 1 receptor/Toll-like receptor superfamily, i.e. in cytokine signalling pathways leading to activation of the transcription factors NF-κB and AP-

1 (Wajant et al. 2001; Chung et al. 2002). With the exception of TRAF1, TRAFs contain a RING domain followed by various zinc finger domains, a coiled-coil region and the C-terminal TRAF domain. Thus, the domain structure of TRAF proteins is remarkably similar to the N-terminal half of TRIM37, with the exception that the B-box domains of TRIM37 are replaced by another type of zinc finger motif in TRAF proteins. The TRAF domain of TRIM37 binds six known TRAF proteins and the cytosolic domains of several TNF-family receptors in a GST pull-down assay (Zapata et al. 2001). However, TRIM37 does not co-localize with TRAF2, TRAF6 or the TNF family receptors CD40, Fas and DR5 in immunofluorescence analysis (Zapata et al. 2001). Though the physiological relevance of these findings remains unclear at present, it is of interest that there is some evidence concerning the involvement of TNF-α in the development of central obesity and in the regulation of insulin sensitivity in humans and mice (Moller 2000).

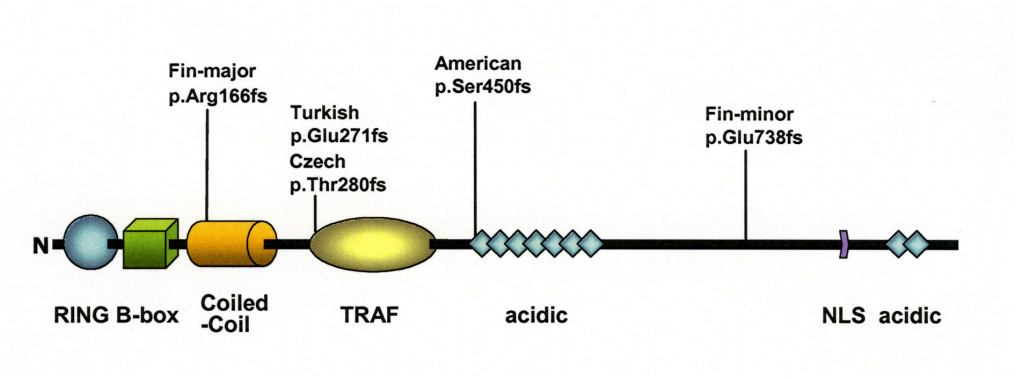

Figure 2. A schematic structure of the TRIM37 protein. The predicted domains and the corresponding positions of the five published mutations associated with mulibrey nanism are depicted on the drawing. NLS denotes a putative nuclear localization signal. According to Avela et al. (2000) and Zapata et al. (2001).

The C-terminal half of TRIM37 has no obvious domain homologies and is also the least conserved part of the protein. There are two regions (amino acids 416-550 and 895-919) with high numbers of acidic amino acids (aspartate and glutamate) the significance of which is unknown (Avela et al. 2000). On the basis of a PSI-BLAST search, the first acidic region contains a 64-amino acid fragment with weak homology (28% identity) to the solute carrier

family 9, isoform 6 (SLC9A6) protein, a sodium/hydrogen exchanger. Two adjacent putative nuclear localization signals are located at amino acids 847 and 851 of TRIM37 (see Figure 2).

3.3 TRIM family proteins in disease

Several TRIM family proteins are implicated in the pathogenesis of diseases in humans and mice (summarized in Table 2). The diversity of the associated diseases suggests that the TRIM family proteins are also functionally diverse and involved in various different cellular processes.

PROTEIN	DISEASE	SUBCELLULAR LOCALIZATION
TRIM5	HIV infection	cytoplasm
TRIM18/midin	X-linked Opitz syndrome	microtubule
TRIM19/PML	promyelocytic leukemia (CT)	nuclear bodies
TRIM20/pyrin	familial Mediterranean fever	microtubule, nucleus, cytoplasmic speckle
TRIM21/SSA/RO	Sjögren syndrome, SLE	cytoplasm, nucleus
TRIM24/TIF	murine leukemia (CT)	nucleus
TRIM25/EFP	breast cancer xenograft model	cytoplasm
TRIM27/RFP	thyroid carcinoma (CT)	nucleus
TRIM32	limb-girdle muscular dystrophy type 2H	cytoplasm
TRIM33	thyroid carcinoma (CT)	unknown
TRIM37	mulibrey nanism	peroxisome, aggregate, nuclear

Table 2. TRIM family proteins implicated in disease. Disorders involving a chromosomal translocation of the indicated TRIM family gene are indicated with CT. PML stands for promyelocytic leukemia protein, SSA for Sjögren syndrome antigen and SLE for systemic lupus erythematosus. The table is updated from Reymond et al. (2001).

Two monogenic disorders with mutations in TRIM family genes, X-linked Opitz syndrome and familial Mediterranean fever, are discussed in more detail below (see sections 3.4 and 3.5). Limb-girdle muscular dystrophy 2H, a mild autosomal recessive myopathy enriched in the Manitoba Hutterite population, is caused by a mutation in *TRIM32* (Frosk et al. 2002). TRIM32 possesses ubiquitin ligase activity and interacts with class V myosins (El-Husseini

and Vincent 1999; Horn et al. 2004). TRIM19/PML and TRIM27/RFP form an oncogenic fusion protein with retinoic acid receptor and the RET tyrosine kinase, respectively, though chromosomal translocation in some promyelocytic leukaemias and thyroid carcinomas in humans (Takahashi et al. 1988; de The et al. 1991). TRIM21/SSA/RO has been identified as an autoantigen in Sjögren syndrome, an autoimmune disorder (Chan et al. 1991). A fascinating role for TRIM5 as a factor restricting human immunodeficiency virus (HIV) infection has recently emerged (Stremlau et al. 2004). HIV readily enters the cells of Old World monkeys but is blocked before reverse transcription. The cytoplasmic blocking factor has been identified as a species-specific variant of TRIM5α (Hatziioannou et al. 2004).

3.4 TRIM18/midin and Opitz syndrome

Opitz syndrome (OS, OMIM 300000) is a congenital disorder of midline development (Opitz et al. 1969). Prominent clinical features include mental retardation, dysplasia of corpus callosum, ocular hypertelorism, cleft-lip palate and defects of the trachea and genitourinary tract. Additionally, cardiac abnormalities are found in the patients (Opitz et al. 1969; Jacobson et al. 1998). Both an X-linked and an autosomal locus have been reported for OS (Robin et al. 1995). The *TRIM18/MID1* gene underlies the X-linked form of the syndrome, also known as the Opitz G/BBB syndrome or hypospadias-dysphagia syndrome (Quaderi et al. 1997). The gene encodes a 72-kDa microtubule-associated protein named TRIM18 or midin (Schweiger et al. 1999). The C-terminal part of TRIM18 comprises a fibronectin type III repeat domain and a B30.2 domain. The B30.2 domain is a 170-amino acid globular protein domain of unknown function. It is found in three very different types of proteins that include RING finger proteins (for example TRIM18/midin and TRIM20/pyrin), proteins with immunoglobulin-like folds (for example butyrophilin involved in lactation), and stonustoxin in the venom of the fish species *Synanceia horrida* (Henry et al. 1998).

Interaction screens in yeast have identified the α4 regulatory subunit of protein phosphatase 2A (PP2A) as a binding partner of TRIM18 (Liu et al. 2001; Trockenbacher et al. 2001). The domain responsible for the interaction seems to be the first B-Box of TRIM18. In transiently transfected cells the α4 subunit colocalizes with wild-type TRIM18 in microtubules, but in perinuclear aggregates with a mutant form of TRIM18 mimicking a patient mutation. Moreover, α4 can be co-immunoprecipitated with TRIM18. The amount of PP2A in mouse

embryonic fibroblasts is regulated by TRIM18 in a proteasome-dependent manner (Trockenbacher et al. 2001). Treatment of wild-type MEFs with a proteasome inhibitor results in the accumulation of polyubiquitinated forms of PP2A, while in MEFs derived from *TRIM18*$^{-/-}$ embryos no such accumulation is detected. Defective ubiquitination of PP2A in the MEFs is rescued by overexpression of TRIM18. However, direct ubiquitination of α4 by TRIM18 *in vitro* was not reported. Hypophosphorylation is observed in two-dimensional SDS-PAGE analysis of microtubule-associated proteins from *TRIM18*$^{-/-}$ cells, which likely results from hyperactivity of PP2A. Taken together these result suggest that disturbed microtubule dynamics could underlie the developmental defects of Opitz syndrome (Trockenbacher et al. 2001).

3.5 TRIM20/pyrin and familial Mediterranean fever

In 1944, a syndrome of benign, recurrent meningitis attacks with characteristic spinal fluid pleocytosis (presence of cells in the spinal fluid) was described in France (Mollaret 1944). The attacks were separated by symptom-free periods lasting from days to years. During the following decades, a condition with recurrent attacks of fever and inflammation in the peritoneum, synovium, or pleura was reported in several ethnic groups around the Mediterranean region. In 1964, Siegal reported several cases with similar symptoms among Ashkenazi Jews in the USA and named the disorder “Familial Paroxysmal Polyserositis” (Siegal 1964). Now named Familial Mediterranean Fever (FMF, OMIM249100), the syndrome is relatively common in several ethnic groups including Arabs, Armenians and Turks (FrenchFMFConsortium 1997). Amyloidosis with renal failure is a complication of the disease. Recurrent pericarditis has been shown in association with FMF (Ercan Tutar et al. 2000).

Mutations in the *TRIM20/pyrin* gene cause FMF (The French FMF Consortium 1997; The International FMF Consortium 1997). The TRIM20 protein is an atypical member of the TRIM family in that it lacks the RING domain. However, it harbours a B-box, coiled-coil region and a B30.2 domain, an arrangement typical of TRIM proteins (Reymond et al. 2001).

The subcellular localization of TRIM20/pyrin is somewhat controversial. Initially, an interaction screen in yeast identified a putative component of a Golgi transport complex as an inretaction partner for TRIM20, suggesting localization to the Golgi complex (Chen et al.

2000). Shortly afterwards, characterization of a *TRIM20* splice variant lacking exon 2 was reported. The corresponding protein isoform was shown to localize exclusively to the nucleus in stably transfected cells, while the full-length isoform shows a diffuse cytoplasmic localization (Papin et al. 2000). Another study reported that TRIM20 rather associates with the cytoskeleton in HeLa cells (Mansfield et al. 2001). Co-localization of TRIM20 with both microtubules and actin filaments was shown by immunofluorescence analysis, and pull-down experiments lent further support to direct interaction of TRIM20 with microtubules (Mansfield et al. 2001). These findings are interesting in the sense that colchicine, a microtubule-disrupting chemical, is an effective prophylactic treatment for FMF. It was proposed that TRIM20 could regulate inflammatory responses through leukocyte cytoskeletal organization (Mansfield et al. 2001). Concurrently, apoptotic speck protein (ASC), identified in a yeast two-hybrid screen, was reported as a novel interaction partner for TRIM20 (Richards et al. 2001). TRIM20 co-localizes with ASC in cytoplasmic speckles, peculiar structures that have the appearance of hollow rings in confocal micrographs. The speckles do not stain for tubulin or actin, suggesting that they do not contain aggregated cytoskeletal proteins, as is the case in aggresomes. TRIM20 seems to protect HeLa cells from apoptosis induced by ectopic ASC expression (Richards et al. 2001). The authors suggest that regulation of cell survival by TRIM20 could be a component of FMF pathogenesis. These data are supported by results from the analysis of *TRIM20*-deficient mice (Chae et al. 2003). Caspase-1 is highly activated, interleukin-1β production increased and apoptosis impaired in *TRIM20*$^{-/-}$ macrophages. Moreover, TRIM20 competes with caspase-1 for binding to ASC (Chae et al. 2003).

4. Ubiquitination

4.1 Biochemistry of ubiquitination

Discovered in the early 1980s, ubiquitination is a post-translational protein modification in which a single molecule or multiple molecules of the 76-amino acid ubiquitin polypeptide is covalently conjugated to target proteins (Hershko et al. 1980; Pickart 2001). Ubiquitin is the prototype and the best characterized member of a group of eukaryotic small ubiquitin-like proteins that are covalently attached to other proteins for various regulatory purposes

(Welchman et al. 2005). These include SUMO-1, -2 and -3 (small ubiquitin-like modifier), NEDD8 (neural precursor cell-expressed, developmentally down-regulated 8) and many other more recently identified proteins. Some of the ubiquitin-like modifiers have very low sequence homology to ubiquitin but their three-dimensional structure forms a characteristic ubiquitin superfold (Welchman et al. 2005).

Ubiquitination involves three steps, each carried out by specific enzymes, designated ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (Pickart 2001) (see Figure 3). In the first step, E1 catalyzes the formation of a thioester bond between the carboxyl group of the C-terminal glycine of ubiquitin and a cysteine residue in the active site of the E1 enzyme. Next, the activated ubiquitin is transferred to an E2 to form a similar transient thioester conjugate. An E3 finally catalyzes the ligation of activated ubiquitin to the target protein. This results in a covalent isopeptide bond between the carboxyl terminus of ubiquitin and the ε-amino group of a lysine residue on the target protein. Ubiquitin can then be repeatedly conjugated to the Lys48 or Lys63 of the previously attached ubiquitin, resulting in a chain of polyubiquitin (Pickart 2001). Whether the ligation of the first ubiquitin and subsequent polyubiquitination are generally catalyzed by the same or separate E2-E3 complexes is not known (Pickart 2001). There is only one type of E1 protein in mammalian cells, a few dozen E2s and hundreds of E3s (Pickart 2001). As suggested by the large number of E3 ligases the target specificity of ubiquitination is mainly determined by the E3s. They can be classified into 1) HECT (homologous to E6-AP carboxyl terminus) domain E3s, 2) RING domain E3s and 3) PHD (plant homeo domain) E3s (Pickart 2001).

Ubiquitination is a reversible process (Pickart 2001). Several classes of deubiquitinating enzymes, i.e. proteases that cleave ubiquitin chains, are known though they are poorly characterized compared to enzymes catalyzing the forward reaction. The two major categories of deubiquitinating enzymes are 1) ubiquitin C-terminal hydrolases (UCHs) and 2) ubiquitin-specific proteases (USPs). Additionally, the OTU-domain Ub-aldehyde-binding protein family and the Jab1/Pad1/MPN-domain metallo-enzyme family have been suggested to represent new classes of deubiquitinating enzymes (Kim et al. 2003). There are a few dozen genes encoding predicted UCHs and more than 80 genes encoding USPs in the human genome (Kim et al. 2003). Ubiquitin itself is translated as a chain of multiple units or fused to ribosomal proteins. The main role of the UCH family proteins seems to be the cleavage of

ubiquitin monomers from these fusion proteins and, perhaps, editing of polyubiquitin chains on other proteins by a similar mechanism (Kim et al. 2003). The USP family proteins deubiquitinate polyubiquitinated substrates, disassemble free polyubiquitin chains and, similarly to UCHs, process ubiquitin precursors (Kim et al. 2003). Deubiquitinating enzymes are involved in numerous cellular processes from regulation of proteasome function to oncogenesis and transcriptional regulation (Kim et al. 2003).

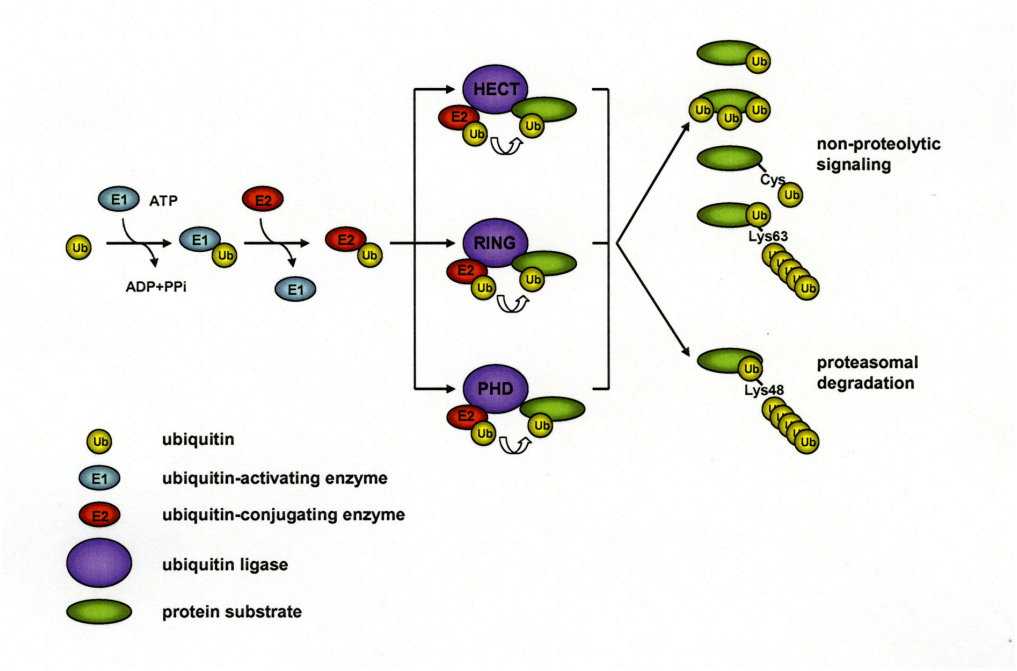

Figure 3. A schematic drawing of the ubiquitin conjugation pathway. Ubiquitin is first activated by an ubiquitin-activating enzyme E1; activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme E2; an ubiquitin ligase E3 facilitates the transfer of ubiquitin from E2 to the protein substrate. The three major classes of E3 ubiquitin ligases are HECT domain, RING domain and PHD E3s. Substrates marked with a Lys48-linked polyubiquitin chain are targeted to 26S proteasome-mediated degradation, whereas mono- and multiubiquitination, ubiquitination at cysteine residues, and Lys63-linked polyubiquitination serve a regulatory function in endocytosis, transcription and DNA repair. The drawing is adapted from Gao and Karin (Gao and Karin 2005).

4.2 Biological functions of ubiquitination

Like phosphorylation, ubiquitination regulates a broad range of eukaryotic cell functions (Sun and Chen 2004; Welchman et al. 2005). In fact, ubiquitination and phosphorylation are sometimes intertwined to regulate protein function, for example in the pathway that activates the NF-κB transcription factor (Sun and Chen 2004). As a rule of thumb, Lys48-linked polyubiquitination serves as a degradation signal whereas monoubiquitination, multiubiquitination (monoubiquitination at several sites in parallel) or polyubiquitination at Lys63 serve as regulatory modifications (Welchman et al. 2005) (see Figure 3). The best studied function regulated by ubiquitination is the degradation of denatured or misfolded proteins and the normal turnover of a large number of intracellular proteins. Proteins marked with a polymer of Lys48-linked ubiquitin chains are selectively targeted to degradation by the proteasome, a giant proteolytic enzyme complex present in the cytosol and nucleus (Miller and Gordon 2005). How proteins that are destined for ubiquitination and/or degradation are correctly recognized by the ubiquitination machinery is a central but as yet poorly understood question (Laney and Hochstrasser 1999). The half-life of a protein *in vivo* is dependent to some extent on its N-terminal residue that is recognized by a specific N-recognin protein. This phenomenon, called the N-end rule, is best characterized in yeast, but homologs of the N-end recognition proteins are present in mammals as well (Kwon et al. 1998). Otherwise, determinants for ubiquitination, such as specific recognition peptides or hydrophobic surface patches have been described in a few cases (Laney and Hochstrasser 1999).

Monoubiquitination or a combination of mono- and polyubiquitination is known to control DNA repair, transcription, signal transduction and endocytosis (Welchman et al. 2005). For example, a fraction of the DNA replication factor proliferating cell nuclear antigen (PCNA) is modified by SUMO-1 at two lysines under normal conditions. DNA damage induces desumoylation and monoubiquitination at Lys164 of PCNA to allow it to bind to sites of DNA damage. The monoubiquitin can be further processed by Lys63-linked polyubiquitination required for subsequent error-free repair of the DNA damage (Hoege et al. 2002; Haracska et al. 2004). Both mono- and polyubiquitination have complex roles in transcriptional regulation in eukaryotes. Histones themselves can be ubiquitinated, nuclear receptors (e.g. estrogen receptors) can be ubiquitinated and targeted for degradation, and transcription factors like p53 can be subject to complex parallel modifications by ubiquitin,

SUMO-1 and NEDD8 (Welchman et al. 2005). Recently, an important role for ubiquitination in the regulation of endocytosis and vesicle sorting has emerged. Several growth factor receptors in mammals and various transporters in yeast are mono- or multiubiquitinated, which promotes their internalization from the plasma membrane and sorting into a subset of late endosomes (Katzmann et al. 2002). Recently, ubiquitination at cysteine instead of lysine residues in the cytoplasmic tails of certain MHC molecules by a viral ubiquitin ligase was reported (Cadwell and Coscoy 2005). The ubiquitination induces internalization of the MHC molecules from the plasma membrane, thus diminishing antigen presentation in infected cells and promoting survival of the virus.

4.2 Disorders of ubiquitination

Defects in all three classes of ubiquitin ligases, the RING finger, HECT and PHD ubiquitin ligases, and in the deubiquitinating enzyme CYLD have been associated with human disease (Jiang and Beaudet 2004). The HECT family ubiquitin ligase E6-AP or UBE3A was first identified as a mediator of papilloma virus infection-induced degradation of p53, a remarkable finding at the time (Scheffner et al. 1993). It was later found that the *UBE3A* gene is mutated in Angelman syndrome, a neurological disorder with severe motor and mental retardation. UBE3A is a rare example of an ubiquitin ligase for which a directly interacting substrate has been identified. The substrate molecule, identified in a yeast two-hybrid screen, is HHR23A, a human homolog of the yeast DNA repair protein Rad23 (Kumar et al. 1999). Angelman syndrome patient mutations disrupt the ability of UBE3A to ubiquitinate HHR23A but, interestingly, autoubiquitination activity and substrate binding are unaffected (Cooper et al. 2004).

AIRE, the protein defective in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a member of the Finnish disease heritage (Nagamine et al. 1997; The Finnish-German APECED Consortium 1997), is among the first identified ubiquitin ligases with the novel PHD type zinc finger. Disease-causing mutations located in the sequence encoding the first PHD abolish the autoubiquitination activity of the protein. Two ubiquitin-conjugating enzymes, Ubc4 and the closely related UbcH5B, support autoubiquitination of AIRE (Uchida et al. 2004).

Familial cylindromatosis or turban tumour syndrome is a peculiar monogenic disorder with skin tumour susceptibility (Bignell et al. 2000). The tumours, known as cylindromas because of their characteristic histological appearance, are believed to arise from the eccrine or apocrine cells of the skin that secrete sweat and scent, respectively. Cylindromas arise predominantly in hairy areas of the body, with approximately 90% on the head and neck (Bignell et al. 2000). Mutations in the gene encoding the deubiquitinating enzyme CYLD1 underlie familial cylindromatosis (Bignell et al. 2000; Brummelkamp et al. 2003; Kovalenko et al. 2003; Trompouki et al. 2003). CYLD1 inhibits activation of NF-κB transcription factors downstream of the tumour necrosis factor receptor family molecules CD40, EDAR, and XDAR. The inhibition is mediated, at least in part, by the deubiquitination and inactivation of TNFR-associated factor 2 (TRAF2) and, to a lesser extent, TRAF6 (Trompouki et al. 2003).

Curiously, a pathological variant of ubiquitin itself is linked to neurodegenerative diseases (Jiang and Beaudet 2004). A frame-shift generated by transcriptional deletion has been detected in *UBIQUITIN B* mRNA in the brains of Alzheimer's disease patients. As a consequence, affected neurons accumulate UBB(+1), a mutant ubiquitin carrying a 19-amino acid C-terminal extension (van Leeuwen et al. 1998). UBB(+1) is a potent inhibitor of the proteasome in neuronal cells, suggesting that it contributes to the neurotoxicity elicited by amyloid accumulation (Lindsten et al. 2002).

Aims of the present study

1. To explore the subcellular localization of the TRIM37 protein and its mutant forms (I-III)

2. To investigate the predicted ubiquitin ligase activity of TRIM37 and the effect of patient mutations on this activity (III)

3. To search for TRIM37-interacting proteins by means of yeast two-hybrid screening (III, unpublished)

4. To analyze the distribution of Trim37 protein in embryonic and adult mouse tissues (IV)

Materials and methods

Methods used in this study are summarized below in Table 3.

METHOD	ORIGINAL PUBLICATION
DNA cloning	I, II, III, IV
Polymerase chain reaction	I, II, III, IV
DNA sequencing	I, II, III, IV
Site-directed mutagenesis	I, II, III
Patient mutation analysis	II, III
Northern blot analysis	IV
SDS-PAGE	I, II, III, IV
In vitro translation	I and this thesis
In vitro ubiquitination assay	III
Enhanced chemiluminiscence detection	I, II, III, IV
Immunoblot analysis	I, II, III, IV
Immunoprecipitation	III
Cell culture	I, II, III, IV
Establishment of primary skin fibroblast cultures	I
Cell fractionation	I
Transient transfection of cells	I, II, III, IV
Raising of antibodies	I
Antibody purification	I, IV
Immunofluorescence staining	I, II, III
Immunohistochemical staining	I, IV
Fluorescence microscopy	I, II, III
Production of GST fusion proteins	III
GST pull-down assay	this thesis
Yeast two-hybrid screening	III and this thesis
Database and computer analysis	I, II, III, IV

Results and Discussion

1. Identification of novel *TRIM37* mutations (II, III)

Novel *TRIM37* mutations were sought in a Finnish patient who is heterozygous for the Fin_{major} mutation, and in several non-Finnish patients who fulfilled the diagnostic criteria for mulibrey nanism, i.e. growth failure, characteristic dysmorphic craniofacial features, J-shaped sella turcica, hepatomegaly and signs of heart disease. All 24 coding exons were amplified by PCR

from genomic DNA and sequenced. In addition, 300 bp of the putative promoter region was analyzed. Altogether seven new *TRIM37* mutations were identified, five of which were frameshift mutations predicting a truncated protein and two were missense mutations. A total of 190 control alleles were screened for the mutations and found to be negative. A heterozygous missense mutation, c.965G>T, was found in a Canadian patient who had died at the age of four years of renal and cardiac failure. The other mutation in this patient was a frameshift mutation (p.Arg471X). This is the first identified missense mutation in mulibrey nanism patients and predicts the amino acid change Gly322Val, located in the TRAF domain of the protein. The mutation is, to our knowledge, the first disease-causing TRAF domain mutation found in any protein.

Another heterozygous missense mutation (c.227T>C) was identified in the Finnish patient who carried the Fin_{major} mutation. The mutation predicts the change of Leu76 to a proline (p.Leu76Pro). It is located in a predicted alpha-helical region between the RING finger and the first B-Box domain of TRIM37. It is likely that the change from leucine to the cyclic amino acid proline disrupts the integrity of the TRIM unit and thus may affect the function of both the RING and B-box domains. Few missense mutations affecting RING or B-box domains have been identified in TRIM family genes. In *TRIM18/midin*, for example, nearly all of the few dozen or so disease associated mutations are clustered in the C-terminal part of the protein, outside the TRIM unit. Only one missense mutation has been identified in the sequence encoding the coiled-coil region in *TRIM18/midin*. However, RING finger mutations are relatively common in other types of RING finger proteins. For example, in the gene encoding parkin, the protein defective in autosomal recessive juvenile parkinsonism, altogether six missense mutations affecting the two RING finger domains of the protein have been identified (Sriram et al. 2005). Mutant transcripts with missense mutations, in contrast to transcripts predicting truncated proteins, are more likely to be translated normally and be stable. Therefore, a missense mutation that causes a disease often points to a functionally important part of a protein. It seems that this is the case for the two novel missense mutations identified in *TRIM37* (see below).

2. Characterization of the TRIM37 polypeptide and antibodies (I, III, unpublished)

Polyclonal antibodies against TRIM37 were raised in rabbits by immunization with the synthetic peptides FPDGEQIGPEDLSFNTDENSGR (C-terminal amino acids 942-963, peptide C1) and SVREAKEDEEDEEKIQNEDYHHE (internal amino acids 490-512, peptide M1) coupled to keyhole limpet hemocyanin. A polyclonal antibody against the peptide SYSRKDKDQRKQQAMWRVPSD (amino acids 651-671, peptide G6), identical in human and mouse TRIM37, was raised in rabbits against a multiple antigen peptide. The specificity of the anti-TRIM37 antisera was verified by immunoblotting and immunofluorescence staining of cell lines transiently transfected with *TRIM37* expression constructs.

The antisera against all three TRIM37 peptides recognized a protein with an apparent molecular mass of 130 kDa in immunoblots (Figure 4). Additionally, the M1 antiserum detected protein species of ~70 kDa and ~110 kDa that were absent in non-transfected cells. The 70-kDa band may represent a non-specific degradation product or, a more interesting possibility, a fragment resulting from specific proteolytic cleavage upstream of the putative nuclear localization signal of TRIM37. The M1 and C1 antisera also recognized the transiently expressed protein in immunofluorescence analysis of paraformaldehyde fixed, Triton X-100-permeabilized cells.

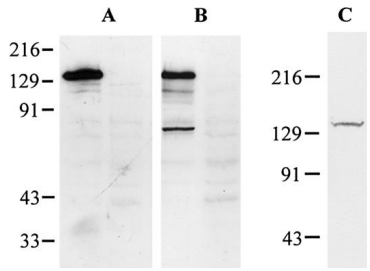

Figure 4. Immunoblot and *in vitro* translation analysis of the TRIM37 polypeptide. Polyclonal antisera raised against (A) the C-terminal TRIM37 peptide (C1) and (B) the internal TRIM37 peptide (M1) detected a band at ~130 kDa in transiently transfected COS-1 cells. The M1 peptide antibody also detected less intense bands at ~110 and ~70 kDa. Nontransfected control lanes are shown on the right in panels A and B. *In vitro* transcription-translation of the TRIM37 cDNA in the presence of [^{35}S]methionine produced a polypeptide migrating at ~130 kDa (C).

Because the apparent molecular mass of full-length TRIM37, deduced from immunoblots, was higher than the calculated value (109 kDa), *in vitro* transcription-translation of *TRIM37*

cDNA was performed, followed by SDS-PAGE and autoradiography. The *in vitro* translation product migrated at ~130 kDa (Figure 4), similarly to the protein expressed in COS-1 cells, indicating that post-translational modification, e.g. glycosylation, was likely not responsible for the higher-than-expected molecular mass of TRIM37. It was later found that TRIM37 is polyubiquitinated in COS-1 cells, which markedly affects its mobility. The stoichiometry of ubiquitination is low, however, and the 130-kDa form of TRIM37 does not react with a ubiquitin antibody in immunoblots. Thus, the discrepancy between the theoretical and apparent molecular weight of TRIM37 probably reflects natural amino acid composition-dependent differences in the migration of proteins in SDS-PAGE.

In order to characterize endogenous TRIM37 biochemically, immunoblots of lysates from various human and mouse tissues and cell lines were probed with the anti-TRIM37 peptide antibodies. Despite repeated attempts, endogenous TRIM37 could not be detected unambiguously by means of immunoblotting (data not shown). This suggests that the peptide antibodies recognize the denatured antigen poorly. A combination of low levels of the antigen and the relative low affinity of anti-peptide antibodies may also contribute to the failure to detect endogenous TRIM37 in immunoblotting. Raising antibodies against larger protein fragments, for example GST fusion proteins, could yield high-affinity antibodies that could prove more suitable for immunoblotting and immunoprecipitation. However, by means of immunofluorescence and immunohistochemical staining, endogenous TRIM37 immunoreactivity was detected in several cell lines and human and mouse tissues sections, respectively (see sections 3.2, 6.1 and 6.2).

3. Analysis of the subcellular localization of TRIM37 and its mutants (I, II, III)

3.1 Subcellular localization of ectopically expressed TRIM37 and its mutants

The subcellular localization of ectopically expressed TRIM37 and its mutant forms was studied using immunofluorescence analysis in transiently transfected cultured cells. For a summary of the wild-type and mutant expression constructs used in these experiments, and the subcellular localization of corresponding proteins, see Table 4.

CONSTRUCT	PROTEIN	SUBCELLULAR LOCALIZATION	AUTO-UB
TRIM37	aa 1-964	cytoplasmic granular, aggregate	++
TRIM37(L76P)	p.Leu76Pro	cytoplasmic granular	+
TRIM37(SS)	p.Cys35Ser;Cys36Ser	cytoplasmic granular	+
TRIM37(G322V)	p.Gly322Val	cytoplasmic homogenous	+++
TRIM*	aa 1-252	cytoplasmic granular, rings	+++
TRIM(L76P)*	aa 1-252 (L76P)	n.d.	+
TRIM(SS)*	aa 1-252 (C35S;C36S)	n.d.	+
TRAFd	aa 205-454	cytoplasmic homogenous	n.a.
C-terminal half	aa 417-964	nuclear, cytoplasmic homogenous	n.a.
Fin-major	p.Arg166fsX10	cytoplasmic homogenous	-
Fin-minor	p.Glu738fsX31	cytoplasmic granular, aggregate	n.d.

Table 4. Summary of the wild-type and mutant TRIM37 expression constructs used in this study. TRAFd denotes TRAF domain. The column SUBCELLULAR LOCALIZATION refers to the localization of the protein as determined by immunofluorescence analysis of transiently transfected cells. The column AUTO-UB refers to the degree of autoubiquitination of the protein observed in COS-1 cells and additionally, with the constructs marked with (*), in an *in vitro* assay. (n.a.) not applicable, (n.d.) not determined.

Both tagged and non-tagged TRIM37 showed a punctate cytoplasmic staining pattern when transiently expressed in COS-1, BHK and HeLa cells. Extended expression time lead to the formation of large cytoplasmic clumps or aggregates that were concentrated around the nucleus (perinuclear aggregates, see section 3.3). In order to identify the punctate structures to which TRIM37 localized, co-staining for various organellar markers was performed. On the basis of these experiments TRIM37 staining did not localize to late endosomes/lysosomes,

Golgi, endoplasmic reticulum, mitochondria or microtubules. These data are in agreement with the results of another group who excluded transiently expressed TRIM37 from mitochondria, Golgi and lysosomes (Zapata et al. 2001). However, the TRIM37-positive granules stained with antibodies against the peroxisomal markers SKL (Ser-Lys-Leu, peroxisomal targeting signal 1, PTS1) and PMP70 (peroxisomal membrane protein 70), suggesting that ectopically expressed TRIM37 localizes to peroxisomes. A mutant protein representing the Fin-major mutation (p.Arg166fsX10), and truncated at the coiled-coil region, localized homogenously over the cytoplasm. In contrast, a mutant protein representing the Fin-minor mutation (p.Glu738fsX31), truncated after amino acid 737, retained punctate localization and co-localized with the peroxisomal protein adrenoleukodystrophy protein (ALDP). The p.Gly322Val mutant showed homogenous cytoplasmic localization in transfected COS-1 cells, indicating that is not targeted to peroxisomes. The mislocalization of the p.Gly322Val mutant is unexpected because the data of Zapata et al. (2001), as well as our unpublished data, suggest that the TRIM domain is sufficient for correct localization of TRIM37. It is possible that the p.Gly322Val mutation induces a conformational change that affects the adjacent coiled-coil region and thus leads to loss of correct localization. The novel p.Leu76Pro mutant was employed later in the characterization of the TRIM37 aggregates (see section 3.3). It localized to granular cytoplasmic structures that were somewhat different morphologically from those to which the wild-type protein localized. As no co-localization experiments with peroxisomal or other organellar markers have yet been performed, the identity of the structures to which the p.Leu76Pro mutant localizes remains to be investigated.

In order to assess the subcellular localization of transiently expressed TRIM37 biochemically, lysates from cells transfected with a TRIM37 expression construct were subjected to centrifugal fractionation and density gradient centrifugation. The light mitochondrial fraction (23,500g pellet) was applied on top of a discontinuous Nycodenz gradient and centrifuged to separate the peroxisomal fraction from mitochondria and lysosomes. In an immunoblot analysis of the fractions collected from the gradient, TRIM37 was enriched, relative to protein concentration, in the heavy peroxisomal fractions with a density of 1.22-1.25 g/ml.

Taken together, these results suggest that ectopically expressed TRIM37 localizes to peroxisomes in cultured cells, and that the localization is dependent on intact TRIM and TRAF domains. This finding is contrary to our original hypothesis, based on computer

predictions that TRIM37 is a nuclear protein. However, despite the fact that the full-length protein never showed nuclear localization we found that the C-terminal half of TRIM37 is targeted mainly to the nucleus when expressed in COS-1 cells (unpublished data). This is in line with the data of Zapata et al. (2001) who showed similar localization results with C-terminal TRIM37 deletion constructs. Moreover, in an immunohistochemical analysis, nuclear TRIM37 staining was later detected in a subset of cells in mouse tissues, such as in the epithelium of the stomach and small intestine (see section 6.2). These data suggest that the nuclear localization signal of TRIM37 may be functional under certain conditions and/or in certain cell types. Finally, it should be noted that considerable amounts of TRIM37 might actually reside in the cytosol, but such a diffuse localization is difficult to discriminate from the more conspicuous peroxisomal localization in immunofluorescence experiments.

3.2 Subcellular localization of endogenous TRIM37

In order to confirm the localization data on the ectopically expressed protein we searched for cell lines expressing endogenous TRIM37. Immunofluorescence staining using the two anti-TRIM37 peptide (M1 and C1) antisera revealed granular cytoplasmic staining in baby hamster kidney (BHK) cells, human skin fibroblasts, human liver carcinoma (HepG2) cells and human intestinal smooth muscle cells. The TRIM37-positive granules were positive for ALDP indicating that they are peroxisomes. Primary fibroblasts derived from three mulibrey nanism patients lacked TRIM37 immunoreactivity, which strongly suggests that the granular staining observed in wild-type fibroblasts is specific. In addition to immunofluorescence analysis, immunohistochemical staining of various human tissues showed a granular cytoplasmic staining compatible with peroxisomal localization, as assessed by catalase staining of consecutive sections. However, it is not possible to conclude from the immunohistochemical data that the granular staining represents peroxisomes. Provided that suitable antibodies are available, immunoelectron microscopy or immunofluorescence staining of tissue sections could allow co-staining with marker antibodies and determination of the subcellular localization of TRIM37 *in vivo*.

In order to gain insight into the mechanisms that target TRIM37 to peroxisomes, immortalized fibroblasts derived from patients with known peroxisomal disorders were

stained with the TRIM37 antibodies. The mutant cell lines used here have different defects in the transport of newly translated proteins from the cytosol into the lumen of the peroxisome. The transport is mediated by either PTS1 (C-terminal tripeptide Ser-Lys-Leu) or PTS2 (a short N-terminal sequence). The cell lines included *PEX1*$^{-/-}$ cells (Zellweger syndrome, a generalized defect in peroxisomal matrix protein import), *PEX5*$^{-/-}$ cells (autosomal neonatal adrenoleukodystrophy, a defect in both PTS1- and PTS2-mediated import into peroxisomes) and *PEX7*$^{-/-}$ cells (rhizomelic chondrodysplasia punctata, a defect in PTS2-mediated import). By immunofluorescence analysis, loss of peroxisomal TRIM37 immunoreactivity was evident in *PEX1*$^{-/-}$ and *PEX5*$^{-/-}$ cells, whereas *PEX7*$^{-/-}$ cells showed granular cytoplasmic TRIM37 staining similar to wild-type fibroblasts. These findings indicate that the localization of TRIM37 immunoreactivity to peroxisomes in cultured fibroblasts is dependent on PEX5 but independent of PEX7. Thus, it appears that TRIM37, which itself lacks known peroxisomal targeting signals, may be imported into peroxisomes as a complex with a PTS1-containing protein.

Finally, primary fibroblasts derived from three mulibrey nanism patients were stained for peroxisomal markers to investigate if the peroxisomes are morphologically affected in mulibrey nanism. The fibroblasts displayed apparently normal staining for the peroxisomal markers PMP70 and catalase, suggesting that there is no gross defect in peroxisomal structure in the patient cells. Further investigation of the peroxisomal morphology and function in cell types that are likely to be more relevant for the mulibrey phenotype than fibroblasts is worthwhile in the future.

On the basis of the subcellular localization of TRIM37 we have tentatively classified mulibrey nanism as a peroxisomal disorder. Because of some overlapping clinical features, including prenatal-onset growth failure, facial dysmorphism, hepatomegaly, pigmentary changes in retina and muscular weakness, the possibility of a peroxisomal defect in mulibrey nanism has been considered previously (Schutgens et al. 1994). Peroxisomes are single-membrane-bound organelles present in nearly all eukaryotic cells (Sacksteder and Gould 2000). The peroxisomal lumen contains numerous metabolic enzymes involved in β-oxidation of long- and very long-chain fatty acids, the biosynthesis of plasmalogens, cholesterol, and bile acids, as well as degradation of certain amino acids and purine (Sacksteder and Gould 2000, see also www.peroxisome.org). In peroxisomal disorders, which can be classified into

peroxisomal biogenesis disorders and single-enzyme disorders, mutations in genes encoding protein components essential for the biogenesis of the entire organelle, or in genes encoding single peroxisomal enzymes, respectively, lead to a metabolic defect and subsequent clinical manifestations (Wanders 2004). In the case of mulibrey nanism, no metabolic defect linking TRIM37 and peroxisomal function has been identified as yet, and no interactions of TRIM37 with peroxisomal proteins have yet been discovered. A striking difference between mulibrey nanism and peroxisomal disorders, like the Zellweger syndrome and X-linked adrenoleukodystophy, is that patients with mulibrey nanism are neurologically intact. Thus, additional biochemical and cell biological approaches, as well as more detailed clinical investigation, are needed to clarify the putative role of peroxisomal function in mulibrey nanism.

3.3 Characterization of TRIM37 aggregates

In transiently transfected cells with a high expression level, TRIM37 rapidly formed aggregates that did not stain for peroxisomal or other organellar markers. These were apparently similar to ribbon-like aggresomes described previously (Garcia-Mata et al. 2002). The tendency of transiently expressed TRIM37 to aggregate was dependent on the intact RING domain because a mutant protein with disrupted RING domain architecture (p.Cys35Ser;Cys36Ser) and the p.Leu76Pro mutant protein did not form such aggregates. Moreover, the p.Gly322Val mutant protein remained homogenously distributed over the cytoplasm in the majority of cells. In order to characterize the TRIM37 aggregates, COS-1 cells transiently transfected with *TRIM37* expression constructs were stained with antibodies against markers for aggresomes and inclusion bodies. The small punctate TRIM37-positive structures that apparently precede the perinuclear aggregates were positive for Rpt6, a subunit of the 26S proteasome, whereas the large perinuclear aggregates were negative. The large aggregates were positive for Hsp70, a cytoplasmic chaperone, and ubiquitin, both of which are known to associate with aggresomes (Garcia-Mata et al. 2002). Aggresomes tend to cause reorganization, or collapse, of vimentin filaments around the perinuclear aggresome focus. We detected only partial reorganization of vimentin around the TRIM37 aggregates in a fraction of the TRIM37-expressing cells. Though the reason for this discrepancy remains

unclear, aggresomes that do not trigger vimentin reorganization have been reported previously (Garcia-Mata et al. 2002).

The aggresome is defined as a perinuclear region where misfolded and aggregated proteins are sequestered for proteasomal and/or autophagosomal degradation in situations when the cell's degradative capacity is exceeded (Johnston et al. 1998; Webb et al. 2003). A dramatic increase in half-life and poor solubility of aggregated proteins have been reported (Johnston et al. 1998). For many of the TRIM family members, immunofluorescence analysis of the ectopically expressed proteins has demonstrated cytoplasmic speckles or aggregates that do not co-localize with organelle markers (Reymond et al. 2001). This suggests, together with our results on TRIM37, that aggresome formation, at least in conjunction with overexpression, is a common propensity of TRIM family proteins. Generally, aggregate formation by the mutant rather than the wild-type proteins has been observed in transfection experiments of disease-associated proteins, for example TRIM18 (Cox et al. 2000). This is easily conceivable as disease-associated mutant proteins are often prone to misfolding and, consequently, are targeted for degradation by the proteasome. What we found for TRIM37 is contrary to this and raises the possibility that the aggresome formation reflects a physiological function of TRIM37, such as interaction with the proteasomal degradation machinery. Although aggresomes are almost exclusively described in conjunction with ectopic expression and various stress conditions like proteasome inhibition, emerging data supports the existence of aggresome-like protein complexes under physiological conditions. For example, inducible nitric oxide synthase forms aggresomes in a urinary bladder papilloma cell line that expresses the enzyme upon cytokine stimulation (Kolodziejska et al. 2005).

4. Analysis of TRIM37 ubiquitination (III)

4.1 Structure of the RING domain of TRIM37

On the basis of a BLAST search, the tripartite motif of TRIM37 shares 20-25% amino acid sequence identity with the other members of the TRIM family. The amino acid sequence of the RING domain differs from the predicted consensus sequence in that it has a lysine (K) in the place of the conserved histidine at position 32 (Figure 5). There are additional cysteines and histidines (amino acids 28, 30, 35) in close proximity, and therefore the actual identity of

zinc-coordinating residues remains elusive (Figure 5). The RING finger of TRIM37 has highest similarity to those of RAD18, HRD1, RNF5 and PEX10.

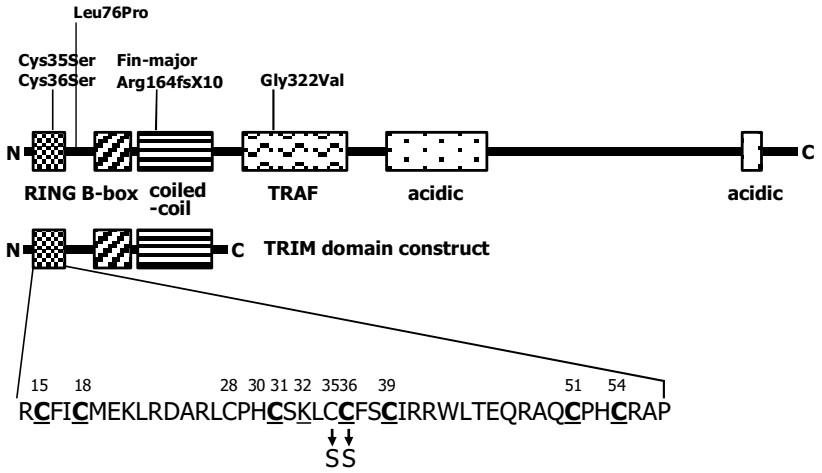

Figure 5. Schematic structure of TRIM37 and the TRIM fragment used in the ubiquitination experiments, and the amino acid sequence of the RING domain. The positions of mutations in the mutant proteins used in the ubiquitination experiments are marked above the schematic structure. In the amino acid sequence of the RING domain, the conserved cysteine residues are underlined and bolded according to a prediction using the NCBI Conserved Domain Search (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Note the lysine (K32) in the position that normally has a conserved histidine residue.

4.2 Analysis of TRIM37 autoubiquitination in cultured cells

The RING domain is essential for ubiquitin E3 ligase activity in several proteins (Pickart 2001). RING-dependent autoubiquitination, i.e. ligation of ubiquitin to the molecule itself *(in cis*), or to an adjacent molecule on a homodimer/multimer (*in trans*), is an indication of E3 ligase activity. For a summary of the results of the ubiquitination assays presented here and in sections 4.3 and 4.4, see Table 4. In order to investigate the putative E3 activity of TRIM37, the ubiquitination of full-length TRIM37 or its TRIM fragment in COS-1 cells was studied. Various TRIM37 expression constructs and a hexahistidine-ubiquitin expression plasmid were co-transfected into COS-1 cells. Ubiquitinated proteins were subsequently enriched from the cell lysates by use of nickel affinity chromatography and guanidium hydrochloride

lysis. The recovery from this enrichment method was low, probably due to the poor solubility and hydrophobicity of the ectopically expressed protein. Therefore, immunoprecipitation by means of an aminoterminal HA tag was subsequently used to enrich the protein. This allowed cell lysis in 0.4% SDS and apparently total solubilization of the ectopically expressed protein, which also diminishes the possibility of deubiquitination and protein degradation after lysis. To avoid interference from immunoglobulin chains in subsequent immunoblot analysis, an affinity matrix with covalently bound anti-HA IgG was used for the immunoprecipitation of the TRIM fragment. The immunoprecipitates were analyzed by immunoblotting. Probing the blots with an anti-ubiquitin antibody showed the presence of a high-molecular-mass ubiquitin-positive smear in the TRIM fragment samples. Accumulation of ubiquitinated species was markedly decreased in a mutated TRIM fragment in which two adjacent cysteines at a conserved zinc-binding position were mutated to serines (p.Cys35Ser;Cys36Ser). Reprobing of the filters with an HA antibody showed the non-modified TRIM fragment at ~30 kDa and, in the presence of ubiquitin, a ladder of high-molecular-mass protein. Ubiquitination of full-length TRIM37 was studied in COS-1 cells using a similar experimental approach, except that successful immunoprecipitation of full-length TRIM37 required the presence of N-ethylmaleimide, a chemical that alkylates cysteine residues. Immunoblot analysis showed accumulation of high-molecular-mass ubiquitinated TRIM37 in the immunoprecipitates. The mutant protein with a disrupted RING domain (p.Cys35Ser;Cys36Ser) showed markedly decreased ubiquitination. Reprobing of the filter with an HA antibody showed a high-molecular-mass smear representing polyubiquitinated TRIM37.

4.3 Analysis of TRIM37 autoubiquitination *in vitro*

In order to rule out the possibility that the polyubiquitination of TRIM37 observed in intact cells was a result of aggregate formation and subsequent proteasomal degradation, ubiquitination of bacterially-produced glutathione-S-transferase (GST)-TRIM fusion proteins was studied *in vitro* (for a summary of the results see Table 4). Rabbit reticulocyte lysate, a source of ubiquitin-activating enzyme and several ubiquitin-conjugating enzymes, is used as a cell-free system to study ubiquitination (Hershko et al. 1980). Due to its poor solubility,

purification of the GST-TRIM fusion protein from bacteria according to the standard protocol did not succeed as extraction from bacteria expressing the GST-TRIM fusion proteins with 1% Triton X-100 yielded only minute amounts of soluble protein. However, addition of the ionic detergent N-lauroylsarcosine at a concentration of 1.5% in the lysis buffer, followed by 3% Triton X-100, dramatically improved the yield and was subsequently used for all purifications. Full-length TRIM37 could not be produced in bacteria, probably due to its relatively large size and poor solubility.

For the *in vitro* ubiquitination assay, wild-type GST-TRIM fusion protein and its mutant forms (p.Cys35Ser;Cys36Ser and p.Leu76Pro) bound to glutathione sepharose beads, were incubated in reaction mixtures containing nuclease-treated rabbit reticulocyte lysate, ubiquitin, ATP, $MgCl_2$, $ZnSO_4$, DTT and the proteasome inhibitor MG-132. The samples were analyzed by immunoblotting using antibodies against ubiquitin or GST. In this assay, wild-type GST-TRIM was polyubiquitinated in a RING domain-dependent manner verifying the result from transfection experiments. This strongly suggests that the ubiquitination detected in intact cells and in the cell-free assay is, indeed, RING-catalyzed autoubiquitination and indicative of ubiquitin E3 ligase activity.

Further evidence to support the participation of TRIM37 in protein ubiquitination was obtained from a yeast two-hybrid screen using the TRIM fragment as bait (see also section 5). Of the clones that were in the correct reading frame with the GAL4 activation domain, four clones encoded ubiquitin and two clones encoded ubiquitin-ribosomal fusion proteins. In contrast, with the TRAF domain or the C-terminal half of TRIM37 as bait, no ubiquitin-encoding clones were identified. We further tested whether the E2 enzyme UbcH5 could support autoubiquitination of GST-TRIM *in vitro*, but the result was negative. Yeast two-hybrid screens also failed to disclose an interacting E2 for TRIM37. Thus, identification of a specific E2 that functions with TRIM37 to catalyze ubiquitin transfer awaits further study.

4.4 Effect of patient mutations on TRIM37 autoubiquitination

The identification of the first two mulibrey nanism-associated missense mutations prompted us to study the effect of the various patient mutations on the autoubiquitination of TRIM37. We first found that, in COS-1 cells, autoubiquitination of TRIM37 was completely abolished

in a protein representing the Fin-major mutation, truncated at the coiled-coil region. This finding is compatible with the hypothesis that coiled-coil region-mediated oligomerization is essential for the function of TRIM family protein (Reymond et al. 2001). Further, similarly to the artificial RING domain mutant (p.Cys35Ser;Cys36Ser), the p.Leu76Pro mutant protein affecting the TRIM unit showed markedly decreased autoubiquitination. It is likely that the Leu76Pro amino acid change affects the integrity of both the RING and B-Box domains thus leading to diminished autoubiquitination. The p.Leu76Pro mutation had a similar effect to the autoubiquitination of a GST-TRIM fusion protein in an *in vitro* assay. In contrast, the p.Gly322Val mutant protein was ubiquitinated similarly to the wild-type protein. The Gly322Val amino acid change is located in the TRAF domain and is unlikely to disturb the TRIM unit, which explains the unaffected autoubiquitination of this mutant protein. It is tempting to speculate that even though the p.Gly322Val mutation does not affect the autoubiquitination activity of TRIM37 it may affect the ubiquitination of the actual (unidentified) substrate because of a disrupted TRAF domain-mediated protein-protein interaction.

In conclusion, the ubiquitination experiments strongly suggest that TRIM37 functions as an ubiquitin E3 ligase. The fact that the autoubiquitination of TRIM37 demonstrated in these experiments is polyubiquitination does not allow a conclusion to be drawn about the nature of the actual substrate ubiquitination, i.e. if it is mono- or polyubiquitination. Ubiquitin ligase activity has not been demonstrated in any mammalian peroxisomal proteins, but in yeast, the peroxisomal membrane protein Pex4p acts as a ubiquitin-conjugating enzyme (Crane et al. 1994), suggesting that ubiquitination takes place at the peroxisomal membrane. In fact, it has been shown recently that yeast Pex5p is polyubiquitinated at the peroxisomal membrane and thereby targeted for proteasomal degradation, which may serve as a quality control system in peroxisomal biogenesis (Platta et al. 2004; Kiel et al. 2005). Additionally, yeast Pex18p is known to be subject to mono- and diubiquitination (Purdue and Lazarow 2001). There are three peroxisomal transmembrane proteins (PEX2, PEX10, and PEX12) that each harbour a RING domain facing the cytosol, but they have not been reported to possess E3 ligase activity. How the ubiquitin ligase activity of TRIM37 is associated with a peroxisomal function remains to be investigated. Nevertheless, dysregulated ubiquitin-dependent proteolysis has previously been implicated in several inherited disorders (Jiang and Beaudet

2004). Ubiquitination may also serve other functions than protein degradation. For example, monoubiquitination has been shown to regulate membrane protein trafficking, endocytosis, and the function of diverse proteins including histones and transcription factors (Schnell and Hicke 2003). Therefore, identification of the substrates for TRIM37 ubiquitin ligase activity will be crucial for understanding the molecular pathways that are affected in mulibrey nanism.

5. Identification of TRIM37-interacting proteins (III, unpublished)

Because the cellular pathway(s), signalling route(s) or structural protein complex(es) to which TRIM37 may belong are not known, we set out to search for proteins interacting with TRIM37 by means of yeast two-hybrid screening. The yeast two-hybrid screens were performed according to the Fields' method (Fields and Song 1989). cDNA fragments encoding amino acids 1-252 (TRIM), 205-454 (TRAF) or 417-964 (C-terminus) of human TRIM37 were used as baits.

With the TRIM bait a total of 42 positive yeast colonies, some of which contained two different prey plasmids, were obtained. Sequencing of the recovered plasmids yielded 38 sequences, of which 18 were in the correct reading frame with GAL4. Probably due to the intrinsic properties of the TRIM bait the efficiency of the screen was relatively low, and a total of only 200 000 colonies were screened, while the recommended minimum is one million colonies. With the TRAF domain bait only four positive yeast colonies, all of which contained the same cDNA fragment, were obtained. With the C-terminal bait 37 positive yeast colonies were obtained. Sequencing yielded 13 sequences, of which eight were in the correct reading frame with GAL4. A list of the putative interaction partners of TRIM37 found in the screens is presented in Table 5. Some of the putative interactions are discussed in more detail below.

BAIT	CLONES	IDENTITY	PULL-DOWN
TRIM	N6,15,42	ubiquitin B	n.d.
“	N9	NICE-3	+
“	N10,22,26	ribosomal-ubiquitin fusion	n.d.
“	N12,34	UBE3A ubiquitin ligase	n.d.
“	N14,39,40	vitamin D-binding protein	n.d.
“	N20	SULT1A2 variant	+
“	N27	RNU2	n.d.
“	N28	REA / prohibitin 2	+
“	N33	elongation factor EEF1A1	n.d.
“	N35	predicted chaperone	n.d.
“	N37	transferrin	n.d.
TRAF	M1,2,3,4	predicted mitochondrial protein	n.d.
C-term.	C5	cyclin G1-binding protein 1	n.d.
“	C6	human factor X	n.d.
“	C9	RPS7 ribosomal protein	n.d.
“	C13	activating transcription factor 5	n.d.
“	C17	MCRS1	n.d.
“	C20	RING finger protein 10	-
“	C32	ABCB6 mitochondrial transporter	+
“	C35	clusterin	-

Table 5. Putative protein interactions of TRIM37 identified in yeast two-hybrid screens. The first column indicates the TRIM37 fragment used as a bait in the screen. Some of the putative interactions have been further studied by means of GST pull-down experiments (PULL-DOWN). Plus (+) denotes a positive result in GST pull-down assays. (n.d.) not determined.

In the yeast two-hybrid screens, the TRIM bait picked up several clones encoding ubiquitin or ubiquitin-ribosomal protein fusions (see Table 5), which is in agreement with the ubiquitin ligase activity of TRIM37. Moreover, two clones with cDNA fragments that encode the HECT domain of the UBE3A ubiquitin ligase, implicated in Angelman syndrome, were recovered in the screen. This interaction, as well as the others listed in Table 5, remains to be verified in further studies. The TRIM bait also picked up two cDNAs that encode proteins with C-terminal tripeptides similar to PTS1 (unpublished data), which prompted us to inspect these clones further. One of the cDNAs encodes a putative splice variant of *hast4/SULT1A2* cDNA. Cytosolic sulphotransferases (SULTs) are enzymes that catalyze sulfonation, an

important step in the metabolism of many drugs, xenobiotics, neurotransmitters, and steroid hormones (Kauffman 2004). The C-terminus of SULT1A2, SEL (Ser-Glu-Leu), differs by only one amino acid from the consensus PTS1 (SKL, Ser-Lys-Leu). However, it is unlikely that SEL functions as a PTS1 because the lysine of SKL is replaced by the negatively charged glutamic acid (Emanuelsson et al. 2003). GST pull-down experiments confirmed that both the alternative isoform encoded by the yeast clone and full-length SULT1A2 (hast4 and hast4v) bind the TRIM fragment of TRIM37 (Figure 6).

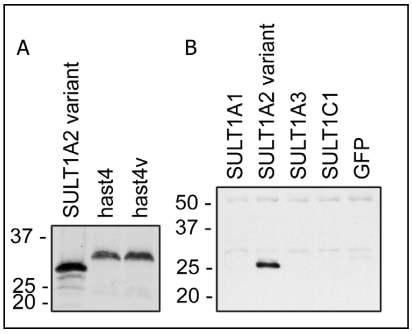

Figure 6. GST pull-down verification of a the putative TRIM37-SULT1A2 interaction. Various *SULT* cDNAs were translated *in vitro* and the reaction products were incubated with GST-TRIM protein coupled to glutathione-sepharose beads. The beads were washed and the bound proteins were analyzed by means of immunoblotting with an antibody against an HA tag (unpublished data). Both the alternative isoform encoded by the yeast clone (A, SULT1A2 variant) and full-length SULT1A2 (A, hast4 and hast4v) bind the TRIM fragment of TRIM37. The closely related enzymes SULT1A1, SULT1A3 and SULT1C1 did not bind the TRIM fragment, and neither did a green fluorescent protein (GFP) control (B).

The other protein with a PTS1-like sequence identified in the TRIM bait screen was NICE-3. This is an uncharacterized protein with homology to 6-phosphogluconate dehydrogenase, an enzyme of the pentose phosphate pathway. The C-terminal tripeptide of NICE-3 is STL (Ser-Thr-Leu). It is unlikely that STL could function as a PTS1 either because of the unusual amino acid, threonine at position 2. NICE-3 also bound to the TRIM fragment in a GST pull-down assay (data not shown).

Three clones encoding mitochondrial proteins (prohibitin 2, an uncharacterized B30.2-domain protein and ABCB6) were found in the yeast two-hybrid screens (unpublished data, see Table 5). Prohibitin 2 is a peculiar protein that apparently localizes to both the nucleus, where it functions as a transcriptional regulator, and to mitochondria where it may function as a chaperone and regulator of cellular senescence (Mishra et al. 2005). GST pull-down experiments verified that the TRIM fragment binds prohibitin 2 *in vitro* (data not shown). In

the TRAF domain bait screen all four positive clones contained the same ~1.3-kb fragment of an uncharacterized cDNA encoding a predicted B30.2-domain protein with a 28-amino acid N-terminal mitochondrial leader sequence. The third mitochondrial protein fragment found in the screens, with the C-terminal TRIM37 bait, was the ATP-binding domain of ABCB6. This interaction was verified by a GST pull-down assay (data not shown). ABCB6 is a mitochondrial membrane protein belonging to the ABC transporter family (Mitsuhashi et al. 2000).

The pleiotrophic clinical manifestations in mulibrey nanism, and the fact that the molecular pathway(s) to which TRIM37 belongs are as yet unknown, make the evaluation of the significance of the interactions presented above difficult. Interaction screens in yeast are prone to false positives and, therefore, extensive verification of the putative interactions by other methods has to be performed. One of the more rigorous methods to confirm an interaction between two proteins under physiological conditions is co-immunoprecipitation of the endogenous proteins. This requires that antibodies suitable for immunoprecipitation are available, which is not the case for TRIM37 and many of the putative interacting proteins listed in Table 5. Nevertheless, as technically challenging as it may be, further analysis of the interactions could shed light on the function of TRIM37 and on the pathways that are defective in mulibrey nanism.

6. Distribution of Trim37 in mouse tissues (IV)

6.1 Distribution of Trim37 in embryonic tissues

Expression of *TRIM37* during early and middle stages of embryonic development, up to E15 in mice, has been studied previously by means of *in situ* hybridization (Lehesjoki et al. 2001). In order to gain further insight into the expression of the *Trim37* gene during ontogenesis, we analyzed the distribution of Trim37 mRNA and protein in mouse tissues by Northern blot analysis and immunohistochemical staining, respectively. For the immunohistochemical analysis, antigen affinity-purified fractions of the anti-human TRIM37 antisera (raised against the M1 peptide) were used. Antigen retrieval by a 15-minute microwave heating in citrate buffer produced a drastic increase in the staining intensity and was included in the staining protocol. To further enhance the staining intensity in some tissues, tyramide signal

amplification (Adams 1992) was utilized. The overall distribution of the Trim37 protein in embryonic tissue was very similar to that reported for the *Trim37* mRNA (Lehesjoki et al. 2001), lending further support to the specificity of the antibody.

By immunohistochemical analysis, a weak staining for Trim37 appeared at E9.0, the earliest stage analyzed. At E10-E12.5, epithelial Trim37 staining in many tissues became apparent. Highest staining was observed in the heart, the somites, surface ectoderm of the embryo, and the developing esophagus and lung. At E15.5 strong epithelial Trim37 staining was observed in the intestine, kidney and tips of the developing pancreatic buds. The surface ectoderm and dorsal root ganglia were intensely positive for Trim37, whereas the liver showed a weak diffuse staining. Taken together, Trim37 is highly expressed in many neural crest-derived tissues and in several epithelia, particularly at sites of mesenchymal-epithelial interaction during mouse embryonic development.

6.2 Distribution of Trim37 in adult mouse tissue

Tissues included in the mulibrey acronym

Expression of Trim37 was detected in all muscle types, including smooth muscle, skeletal muscle and heart muscle. Among the muscles studied, the expression was highest in the muscle of the abdominal wall, in which the staining was apparently fiber-specific and striated (Figure 7A). The only manifestation of mulibrey nanism relating to the skeletal muscle is mild hypotonia. It is implausible that the muscular hypotonia is related to the expression of Trim37 in the muscle. Rather, it likely reflects a mild neurological dysfunction, as has previously been suggested (Karlberg et al. 2004a). It may, for example, be a result of disturbed Trim37 function in the ganglia that innervate muscles, and showed intense Trim37 staining in mouse tissues.

A relatively weak nuclear Trim37 staining was detected in the mouse liver (Figure 7B), which is in contrast to the granular cytoplasmic staining that was previously (Kallijärvi et al. 2002) observed in human liver sections. This discrepancy could be explained by a species-specific difference in the distribution of the protein. Nuclear Trim37 staining was even more evident in some other tissues than the liver, for example in the oesophagus and stomach, suggesting

that the two predicted NLSs in mouse Trim37 are functional in certain cell types or under certain conditions.

Intense Trim37 staining was detected in restricted neuronal cell populations in many regions of the brain (data not shown). Mulibrey nanism patients have normal intelligence, but show mild structural defects in the central nervous system (CNS), i.e. enlarged ventricles and basal cisternas, as well as mild neurological symptoms, including muscle hypotonia and a mild delay in motor and speech development (Karlberg et al. 2004). In the light of our immunohistochemical findings it is somewhat surprising that the patients clinically display only minor neurological defects.

In the retina (Figure 7C), Trim37 staining was detected in the photoreceptor layer (pr) and, to a lesser extent, in the inner (ip) and outer (op) plexiform layers, and nerfe fibre layer (nf) that comprise processes of photoreceptor cells and neurons. Yellowish dots in retinal mid peripheral region are one of the major diagnostic signs of mulibrey nanism (Karlberg et al. 2004). In addition, atrophy of the corneal epithelium, thickening of the Bowman's membrane, and atrophy of the retina and of the pigment epithelium has been reported (Tarkkanen et al. 1982). The high cell-layer specific expression of Trim37 could account for the ocular changes present in mulibrey nanism patients. Interestingly, the vision is only modestly, if at all affected in these patients.

The digestive system

Intense Trim37 staining was observed in many tissues of the digestive system. The mucinous acini of the salivary gland stained intensely for Trim37 while the serous part was almost negative (Figure 7D). Goblet cells along the gastrointestinal tract stained intensely for Trim37 (Figure 7E). Moreover, nuclear Trim37 was observed in the epithelium of the esophagus and stomach. In the stomach, the nuclear staining was strongest towards the base of the gastric glands (Figure 7F). Intense Trim37 staining was also observed in enteric ganglia in stomach and all parts of the intestine from duodenum to colon (data not shown). Half of mulibrey nanism patients suffer from poor feeding during infancy, presenting as vomiting and a delay in switching to solid food (Karlberg et al. 2004). Interestingly, the feeding difficulties disappear in the great majority of the patients during early childhood. In the light of the Trim37 expression pattern in the digestive system, it is tempting to speculate that functions

regulated by enteric ganglia (e.g. bowel movement) or a function performed by the goblet cells (e.g. secretion of mucus), or both, contribute to the feeding difficulties in infants with mulibrey nanism.

The endocrine organs

Strong Trim37 staining was detected in a subset of cells in the adenohypophysis, the endocrine part of the pituitary gland (Figure 7G). The identity of the Trim37-expressing cell type remains to be investigated. Intense Trim37 staining was also detected in the adrenal medulla, while the adrenal cortex was negative (Figure 7H). The staining was present in most but not all cells of the adrenal medulla, suggesting that it is cell-type specific. *Trim37* mRNA is also restricted to the medulla in the developing adrenal gland in embryonic mice (Lehesjoki et al. 2001). Elevated levels of plasma ACTH is seen in mulibrey nanism but the adenocortical function appear to be normal (Karlberg N, unpublished data). The strong expression of Trim37 that we dectected in a subset of unknown cell in the pituitary gland of the mouse, however, is not contradictory to the clinical presentation. Adrenal medullary cells are modified postganglionic neuronal cells that secrete mainly catecholamines (the sympathoadrenal system). The serum levels of catecholamines are normal in mulibrey nanism patients and no changes have been noted in the adrenal medulla (Karlberg N et al., unpublished data). However, recent data show that the adrenal medulla also secretes peptide hormones, for example pancreastatin that inhibits glucose uptake and activates hepatic glycogenolysis in humans (O'Connor et al. 2005). The expression of Trim37 in both the adrenal medulla and endocrine pancreas, combined with recent clinical data showing that subjects with mulibrey nanism develop severe insulin resistance and metabolic syndrome early in adulthood (Karlberg et al. 2005), is of great interest. Te exact identity of the Trim37-positive cells in the endocrine tissues is an important subject of further study that could shed light on the pathogenetic mechanism underlying mulibrey nanism.

The gonads

In post-pubertal testis, a stage-specific cytoplasmic staining of spermatids was detected (Figure 7I). Developing sperm from type B spermatogonia to early spermatids were strongly

positive, while the rest of the cell types in the testis, including Leydig cells, Sertoli cells and spermatogonia, were negative. In post-pubertal ovary, intense Trim37 staining was detected in the oocytes. Staining was also present in the granulosa cells, luteal gland, and in the epithelium of the fallopian tube (data not shown). Female patients with mulibrey nanism present incomplete sexual maturation and develop premature ovarian failure. Their uteri and ovaries are small and only a few or no follicles are present in ultrasonography (Karlberg et al 2004b). In males, testicular hypoplasia and a reduced number of sperm has been encountered (Lipsanen-Nyman M, unpublished data). Interestingly, the highest level of *Trim37* mRNA is detected in the testis in both humans (Avela et al. 2000; Hämäläinen et al. 2005) and mice (see above). In post-pubertal mouse testis, we detected a strong stage-specific Trim37 staining of the germ cell cytoplasm. Our immunohistochemical findings in mouse gonads are in agreement with the clinical findings seen in postpubertal males and females with mulibrey nanism.

In summary, the tissue distribution of Trim37 during ontogenesis suggests that it has a function in many neural crest-derived tissues. These include the adrenal medulla and sympathetic and parasympathetic nerves. Moreover, the facial dysmorphism typical of mulibrey nanism points to a function of human TRIM37 in the development of the craniofacial cartilage and bone that are derived from the neural crest as well (Helms and Schneider 2003). The high expression of Trim37 that was detected in a subset of the presumably hormone-secreting cells of the adenohypophysis suggests that Trim37 plays a role in the regulation of an endocrine function. Trim37 may also be important for the function of a number of secretory cell types in the salivary glands and in the intestine, as well as for sympathoadrenal function and gametogenesis.

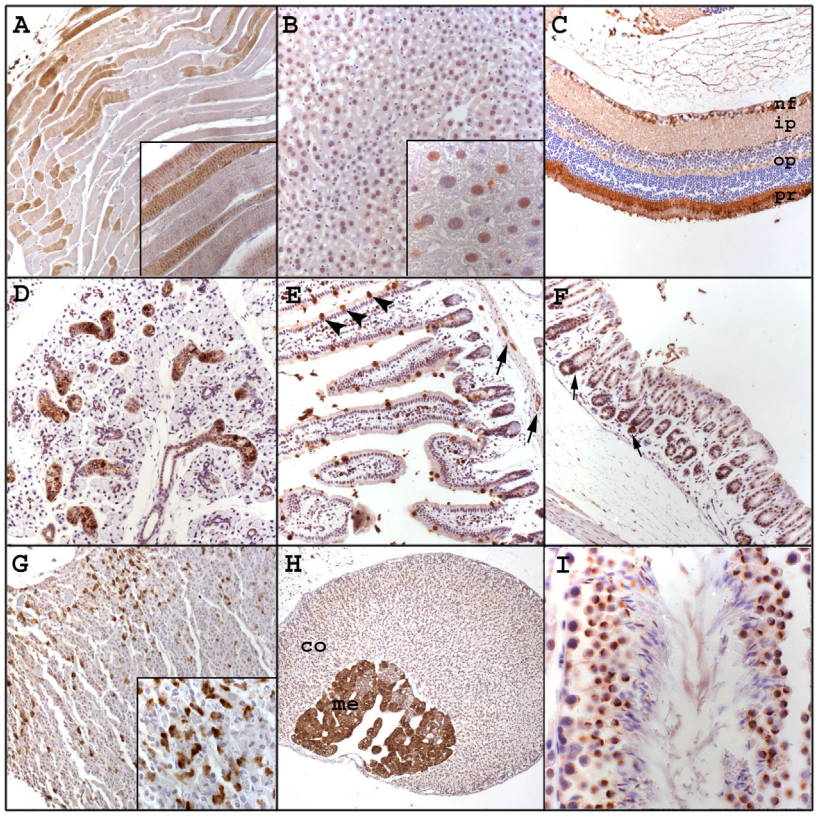

Figure 7. Immunohistochemical analysis of the distribution of Trim37 in various organs of adult mice. Skeletal muscle from the abdominal wall (A) shows an apparently fibre-specific staining that is striated when inspected at a higher magnification (inset). In the liver (B), a weak nuclear staining was observed in the hepatocytes (inset). The retina (C) was intensely positive for Trim37. The staining was strongest in the photoreceptor layer (pr) but was also present in the inner (ip) and outer (op) plexiform layers and in the nerve fibre layer (nf). Intense Trim37 staining was detected in many tissues of the digestive system (D-F). The mucinous acini and the excretory ducts of the salivary glands were strongly positive (D). In the duodenum (E) a strong staining of the goblet cells (arrow heads) and intestinal ganglia (arrows) emerged. In the stomach (F) nuclear Trim37 staining in the epithelium was observed (arrows). The adenohypophysis (G) shows an intense staining in a subset of the cells that is mainly cytoplasmic. The adrenal medulla (H, me), the neuroendocrine part of the adrenal gland, was among the most intensively Trim37-positive tissues, while the adrenal cortex (co) was essentially negative. Within the adrenal medulla there was heterogeneity in the staining pattern, with a minority of the cells showing weak staining. In the round spermatids in the testis, Trim37 staining was detected in the polarized cytoplasm (I). Magnification is 630X in I, 200X in the other figures, and 1000X in the insets.

Conclusions and future prospects

In this study the functional characterization of the TRIM37 protein was initiated. The work aims at understanding the molecular pathogenetic mechanisms that underlie mulibrey nanism, a peculiar pleiotrophic disorder with a number of distinct clinical features that become manifest at different time points. The complexity of the clinical picture that includes symptoms as diverse as growth failure, cardiopathy, type-2 diabetes, and susceptibility to Wilms tumour, raises the possibility that TRIM37 has several distinct molecular functions. Alternatively, it can be hypothesized that TRIM37 has a single function that serves different purposes depending on the cell type and developmental stage. Dissecting the function of a previously uncharacterized protein is, in many cases, a demanding task. Both inadequacy of the molecular tools available (e.g. antibodies, biologically relevant cell lines) and complexity of the biological processes studied may contribute to the challenge. Given that the underlying genes for most monogenic disorders, including mulibrey nanism, have been known for only a short time, further study is the only cure for this problem.

This thesis, in which the TRIM37 protein has been viewed from several different angles, provides a basis towards understanding the molecular pathogenesis of mulibrey nanism. In the course of the study, a number of new mulibrey nanism-associated mutations were identified, among these the first missense mutations identified in the *TRIM37* gene. The novel missense mutations turned out to be important for the subsequent functional studies of TRIM37. Furthermore, the subcellular localization of the TRIM37 protein and its mutants was investigated. On the basis of the localization studies we have tentatively classified mulibrey nanism as a peroxisomal disorder. However, the finding that nuclear Trim37 staining was observed in some mouse tissues and the fact that several mitochondrial proteins were identified as putative interaction partners for TRIM37 raise the possibility that the protein may localize to more than one subcellular compartment. The differential localization could depend on the cell type, the phase of the cell cycle, or the metabolic status of the cell. Clearly, more experiments are needed to clarify the role of peroxisomal function in mulibrey nanism, and to shed light on the significance of the other putative subcellular localizations. Analysis

of patient samples using high-throughput methods, such as expression profiling with cDNA arrays and lipid profiling with mass spectrometry, could prove useful in this respect. The generation of *TRIM37* knock-out mice will, if the human phenotype is replicated in mice, likely aid the inquest into the putative metabolic defects in mulibrey nanism.

A biochemical function, ubiquitin E3 ligase activity, was demonstrated for TRIM37 in this study. The finding that a patient missense mutation disrupts the ubiquitin ligase activity suggests that the loss of the E3 activity is crucial to the pathogenesis of mulibrey nanism. In the light of our findings it is tempting to speculate that the function of TRIM37 is to induce ubiquitin-dependent degradation of a target protein. Loss-of-function mutations in *TRIM37* would thus lead to an excess of the target protein and subsequent downstream effects depending on the identity of the target protein. However, non-proteolytic regulatory ubiquitination by TRIM37 remains a possibility to be reckoned with. In any case, the identification of substrates of TRIM37-mediated ubiquitination is a prerequisite for the progression of this line of investigation. A set of putative interaction partners of TRIM37 was identified in yeast two-hybrid screens. While none of the interactions were solidly confirmed in this study, the data provide material for further functional studies. Characterization of the putative interacting proteins identified in this study, as well as mass spectrometric identification of affinity-purified proteins that bind to different parts of TRIM37, could help identify substrates of TRIM37.

Immunohistochemical analysis of embryonic and adult mouse tissues indicated that Trim37 is expressed in a highly tissue-specific manner during ontogenesis, which suggests that it has a cell type-specific regulatory function rather than a general role in cell maintenance. These findings are in agreement with the multiorgan manifestation of mulibrey nanism. Putative cell type-specific functions of TRIM37, both during embryonic development and in the adult organism, are a fascinating area of research and could shed light on the development of the complex mulibrey nanism phenotype.

The recent finding that mulibrey nanism patients are prone to develop insulin resistance and type 2 diabetes mellitus is of utmost interest (Karlberg et al. 2005b). There are few monogenic models for insulin resistance, which is why research on mulibrey nanism could have a great impact on the study of diabetic mechanisms. It will be interesting to see whether *TRIM37*$^{-/-}$ mice will phenocopy the insulin resistance of mulibrey nanism patients. Another

important question that remains open at present is the mechanisms that lead to tumour susceptibility in mulibrey nanism patients. Particularly, investigation of the mulibrey nanism-associated Wilm's tumours could uncover the development of this tumour type in general. More studies have to be conducted to clarify both the clinical, developmental and biochemical aspects of the disorder. Thorough understanding of the basic pathogenetic mechanisms is also essential for the development of therapies for mulibrey nanism in future.

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