Identification and characterisation of a novel integral membrane protein, shrew-1 that complexes with *adherens junctions* in polarised cells

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

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vorgelegt
beim Fachbereich Biologie und Informatik
der Johann Wolfgang Goethe-Universität

Frankfurt am Main, Oktober, 2003

Acknowledgements

I would like to express my deepest gratitude to Prof. Dr. Anna Starzinski-Powitz, not only for providing me the opportunity to work in her lab but also for being a constant source of encouragement and motivation throughout this work. Her expert supervision and timely criticism were immensely invaluable for the completion of this work.

My special thanks to Ela for helping me at the most crucial time of this project and for giving me a new approach to analyse my results.

For most of the friendly technical help during this work, I thank Beata Krebs.

I would like to thank Andreas not only for the critical reading of my thesis but also for giving me excellent supervision for my experiments in cell culture as well as the initial molecular biology experiments, and of course the "charming" criticisms :)

For providing a warm and lively atmosphere combined with the unbeatable enthusiasm about cooking which resulted in quite delicious products, I thank Christine. And also thanks for the friendly shoulder who was always there in both good and bad times.

For providing a friendly working atmosphere in the lab, I thank Andi, Frank and Rene.

I thank Viktor for his cheerful assistance during the last part of my experiments, and Alex for always being willing and enthusiastic to discuss experiments with me.

I thank Heinz Schewe for teaching me confocal microscopy and for helping me take the most beautiful pictures of my work.

I owe this work to my beloved husband, Kapil, for his constant support, encouragement and unlimited patience especially during the most rough times, as well as for providing valuable suggestions during the times when I needed them the most.

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1. Introduction

1.1. Adherens junctions

Cell-cell adhesion is an important process that is dynamically rearranged and regulated in various situations including tissue development, the establishment of epithelial cell polarity, and wound healing (Adams and Nelson, 1998; Gumbiner, 2000; Takeichi, 1995). In mammals, adhesion between epithelial cells is generally mediated by three types of junctions: namely tight junctions (TJs), adherens junctions (AJs) and desmosomes, which together constitute the Intercellular Junctional Complex. The complexes contain transmembrane receptors, usually glycoproteins that mediate binding at the extracellular surface and determine the specificity of the intracellular response. The associated cytoplasmic proteins of these receptors structurally link them to the cytoskeleton, hence establishing communication to other cell-cell junctions and to cell-substratum junctions. The linkage of cell-cell junctions allows single cells of an epithelial sheet to function as a coordinated tissue. Additional proteins assist in connecting structural and signaling elements, and thus intercellular junctions function to integrate a number of cellular processes ranging from cytoskeletal dynamics to proliferation, transcription, and differentiation (reviewed in Perez-Moreno et al., 2003).

1.1.1. E-Cadherin and its associated proteins

The transmembrane core of AJs consists of cadherins, which cluster at sites of cell-cell contacts in most solid tissues. The best characterized member of the cadherin family is E-cadherin, an epithelial cadherin which is important for the induction of epithelial cell-surface polarity and mediates the establishment of AJs. It is also required for the compaction of preimplantation embryos in early development (Larue et al., 1994). E-cadherin has been characterised as a tumor-suppressor molecule. Any mutation in the E-cadherin gene, its downregulation, or the hypermethylation of its promoter are associated with tumor progression (Takeichi, 1993; Birchmeier et al., 1993; Christofori and Semb, 1999).

E-cadherin exhibits calcium dependent homophilic interactions with other cadherin molecules on the neighbouring cells via its extracellular portion. It is connected to the cytoplasm by its cytoplasmic tail that binds to β-catenin/plakoglobin which is further connected via α-catenin (an adaptor protein) to the cytoskeleton (Fig. 1).

Alternatively, α-catenin binds to the Rac1/Cdc42 effector protein, IQGAP1, when displaced from the cadherin-catenin complexes (Kaibuchi et al., 1999), which occurs only in the case of negative regulation of cell-cell adhesion (Kuroda et al., 1998). The linkage of E-cadherin to the cytoskeleton via the catenins stabilises the cadherins at the junctions hence facilitating cell-cell adhesion. The juxtamembrane domain of the cadherins binds to p120 (Reynolds et al., 1994), although the functional implication of this binding is not really clear. The protein $p120^{ctn}$ has been shown to act both as a positive and negative regulator of cadherin adhesiveness (Anastasiadis and Reynolds, 2001). Recent evidence using a p120ctn-deficient cell line has implicated a crucial role for p120-E-cadherin interaction not only for the proper localisation and function of E-cadherin but also an increase of protein expression (Ireton et al., 2002).

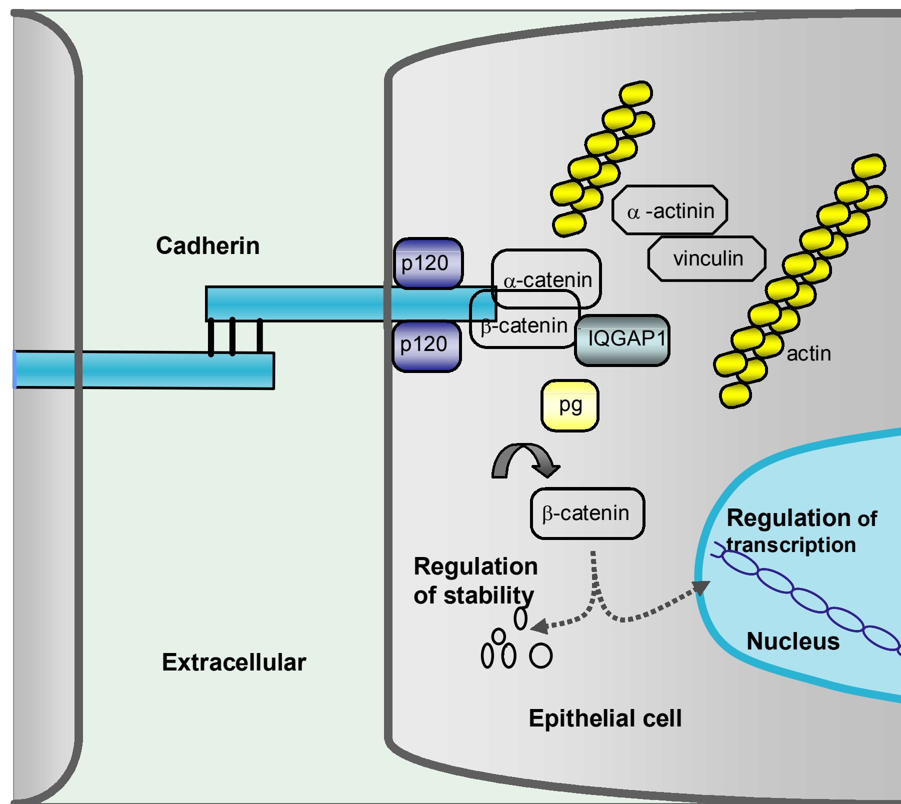


Figure 1. E-cadherin based adherens junctions.

Apart from being an important component of the cell adhesion machinery by linking cadherins to the cytoskeleton, β-catenin is also a pivotal component of the Wnt/Wingless (Wg) signaling pathway which plays a key role in various developmental processes (Cox and Peifer, 1998; Willert and Nusse, 1998). The

cellular level of the β-catenin protein, as well as its subcellular localization are very tightly regulated. When not associated with cell-cell junctions, β-catenin is incorporated into a large complex that includes the APC tumor suppressor gene, the GSK-3β serine/threonine protein kinase, and the axin/conductin protein (Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998). Activation of the Wnt/Wg pathway blocks this degradation, giving rise to an increased pool of free β-catenin that translocates into the nucleus and subsequent on binding to TCF/LEF family of transcription factors, stimulates transcription of some oncogenes and a variety of unknown target genes (Behrens et al., 1996; Molenaar et al., 1996; Riese et al., 1997; van der Wetering et al., 1997). The deregulation of β-catenin leading to an increased free pool has been associated with various types of cancer (Clevers and van de Wetering, 1997; Gumbiner, 1997; Peifer, 1997; Bullions and Levine, 1998).
The important role of stabilising intercellular junctions and coordinating actin dynamics at these sites is performed by α-catenin, a key regulator of the cadherin-catenin complex. α-catenin binds to other cytoskeletal elements such as vinculin, α-actinin, ZO-1 and actin (Imamura et al., 1999). The binding of α-catenin to the nectin-afadin complex (Mandai et al., 1999) is necessary for the recruitment of E-cadherin to the nectin-based cell-cell adhesion sites and is involved in the formation of E-cadherin based AJs (Fig. 2). Vezatin, another newly identified member of the AJs interacts with α-catenin and bridges myosin VIIA to the cadherin-catenins complex, probably strengthening cell-cell adhesion (Küssel-Adermann et al., 2000). The binding of α-catenin to these newly identified members of the AJs contributes to the organisation of the junctional complex by being responsible for the proper localisation of these molecules (Küssel-Anderman et al., 2000; Tachibana et al., 2000).

Additionally, some novel PDZ proteins have been identified that may form complexes with basic AJ components to establish mature AJs in non-epithelial and epithelial cells. PDZ domains are basically protein-protein interactions domains that can interact with short carboxyl-terminal peptide motifs or with other PDZ domains (Fanning and Anderson, 1999). PDZ proteins localized at AJs may play a role in regulating the localization of interacting proteins, including adhesion molecules, receptors and signaling proteins. One of the two newly identified PDZ proteins of the adherens junctions include LIN-7 that occurs in a LIN-2-LIN-7-LIN-10 protein

complex that is involved in the organization of epithelial and neuronal junctions in *C. elegans* and mammals (Margolis et al., 1999). The other protein is Arc protein (Liu et al., 2000) which is also present in AJs in *Drosophila,* the exact function for which is not yet deciphered although it exhibits a highly regulated pattern in developing embryonic epithelial tissues.

Another potential partner identified for E-cadherin is the presinilin 1 (PS1) protein involved in Alzheimers disease. It has been reported that PS1 directly binds to E-cadherin, although whether this association stabilises E-cadherin (Baki et al., 2001) or targets E-cadherin for cleavage and AJ disassembly (Marambaud et al., 2002) is not yet clear. Additionally, PS1 facilitates the stepwise phosphorylation of β-catenin that targets it for degradation, and conversely, loss of PS1 leads to stabilisation of β-catenin, enabling it to function outside AJs (Kang et al., 2002). These findings as well as its broad expression in epithelia suggest that its regulation may impact on AJ-cytoskeletal dynamics.
Understanding of this complex network of proteins and their regulation at the AJs will provide much deeper insight into understanding the dynamic regulation of AJs during development and disease.

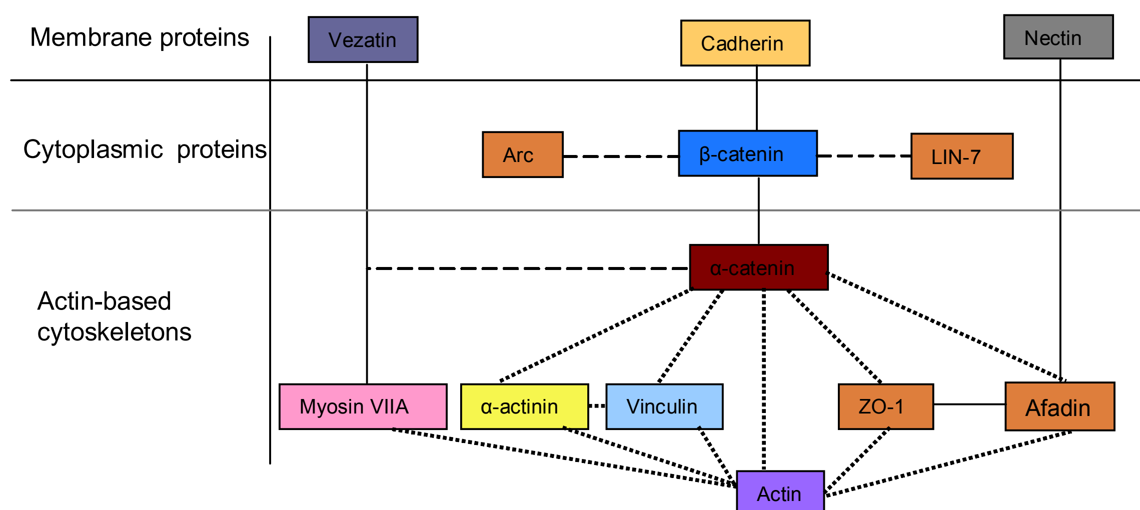


Figure 2. Protein complexing at the AJs. Stable interactions are depicted by a straight line , colocalisation and/or possible direct interactions are depicted by a dashed line.

1.1.2. Regulation of the adherens junctions

Adherens junctions are highly dynamic structures that can undergo rapid but nevertheless regulated assembly and disassembly, as in epithelial-mesenchymal transitions. Such processes are needed, for example, when cells decrease the strength of their adhesion in order to move. If deregulated in pathological situations these phenomena may participate in establishing invasion and metastasis of carcinomas, as has been shown in many cases (Shimoyama and Hirohashi, 1991; Shiozaki and Mori, 1991; Takeichi, 1991).

As mentioned above, E-cadherin plays a central role in the establishment of epithelial adherens junctions. The dynamics of the regulation of the AJs are quite dependent on the cadherin-catenin complexes and their associated proteins. The major factors that are involved in the regulation of these components and hence in the regulation of the AJs are summarised below:

a) Posttranslational modifications

The phosphorylation of E-cadherin by casein kinase II (CKII) and glycogen synthase kinase-3β (GSK-3 β) increases the affinity of E-cadherin for β-catenin, thus enhancing E-cadherin mediated cell-cell adhesion (Huber and Weis, 2001; Lickert et al., 2000). CKII has also been shown to phosphorylate β-catenin at serine/threonine residues and in addition to stabilising the protein, it also increases its adhesion with α-catenin, thus stabilising the cadherin adhesion complex (Bek and Kemler, 2002).

Tyrosine phosphorylation is also known to play a major role in the regulation of E-cadherin function: β-catenin is tyrosine phosphorylated by the non-receptor tyrosine kinase SRC, this modification leads to disassembly of the cadherin-catenin complexes and subsequent loss of cell adhesion (Behrens, 1993; Hamaguchi et al., 1993). Moreover, β-catenin has also been shown to be phosphorylated by receptor-tyrosine kinases, epidermal growth factor receptor (EGFR) and c-met (also known as scatter factor or hepatocyte growth factor receptor) (Hoschuetzky et al., 1994). Increased tyrosine phosphorylation of β-catenin is frequently found in colorectal cancer (Takayama et al., 1998).

An interaction partner of E-cadherin-Hakai, has been identified that binds to E-cadherin in a phosphotyrosine dependent manner causing the shuttling of internalised E-cadherin to the lysosome rather than recycling it back to the plasma

membrane (Fujita et al., 2002). Thus, it has been concluded that Hakai has the ability to regulate adhesion by modulating the amount of cell surface cadherin.

b) Role of small GTPases

The Rho family of small GTPases (includes RhoA, Rac1 and Cdc42) is known to regulate cell shape, cell growth and cell polarity (Tapon and Hall, 1997). Rac1 and Cdc42, together with their exchange factor Tiam-1, also regulate cadherin-mediated cell-cell adhesion (Braga et al., 1997; Jou and Nelson, 1998). This has been shown to be regulated by IQGAP1, a target molecule of Cdc42 and Rac1. IQGAP1 induces the dissociation of α-catenin from the E-cadherin cell-adhesion complex by competing with α-catenins binding to β-catenin.; this results in the loss of E-cadherin mediated adhesion by inhibiting its association to the cytoskeleton (Kuroda et al., 1998).

Rac1 has additionally been implicated in the regulation of adherens junctions via clathrin independent (involving caveolae) endocytosis of E-cadherin (Akhtar and Hotchin, 2001) and has a possible role in the dynamic rearrangement of E-cadherin-mediated cell-cell adhesion since the E-cadherin endocytosed via activated Rac1 is not fated for degradation albeit for recycling. This group highlighted for the first time the emerging role of caveolin-1, a major coat protein of caveolae, in the dynamics of AJs.

c) Transcriptional regulation

The transcriptional regulation of E-cadherin controls E-cadherin levels and hence the adhesive interactions during tissue remodelling events. A well studied element of the E-cadherin promoter is an E-box which binds factors such as Snail, Slug, E12/E47, and SIP to promote transcriptional repression of the E-cadherin gene (Bolos et al., 2003). Genetic ablation of snail in mice results in early embryonal abnormalities, including the development of a mesoderm with epithelial characteristics such as AJs and apical-basal polarity (Carver et al., 2001). Conversely, overexpression of snail and its cousin slug results in epithelial to mesenchymal transitions (EMTs) *in vitro* (Bolos et al., 2003). EMTs play a broad role in the normal development of tissues, including kidney and skeletal muscle. Snail has also been implicated in specifying mesodermal fate during gastrulation (Carver et al., 2001; Ciruna and Rossant, 2001). A variety of epithelial cancers show

elevation of these repressor proteins, and this correlates with tumor invasiveness (Comijn et al., 2001).

1.2. Caveolin-1

Caveolae are plasma membrane specializations of 50-100 nm that exist in two different forms: (a) as invaginations of the plasma membrane and (b) as vesicles residing near the membrane. They are found in many cell types, but are notably abundant in fibroblasts, adipocytes, endothelial cells, type I pneumocytes, epithelial cells, and smooth and striated muscle cells (Couet et al., 1997a). Fig. 3 shows the electron microscopy picture of caveolae at the membrane. The chief structural proteins of caveolae are caveolins. Three caveolins genes known so far are: caveolin-1, caveolin-2 and caveolin-3. While caveolin-1 and caveolin-2 are ubiquitously expressed, caveolin-3 occurs specially in muscle tissue.

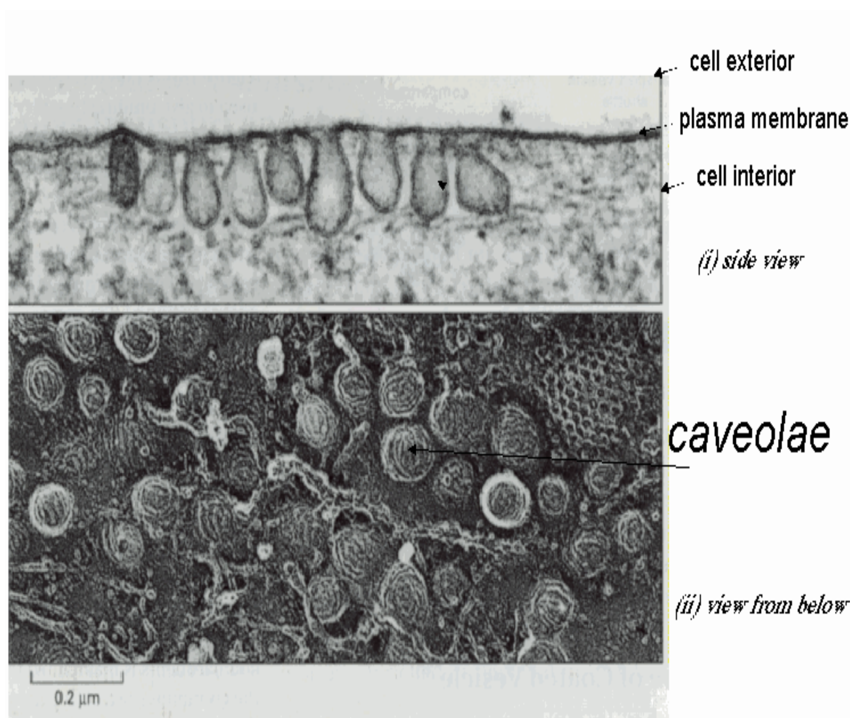


Figure 3. Caveolae at the plasma membrane as visualised by electron microscopy.

Caveolins are integral membrane proteins with a hydrophobic segment of 33-amino acids and form a hair pin like structure with both N- and C- terminus in the cytoplasm (Kurzchalia et al., 1992 and 1994). Three important functional roles have been designated to caveolins:

1. the principle structural components of caveolae membranes

2. shuttle proteins in the biosynthetic trafficking of cholesterol from the ER to the plasma membrane

3. scaffolding proteins to organize and inactivate signaling molecules that are concentrated on the cytoplasmic surface of caveolar membranes.

Caveolin-1 was first identified by Glenney and co-workers as a major v-src substrate in Rous sarcoma virus-transformed chicken embryo fibroblasts (Glenney, 1989; Glenney and Lucille 1992; Glenney and Soppet 1992). Thus, caveolin-1 was first discovered in the context of tyrosine phosphorylation, signal transduction, and cell transformation.

Only later was caveolin-1 found to be a component of the caveolar coat (Rothberg and Artavanis-Tsakona, 1992). It plays an important role in the biogenesis of caveolae through its ability to form homo-oligomers and its direct interaction with cholesterol and probably glycosphingolipids.

Along the lines that caveolin family members function as scaffolding proteins (as mentioned above), caveolin-1 was found to suppress the kinase activity of Src family tyrosine kinases (c-Src/Fyn), epidermal growth factor receptor (EGFR), Neu, and a protein tyrosine kinase C through the caveolin-scaffolding domain, a modular protein domain that recognises a specific caveolin binding motif in various signaling molecules (Couet et al., 1997a; Okamoto et al., 1998; Couet et al., 1997b). In addition, the scaffolding domain of caveolin-1 has been shown to inhibit endothelial nitric–oxide synthase activity and the GTPase activity of heterotrimeric G-proteins (Li et al., 1995; Li et al., 1996; Song et al., 1996; Razani et al., 1999, Garcia-Cardena et al., 1997). The constitutive activation of these signaling molecules leads to cell transformation. Since the interaction of caveolin-1 via its scaffolding domain (Okamoto et al., 1998) with these signaling molecules leads to their inactivation, caveolin-1 has been found to play an important role as a tumour suppressor protein (Koleske et al., 1995; Engelman et al., 1997; Galbiati et al., 1998).

This role of caveolin-1 as a tumor suppressor has been shown in many different reports:

1. It has been seen that caveolin-1 gene is localised to a suspected tumour suppressor locus (7q31.3), a known fragile site (FRA7G) that is deleted in many types of cancer (Engelman et al., 1998). Thus, downregulation of caveolin-1 expression and caveolae organelles might be critical for the induction and the maintenance of the transformed phenotype.

2. The level of caveolin-1 mRNA and protein expression are either lost or reduced during cell transformation by activated oncogene products such as v-Abl and Ha-Ras (Koleske et al., 1995). Induction of caveolin-1 expression in v-Abl and Ha-Ras transformed NIH 3T3 cells abrogated the loss of anchorage-independent cell growth and led to the formation of caveolae (Engelman et al., 1997).

3. It has been shown by the group of Galbiati et al (1998) that antisense mediated functional knockout of caveolin-1 leads to the oncogenic transformation and constitutive activation of the p42/44 mitogen-activated protein kinase (MAPK) cascade.

4. Caveolin-1 has also been shown to regulate contact inhibition and growth arrest in nontransformed cells (Galbiati et al., 1998; Volonte et al., 1999), hence raising the possibility of its being involved in cell adhesion processes.

5. It has been shown by Galbiati et al (2000), that caveolin-1 expression inhibits Wnt/β-catenin/Lef-1 signaling by recruitment of β-catenin to caveolae membrane domains. This is also quite interesting in the context of cell-cell adhesion since caveolin-1 also forms a complex with E-cadherin and β-catenin most probably via its direct interaction with β-catenin. This makes caveolin-1 an additional important candidate for studying the complex mechanism of cell-cell adhesion.

1.3. Endometriosis

The invasive but benign disease endometriosis is one of the most frequent diseases in gynaecology, affecting up to 15-20% of women during the reproductive stage and causes infertility in up to 50% of women (Goldman and Cramer, 1990). Endometriosis is characterized as the ectopic presence of endometrial glands and stroma outside the uterine cavity, which undergo cyclic proliferation and breakdown similar to eutopic endometrium. Ectopic lesions are mainly located in the peritoneal cavity and ovaries (Jenkins et al., 1986; Halme et al., 1998). Furthermore, infiltrating lesions are found for example in the colon, kidney, liver, lungs and pancreas. Thus, the endometriotic cells are able to penetrate organs of the pelvic cavity or more distant organs after spreading via the blood stream or lymphatic system.

The aetiology and pathophysiology of the disease remains poorly understood. Different theories have been proposed to explain the onset and progress of this disease:

- **Implantation theory:** This theory postulates that the majority of endometriotic lesions are derived from viable eutopic endometrial cells that are transported to the peritoneal cavity by retrograde menstruation, which then adhere to the peritoneal wall, proliferate and form endometriotic lesions (Sampson 1940).
- **Metaplasia theory:** According to this theory, endometriosis might also be established by hormone-dependent transformation of peritoneum into Mullerian-type epithelium (Fuji 1991). This metaplasia theory is supported by rare cases of endometriosis in men and in women without retrograde menstruation or abnormal fallopian tubes (Suginami 1991).

Both theories are variations of the initial development of endometriotic lesions.
Until now, no theory has come up which explains the growth and differentiation of endometriosis and also why implantation and endometriosis do not occur in all women, inspite of the fact that retrograde menstruation occurs in almost all women. There is still no explanation to why deep and ovarian endometriosis or endometriotic disease does not develop in all women with subtle and/or minimal endometriosis and why some women develop cystic ovarian and other deep endometriosis, nor why deep lesions can be present as infiltration, or retraction or adenomyosis externa (Koninckx et al., 1991).

1.3.1. Factors involved in endometriosis

Different factors that are involved in endometriosis have been identified such as i) Peritoneal fluid that contains in women with endometriosis an increased number of activated macrophages that secrete a variety of cytokines, including interleukin IL-6, IL-8, vascular endothelial growth factor, and tumor necrosis factor-α TNF-α (reviewed in Giudice et al., 1998), ii) Ovarian steroids , whereby it is shown that high levels of estrogen is related to increased incidence of endometriosis (Halme et al., 1998), iii) Extracellular matrix and its components which involve the matrix metalloproteinases (Osteen et al., 1996), different ligands such as tenascin, fibronectin isoforms and integrins α_3, α_6, and $\alpha_v\beta_3$ (Rai et al., 1996; Lessey and Young, 1997) and iv) the adhesion molecules such as E-cadherin which is expressed in all endometrial samples, but could only be detected in 40% of menstrual effluent samples (van der Linden et al., 1994). This shows that the loss of cell adhesion properties might play a role in the shedding of endometrial tissue

during menstruation and the detached endometrial tissue can attach to the peritoneum, thereby leading to endometriotic lesions.

The study of molecular, cellular and pathophysiological parameters governing endometriosis is hampered by a lack of *in vitro* model systems, such as endometriotic cell lines. To overcome this problem, cell lines were established successfully for the first time in our group, from peritoneal biopsies and were characterised at the molecular and cellular level (Zeitvogel et al., 2001). For the preparation of the cell lines, cells from peritoneal endometriotic biopsies were taken into culture and are subsequently transformed with SV40 T antigen for immortality. Two types of cells could be transformed i) exhibiting stromal cell features (cytokeratin/E-cadherin negative) ii) epithelial-like (cytokeratin positive/E-cadherin negative), which proved as invasive as metastatic carcinoma cells, as measured with a matrigel invasion assay. This could be the influence of N-cadherin implicated as a path finding cadherin allowing cellular invasion and migration in both normal and pathophysiological processes. These results supported the idea that endometriosis, although not neoplastic, shares features with malignant cells and that metastasis in endometriosis may include mechanisms proposed for micrometastasis in cancer. Thus, these cell lines could serve as a useful tool for analysing molecular and cellular events relating to endometriosis as well as representing a paradigm for invasion and metastasis in general.

1.4. Shrew-1

One of the cell lines that was established for investigation of molecular and cellular mechanisms involved in the invasiveness of endometriosis, was EEC145T (Zeitvogel et al., 2001). This cell line shared common features with other established endometriotic cell lines in our lab, i.e. was E-cadherin negative, cytokeratin positive and was invasive in a collagen invasion assay (Zeitvogel et al., 2001). However, a peculiar feature of the EEC145T was that at approximately passage 25, this cell line started losing its invasive features and downregulating cytokeratin expression without changing the proliferative potential.

To analyse the molecules differentially expressed in the invasive and the non-invasive passages, Differential Display Reverse Transcriptase PCR (DDRT-PCR) with an invasive (p17) and a non-invasive (p33) passage of this cell line was

performed (Bharti et al., in press). This reproducibly resulted in the isolation of a 391 bp DDRT-PCR fragment (Fig. 4A) that was differentially expressed in the invasive form of EEC145T cell line. Cell lines EJ28, RT112 and the late passage of EEC145T did not seem to have any shrew-1 mRNA (Fig. 4B). It could also be shown that shrew-1 mRNA was not detected in normal endometrium, but only in endometriosis (Handrow-Metzmacher, unpublished). With Northern blot analysis, it could be shown that this 391 bp fragment hybridised to a transcript of 4 Kb in different tissues such as pancreas, kidney, spleen and brain, in addition to a 9 Kb message in the brain, generating the possibility of two isoforms or two different promoters leading to two different transcription initiation sites (Handrow-Metzmacher, unpublished).

Database searches in NCBI for sequences identical to shrew-1 DDRT cDNA sequences also revealed ESTs with identical sequences from whole brain, kidney, lung epithelia, pooled, spleen, 2 pooled Wilms tumors-one primary and one metastatic to brain, primary lung cystic epithelial cells, synovial membrane and hippocampus. Table 1 also shows some tissues that tested positive for shrew-1 expression in our lab by in situ hybridisation (Klemmt, unpublished).

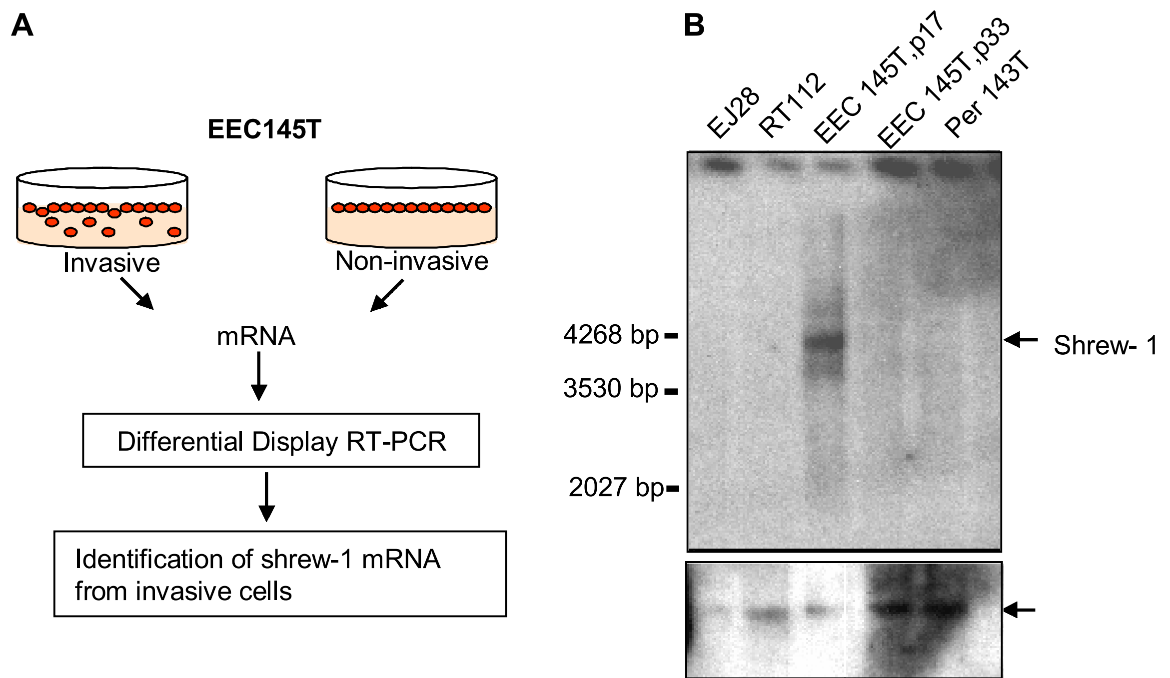


Figure 4. **(A)** Diagram depicting DDRT-PCR performed with invasive and non-invasive passages of the endometriotic cell line EEC145T, leading to the identification of shrew-1 mRNA. (**B**) The 391 bp cDNA was used as a probe to test for the presence of shrew-1 mRNA in endometriotic and carcinoma cell lines. Poly A+ RNA was prepared from the cell lines EJ28 (invasive bladder carcinoma), RT112 (non-invasive bladder carcinoma), EEC145T (p17 = invasive passage 17; p33 = non-invasive passage 33 of the endometriotic cell line) and Per 143T (peritoneal cells immortalised with SV40 T antigen). A Northern blot probed with ^{32}P-labelled shrew-1 probe detected an mRNA of about 4 kb in the invasive endometriotic cell line. Lower panel: the membrane was reprobed with cytochrome C oxidase to check the integrity and loading of the RNA samples.

Screening of a ZAP Express ^{TM} /EcoRI/XhoI custom cDNA phage library constructed from RNA of invasive passage p17 of EEC145T led to the isolation of phagemid clone Q2A containing an insert of 2204 nucleotides including the original DDRT-PCR fragment. The genomic sequence of shrew-1 isolated by NCBI data bank showed shrew-1 to be present on chromosome 1p36.

The exon structure deduced from this shrew-1 nucleotide sequence of 2204 nucleotides consists of 5 exons, with the original DDRT-PCR sequence in the exon 5. The longest open reading frame (ORF) found consisted of a stop codon in the exon 4, leading to the conclusion that the original shrew-1 fragment isolated was a part of the 3´UTR of the shrew-1 mRNA.

TISSUES	CARCINOMAS
Large intestine	Kidney
Embryo	Liver metastatis
Endometriosis	Endometriosis
Endometrium	Pancreatic carcinoma
Heart	Breast cancer(ductular)
Pancreas	Breast cancer(lobular)
Placenta	Lung carcinoma
Spleen	
Prostrate gland	
Ovary	
Skeletal muscle	
Thymus	

Table 1. Tissues positive for shrew-1. Summary of expression analysis of shrew-1 in various epithelial tissues and carcinomas by in situ hybridisation.

A polyadenylation signal in the deduced cDNA sequence at the 3´ end was isolated which was most probably the transcription termination site of the shrew-1 mRNA. Further attempts were done to deduce the sequence more towards the 5´ end to isolate the complete 4 Kb nucleotide stretch. The rest of the cDNA was isolated by exon trapping experiments and 5´ and 3´ RACE experiments using commercially available Marathon-Ready™ cDNA from human brain, which is also positive for shrew-1. The cDNA finally obtained contained 2910 nucleotides and was identical to mRNA sequences in the EEC145T cells as revealed by overlapping RT-PCRs and DNA sequencing (accession number AX009358). This cDNA encoded an ORF of 411 amino acids (Handrow-Metzmacher, unpublished).

1.4.1. Alternative splicing of shrew-1

Different alternatively spliced products of shrew-1 were identified with further RACE and exon trapping experiments (Fig. 5). The spliced variants at the 5´end consisted of exon 0 and exon 0a. Exon 0a does not contain any translation initiation site whereas exon 0 contains one translation initiation site. At the 3´end, exon 4a was

isolated as an alternatively spliced product, in between exon 4 and exon 5. However, the splicing at the 3´end did not seem to be of any significance to the protein encoding region since the translation was terminated in exon 4. However, this could have an influence over the stabilisation of transcript of shrew-1 (Handrow-Metzmacher, unpublished).

Further work in this thesis has been done on the cDNA encoding the longest ORF of 411 amino acids, referred to as shrew-1.

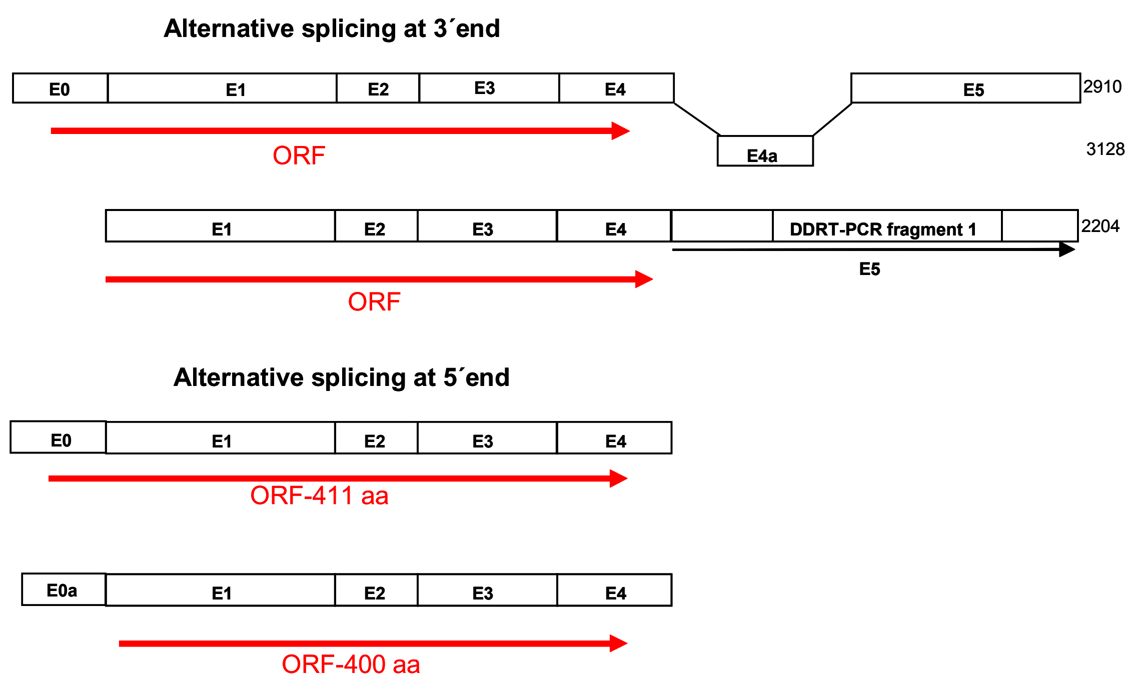


Figure 5. Alternative splicing of shrew-1 at the 5´ and at the 3´ end. The longest open reading frame (ORF) obtained consisted of 411 amino acids.

1.4.2. shrew-1 protein

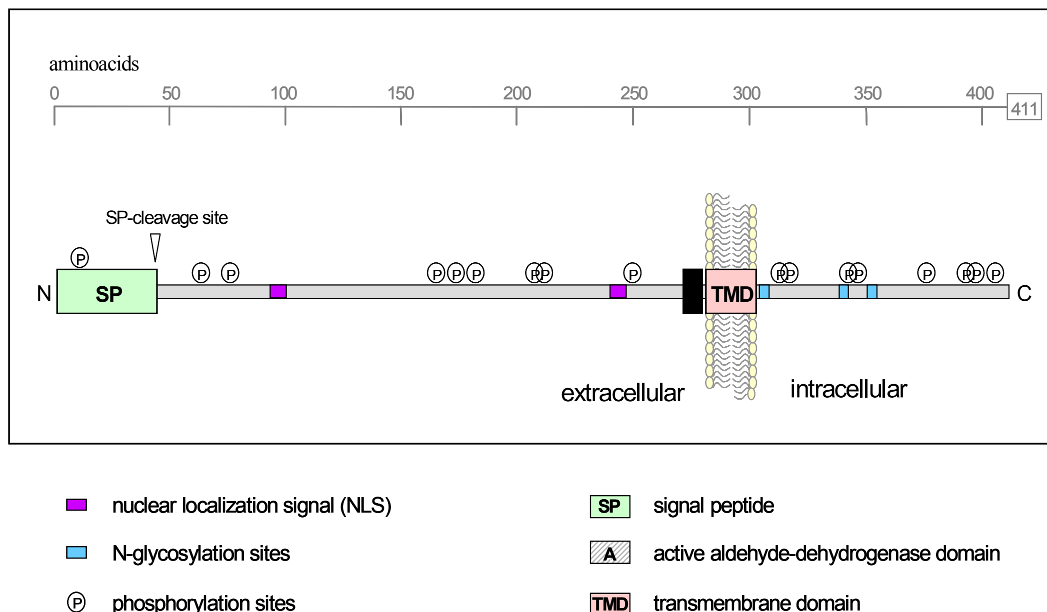
Shrew-1 was predicted by computational analysis to have a cleavable signal peptide of 43 amino acids, a transmembrane domain of 20 amino acids, some low complexity regions within the putative extracellular domain and a putative cytoplasmic domain (Fig. 6A). This points towards the evidence that shrew-1 might be a transmembrane protein (Bharti et al., in press). The relevance of this prediction will be tested further in this thesis.

The amino acid composition (Fig. 6B) of shrew-1 predicts a highly alkaline protein with an isoelectric point of 9.86 and a theoretical molecular mass of 44.5 kDa. No homology to any other known protein or to any protein domains was found (Bharti et al., in press). It seems that shrew-1 does not belong to any known protein family.

Various post translational modifications could be predicted using computer prediction programs.

These are summarised in table 2.

A



B

MWIQQLLGLSSMSIRWPGRPLGSHAWILIAMFQLAVDLPACEALGPGPEFWLL
PRSPPRPPRLWSFRSGQPARVPAPVWSPRPPRVERIHGQMQMPRARRAHRP
RDQAAALVPKAGLAKPPAAAKSSPSLASSSSSSSAVAGGAPEQQALLRRGKR
HLQGDGLSSFDSRGSRPTTETEFIAWGPTGDEEALESNTFPGVYGPTTVSILQT
RKTTVAATTTTTTTATPMTLQTKGFTESLDPRRRIPGGVSTTEPSTSPSNNGEVT
QPPRILGEASGLAVHQ*IITITVSLIMVIAALITTLVL*KNCCAQSGNTRRNSHQRKTN
QQEESCQNLTDFPSARVPSSLDIFTAYNETLQCSHECVRASVPVYTDETLHSTT
GEYKSTFNGNRPSSSDRHLIPVAFVSEKWFEISC

Figure 6. **(A)** Computational analysis of shrew-1. SP is the signal peptide constituted of 43 amino acids, TMD is the transmembrane domain, A is an aldehyde dehydrogenase domain and the various phosphorylation sites are denoted by P. The predicted orientation of the protein is that the amino terminus is extracellular and the carboxyl terminus is cytoplasmic. (**B**) The complete 411 amino acid sequence of the shrew-1 protein. The putative signal peptide is depicted in bold letters and the transmembrane domain is underlined. The cytoplasmic domain is marked in red.

Sequence motif	Amino acids	Position	
		Extracellular	Intracellular
N-glycosylation	NLTD NETL		331 351
cAMP-and cGMP-dependent protein kinase phosphorylation sites	RKTT RRNS	212–215	 314-317
Protein kinase C phosphorylation sites	SIR SFR SPR TRK TRR SAR SDR SEK	13–15 65-67 80-82 211-213	 313-315 337-339 392-394 403-405
Casein kinase II phosphorylation site	SSFD TETE TGDE STTE SSLD TTGE SSSD	165-168 176-179 186-189 251-254	 342-345 376-379 390-393
N-myristoylation site	GSRPTT GGVSTT GVSTTE GNRPSS	171-176 248-253 249-254	 386-391
Amidation site	RGKR	154-157	
Aldehyde dehydrogenases glutamic acid active site	GEASGLAV	273-280	

Table 2. PROSITE analysis of the various posttranslational modifications and their positions in shrew-1 sequence.

No orthologs of shrew-1 were found in yeast, *Drosophila* or *C.elegans*. However, a homology with an unidentified zebra fish protein was found in the putative transmembrane and the cytoplasmic domains of the protein. More than 90% homology was also found with a mouse protein (400 amino acid isoform). This led to the conclusion that shrew-1 might be required only in higher vertebrates and indeed is highly conserved in them.

1.5. AIMS OF THE STUDY

The identification and isolation of shrew-1, a novel protein from the invasive passage of the endometriotic epithelial cell line EEC145T was the first major step taken towards the understanding of the invasive phenotype of endometriotic cells. However, the identification of ESTs similar to shrew-1 sequence from other tumours such as Wilms tumours and from colon carcinomas led to the belief that shrew-1 is not an endometriosis specific protein and is also associated with other carcinomas as well. At the same time of identification of shrew-1, the group of Kudoh et al., (sequence unpublished, accession number AF175409) identified MOT8, a protein with 100% similarity to shrew-1 from invasive fibrosarcoma cells, further strengthening its association with tumors. In addition to being upregulated in some tumors, it is also present in other epithelial tissues such as brain, pancreas, kidney and spleen.

The first computer analysis predicted it to be a transmembrane protein with a signal peptide, a putative ectodomain, a strong hydrophobic segment (probably the transmembrane domain) and a putative cytoplasmic domain. Some posttranslational modifications such as phosphorylation and glycosylation were also predicted. At the same time, it was seen that shrew-1 has no homology to any other known protein or any conserved domain in the sequence. The aim of this work was to characterise this novel protein which obviously is not a member of any known protein family, making it difficult to study. In this work, shrew-1 has been characterised with respect to its cellular localisation, its orientation at the membrane, identification of its interaction partners and its possible role in invasion.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

The chemicals of analytical quality were used from the companies:

Amersham-Pharmacia, Freiburg/Breisgau

Roche, Mannheim

Biorad, München

Fluka Buchs Pharmacia LKB, Freiburg

Merck Eurolab, Darmstadt

Roth, Karlsruhe

Serva Feinbiochemika, Heidelberg

Sigma-Aldrich, Steinheim

2.1.2. Biochemicals

Biochemical	Company
Adenosinetriphosphate(ATP)	Roth
2´-deoxy-adenosine-5´-triphosphate(dATP)	Roth
2´-deoxy-cytidine-5´-triphosphate(dCTP)	Roth
2´-deoxy-guanosine-5´-triphosphate(dGTP)	Roth
2´-deoxy-thymidine-5´-triphosphate(dTTP)	Roth
4-6-Diamidino-2-phenylindol-di-hydrochloride (DAPI)	Sigma
1 kb DNA ladder size marker	Gibco BRL
7b Protein standard ladder(prestained marker)	Sigma
NBT/BCIP-Phosphate stock solution	Roche
Superfect transfection reagent	QIAGEN
Polyfect transfection reagent	QIAGEN
Biotin	Perbio

2.1.3. Stock solutions and buffers

For preparation of all stock solutions and buffers, *aqua bidest* from the "Millipore Q-Water System" (Millipore) was used. All the solutions and buffers, which could

become contaminated with nucleic acids and bacteria or which were to be used in cell culture were autoclaved (for 20 min at 121°C and 1.0 bar pressure) or were sterile filtered (Sterilfilter: 0.22um Poresize, firm Millipore)
All solutions and buffers which were used for RNA handling were made in 0.1% DEPC water. For sterility control of the medium used in cell culture, aliquots from each medium bottle were taken and were incubated for 7-10 d in the incubator and observed under the microscopic to check for any contamination.

X% Agarose solution

Xg Agarose dissolved in 100 ml 0.5X TBE by heating, not autoclaved

Ampicillin stock solution

100mg/ml Ampicillin-Sodium salt. Dissolved in water and sterile filtered. To be stored in dark at 4ºC

AP buffer

100mm NaCl

50mM $MgCl_2$

100mM Tris-Cl pH 9.5

Blocking solution

1X PBS

0.2% Tween 20

5% Skimmed milk powder

Blotting buffer for Western Blot

25mM Tris HCl, pH8.8

150mM Glycine

5% or 10% Methanol

Coomassie solution

42.5% Ethanol(v/v)

10% Acetic acid(v/v)

5% Methanol

2.5g/l Coomassie-blue(Sigma)

Coimmunoprecipitation buffer

10 mM Tris, pH 8.0

150 mM NaCl

5 mM EDTA

1% Triton X-100

60 mM n-octyl-glucoside

Dissolved in 50ml 1XPBS and added 1 tablet of complete^{TM} Protease Inhibitor cocktail (Roche)

DEPC Water

0.1% Diethylpyrocarbonat(v/v) in *aqua bidest.* Autoclaved 12 hours after adding DEPC.

DMEM

13.38 g/l DMEM-Powdered medium (4500 mg/l Glucose; Gibco)

3.7 g/l $NaHCO_3$

pH 6.8 set with 1 M HCl

sterile filtered and stored at 4ºC

DNA ladder

15 µl 1Kb DNA ladder

60 µl DNA Sample buffer

225 µl dd H_2O

DNA-Sample buffer

0.1% Bromophenolblue (w/v)

0.1% Xylencyanol FF (w/v)

10mM EDTA

40% Glycerin(v/v)

Do not autoclave

Ethidium Bromide Stock solution

10mg/ml EtBr

Unautoclaved and kept in dark , 4ºC.

Ethidiumbromide staining solution

100ul EtBr-Stocksolution

in 1000ul 0.5X TBE in dark, RT.

Fetal calf serum

Mycoplasma tested FCS was used from Sigma.

For long time storage was kept at –80ºC and for regular use was kept at 4ºC.

Freezing medium

10% DMSO

90% FCS

Hoechst 33258 stock solution (10mg/ml)

0.5 mg/ml Hoechst 33258 stored in dark at 4ºC

Hoechst 33258 working solution (2μg/ml)

Hoechst 33258 stock solution diluted 1:5000 in 1X PBS

Kanamycin stock solution

10mg/ml Kanamycin monosulphate dissolved in water and stored at -20ºC in aliquots

LB-Medium (1 L)

10g NaCl

10g Casein-Hydrolysate

5g Yeast-Extract

add 1L ddH_2O

set pH 7.0 with 5 N NaOH

LB-Agar (1 Liter)

15 g Agar

1l LB medium

Lysozyme stock solution

10mg/ml Lysozyme dissolved in water and stored at -20ºC

3 M Na-Acetate pH 5.2

Dissolve Na-Acetate in ddH_2O.

Set pH with 96% acetic acid and autoclaved

NENT Buffer

100mM NaCl

20mM Tris HCl, pH 8

1mM EDTA

0.5% NP40

sterile filtered and stored at 4ºC

PAA Gel

Resolving gel	8%	10%	12%	Stacking gel	5%
50% PAA	1 ml	1.2 ml	1.4 ml	**50% PAA**	400 µl
1.88 M Tris-HCl pH 8.8	1.2 ml	1.2 ml	1.2 ml	**0.62 M Tris-HCl pH 6.9**	800 µl
2.5% SDS	250 µl	250 µl	250 µl	**2.5% SDS**	160 µl
H_2O	3.5 ml	3.3 ml	3.1 ml	**H_2O**	2.64 ml
10% APS	30 µl	30 µl	30 µl	**10% APS**	20 µl
TEMED	5 µl	5 µl	5 µl	**TEMED**	4 µl

PAA-Gel destaining solution

40% Methanol (v/v)

10% Acetic acid

50% H_2O

1xPBS

137mM NaCl

2.6mM KCl

8.1mM $Na_2HPO_4.2H_2O$

1.5mM KH_2PO_4

Set pH 7.2-7.4 with HCl

Penicillin/Streptomycin solution

From Gibco BRL

10,000 IU/ml Penicillin(50U/ml)

10,000UG/ml Streptomycin(100ug/ml)

Aliquots stored at 4ºC

RIPA Buffer

150mM NaCl

50mM Tris, pH 7.5

0.5 % Na-Desoxycholate

1% NP 40 (v/v)

1mM EDTA

Dissolved in 50ml 1XPBS and added 1 tablet of complete^{TM} Protease Inhibitor cocktail (Roche).

1x SDS-Electrophoresis buffer

25mM Tris-HCl

250mM Glycin

0.1% SDS(v/v)

2xSDS Sample buffer

0.125 M Tris pH 6.75

4% SDS

10% β-Mercaptoethanol

20% Glycerine

STET solution

50mM Tris-HCl pH 8.0

50mM EDTA

0.5% Triton X-100(v/v)

8% Saccharose (w/v)

Sterile filtered and stored at 4C.

10X TBE

890mM Tris base

890mM Boric acid

25mM EDTA

1x TBS

150mM NaCl

10mM Tris-HCl pH 7.5

Stored at 4ºC

TFB I buffer

30 mM Potassium acetat pH 5.8

(Set pH with 2M acetic acid)

50 mM $MnCl_2$

100 mM KCl

10 mM $CaCl_2$

15 % (v/v) Glycerin

Filter sterilised and stored at 4ºC

TFB II

10 mM MOPS

75 mM CaCl2

10 mM KCl

15 % Glycerin (v/v)

TWB buffer

20 mM HEPES, pH 7.9

60 mM NaCl

6 mM $MgCl_2$

8.2% Glycerin

0.1mM EDTA

Filter sterilized and stored at 4ºC

Trypsin Solution

0.8 g NaCl

0.4 g KCl

1 g Glucose

0.58 g $NaHCO_3$

1 g Trypsin

0.2 g EDTA

upto 1l H_2O

sterile filtered and aliquots kept at 4ºC.

2.1.4. Restriction endonucleases

The Restriction endonucleases were purchased from Gibco BRL and MBI Fermentas. These were stored at –20ºC in glycerin containing buffer. They were used according to the manufacturers protocol with the delivered buffer.

2.1.5. Nucleic acid modifying enzymes

Enzyme	Characteristic	Manufacturer
Taq-DNA-Polymerase	Polymerase	Gibco BRL
Pfu-DNA-Polymerase	Polymerase	Stratagene
M-MLV Reverse Transcriptase	Reverse transcriptase	Gibco BRL

2.1.6. Plasmids

Plasmid	Reference
pGEX 5X1	Pharmacia
pGEM®-T Easy	Promega
pECFP-N1	Clontech

pEGFP-C2, -N3,	Clontech
MOM 70 E-cad	M. Rüdiger, Abt. Prof. Jockusch, TU Braunschweig
pcDNA3.1+/-	Fa. Invitrogen

2.1.7. Bacterial stocks

Stock	Reference
E.coli BL21 (DE3) pLysS	Studier and Moffat., 1986
E.coli DH5α	Bachmann, 1983

2.1.8. Cell lines

Cell line	Description	Reference
COS-7	Monkey kidney cells (SV 40 transfected)	ATCC: CRL-1651
EJ28	Human invasive Bladdercarcinoma cells	Gaetje et al., 1997
HeLa	Humane Cervixcarcinoma cells	ATCC: CCL-2.1
MCF7	Humane Breast cancer cells	ATCC: HTB-22
RT112	Humane, non invasive Bladdercarcinoma cells	Gaetje et al., 1997
MDCK	Canine kidney epithlial cells	ATCC CCl-34

2.1.9. Molecular Weight Marker

2.1.9.1. *Prestained SDS Molecular Weight Marker* SDS-7B (Sigma)

Protein	MW (Da)
α-Macroglobulin	180.000
ß-Galactosidase	116.000
Fructose-6-Phosphate kinase	84.000
Pyruvate kinase	58.000
Fumarase	48.500
Lactate-Dehydrogenase	36.500
Triosephosphate-Isomerase	26.600

2.1.9.2. 1 kb-DNA-*ladder* (Gibco BRL)

Fragment size [bp]
12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 517/506; 396; 344; 298; 201; 154; 134; 75

2.1.10. Oligonucleotides (primers)

All primers were ordered from Roth and Sigma.

1. Oligo(dT)-Primer

The Oligo(dT) Primer is used in reverse transcription for the first strand cDNA synthesis .It hybridises with the poly(A)$^{+}$ tail of every mRNA.

Primer	Nucleotide sequence (5´–» 3´)
Oligo(dT)-*Primer*	TTTTTTTTTTTTTTTTTTTTTTGGATCCC

2. BiP-specific Oligonucleotide

The successful cDNA-first strand synthesis can be checked with the help of BiP-specific oligonucleotides. These oligonucleotides amplify a PCR fragment of 561bp from the household gene. In case of contamination, an additional band of approximately 1.2 kb can be detected with these primers.

Primer	Nucleotide sequence (5´–»3´)
BiP 5´	TACACTTGGTATTGAAACTG
BiP 3´	GGTGGCTTTCCAGCCATTC

3. Oligonucleotides for cloning different constructs

Primer	Nucleotide sequence (5´–»3´)
trkA-Kpn	CGG***GGTACC***ATGCTGCGAGGCGGACGGCG
trkA+Fragoh	GACAGCCAGACCTTCGTCCTTCTTCTCCACCG
oh-cd-Frag	CTTGTGCTCAACTGCTGTGCCCAAAGCGGG
oh-cpdfrag	ATACTGAGC***AAGCTT***CAAATGGGGAACACTCGT CGGAACAGCC
bp-cpdfrag	CGGGCCC***TCTAGA***TTTCCTGAGGCTTAAACTGTGGG
gfp-cpdfrag	CGGGCCCGC***GGTACC***GCAGGAGATTTCAAACC
FP-Cav-EcoR1	ATC***GAATTC***ATGTCTGGGGGCAAATAC
RP-Cav-Sal1	GGCTCGA***GTCGAC***TTATATTTCTTTCTG
FP-Frag-SP	TAGGCTTC***GAATTC***TATGTGGATTC
FP-ED-ohXP	TACGACGATGACGATAAGCTGGGCCCGGGG
RP-SP-ohXP	GTCATCGTCGTACAGATCGGCCTCACAGGCGGGCAGGTC
FragGST-1	ACAACTCTT***GAATTC***AAAAATTGCTGTGCC
FragGST-2	GCTATA***GTCGAC***CAGTCAGCAGGAGATTTCAAA
FP-Frag-TMD	TCC***GAATTC***ATGGCTGTCCATCAG

FP-TCD-HindIII	TTTT***AAGCTT***ATGTTCGGTCTGGCTGTC
RP-FragXba1	CCGACAAGATG***TCTAGA***TCAGCAGGAG
RP-FragED	GGC***GGTACC***CTGATGGACAGCCAG

2.1.11. Plasmid constructs

Construct	Vector	Restriction site	Primers	Description
trk-GFP	pEGFP-N3	Kpn1	trkA-Kpn gfp-cpdfrag	Ectodomain of trkA, transmembrane and cytoplasmic domain of shrew-1. Linear amplification.
trk-BP	pcDNA 3.1(+)	Kpn1, Hind111	trkA-Kpn bp-cpdfrag	Same as above
Frag-XP-GFP	pEGFP-N3	EcoR1, Acc651	FP-Frag-SP FP-ED-ohXP FP-ED-ohXP RP-SP-ohXP	Express tag after signal peptide, c-terminus GFP tag. Linear amplification.
CPD-GFP	pEGFP-N3	Hind111, Acc651	oh-cpdfrag gfp-cpdfrag	Cytoplasmic domain of shrew-1 fused to GFP tag
CPD-BP	pcDNA 3.1(+)	Hind111, Xba1	oh-cpdfrag bp-cpdfrag	Cytoplasmic domain of shrew-1 fused to BP tag
ED-GFP	pEGFP-N3	EcoR1, Acc651	FP-Frag-SP RP-FragED	Ectodomain of shrew-1 fused to GFP tag
NTD-GFP	pEGFP-N3	EcoR1, Acc651	FP-Frag-SP RP-FragNTD	Ectodomain and the transmembrane domain of shrew-1 fused to GFP tag
TCD-GFP	pEGFP-N3	EcoR1, Acc651	FP-Frag-TMD, gfp-cpdfrag	Transmembrane domain and the cytoplasmic domain of shrew-1 fused to GFP tag
GST-CPD-Frag	pGEX-5X1	EcoR1, Sal1	FragGST-1, FragGST-2	Cytoplasmic domain of shrew-1 fused to GST tag
GST-cav	pGEX-5X1	EcoR1, Sal1	FP-Cav-EcoR1, RP-Cav-Sal1	Caveolin-1 fused to GST tag for bacterial expression.

2.1.12. Antibodies

1. Primary Antibodies (Monoclonal)

Antibody	WB	IP	IF	Reference
Mouse -α-PAN-cadherin		1:1000	1:100	Sigma
Mouse-α-A-Cam			1:100	Sigma
Mouse -α-E-cadherin 5H9		1:1000	1:40	Monosan
Rat-α-E-cadherin DECMA	1:500	undiluted	1:1000	Sigma
Mouse-α-E-cadherin, cl 36	1:2500	1:1000		Transduction Laboratories
Mouse-α-β-catenin, cl 14	1:500		1:100	Transduction Laboraties
Mouse-α-GFP	1:1000	1:500	1:200	Clontech
Mouse-α-Birch profilin	1:500		1:1000	M. Rüdiger, Abt. Prof. Jockus TU Braunschweig
Mouse-α-N-cadherin, cl3B9	1:1000			Zymed
Mouse-α-Pyruvate Kinase	1:10			Schebotech
Mouse-α-trkA	1:200			Calbiochem

WB= Western blot; IP= Immunprecipitation; IF= Immunfluorescence

2. Secondary Antibodies (polyclonal)

Antibody	Dilution	Reference
Goat -α-Rabbit IgG (H+L) Alkaline Phosphatase-conjugated	1:7500 WB	Jackson
Goat-α-Maus IgG (H+L) Alkaline Phosphatase-conjugated	1:7500 WB	Jackson
Goat-α-Mouse IgG IgG (H+L) Peroxidase-conjugated	1:7500 WB	Sigma
Cy3™-conjugated F(ab´) 2 Fragment Goat-α-Mouse IgG (H+L)	1:200 IF	Jackson
Alexa flour™ 594 goat-α-mouse IgG(H+L) Red	1:400 IF	Molecular Probes
Alexa flour™ 488 goat-α-mouse IgG(H+L) Green	1:400 IF	Molecular Probes
Alexa fluor™594 goat-α-Rabbit IgG (H+L) Red	1:400 IF	Molecular Probes
Alexa fluor™594 goat-α-Rabbit IgG (H+L) Green	1:400 IF	Molecular Probes
Goat-α-Rabbit IgG (H+L) Alexa Fluor™594 (red)	1:400 IF	Molecular Probes

2.1.13. Kits and Transfection Reagents

CONCERT™ High Purity Plasmid Midiprep System (Gibco BRL, Karlsruhe)

CONCERT™ High Purity Plasmid Miniprep System (Gibco BRL, Karlsruhe)

CONCERT™ Rapid Gel Extraction System (Gibco BRL, Karlsruhe)

Effectene™ Transfection Reagent (QIAGEN, Hilden)

PolyFect® Transfection Reagent (QIAGEN, Hilden)

QIAquick® PCR Purification Kit (QIAGEN, Hilden)

TNT® Coupled Reticulocyte Lysate Systems (Promega)

2.2. Methods

2.2.1. DNA analysis

Restriction digests were performed according to the manufacturers protocol (MBI fermentas). DNA fragments were purified by "Rapid Gel extraction kit" from Gibco BRL, Germany. PCR was performed using Pfu Polymerase (Promega) and Taq polymerase (Gibco). Ligation was performed with T4-DNA Ligase (MBI fermentas, Germany) according to the manufacturers protocol. Standard procedures were used for transformation and amplification in E.coli strain DH5α (Sambrook and Russell 2001).

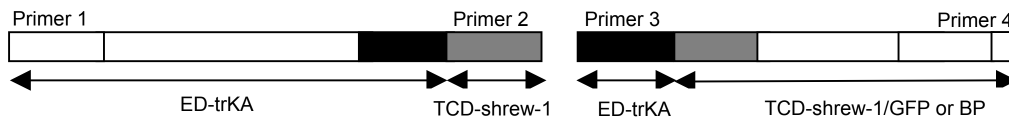
For the quick confirmation of the ligation for cloning, the quick preparation from Holmes and Quigley, 1981 was used. This is a very quick and easy, but unclean method for the isolation of plasmid DNA (high copy number) and gives a yield of 3-5μg from 1 ml of bacterial culture. For the purpose of sending the plasmids for sequencing to sequencing labs plasmid DNA was prepared from the CONCERT™ Rapid Plasmid Miniprep Systems from Gibco BRL. For transfection in eukaryotic cells or for use in higher amounts, the plasmid DNA was prepared from CONCERT™ High Purity Plasmid Midiprep Systems from Gibco BRL.

2.2.1.1. Expression constructs

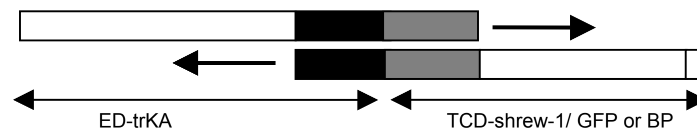
Cloning of shrew-trkA chimaeras

The transmembrane domain and the cytoplasmic domain of shrew-1 was fused to the ectodomain of *transforming tyrosine kinase protein* (trkA) (Sachs et al. 1996). The receptor trkA binds with a very high affinity to its ligand-*nerve growth factor* (NGF, Review: Barker and Murphy 1992). This fragment was synthesized in a three-step PCR involving linear amplification.

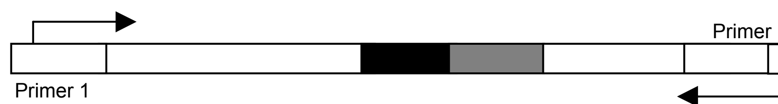
1. PCR amplification of ED-trKA and TCD of shrew-1/ GFP or BP



2. Linear amplification



3. Reamplification



In the first step of PCR amplification, the extracellular domain of trKA (Primer 1 and 2) and the transmembrane and cytoplasmic domain (Primer 3 and 4) were amplified. The primers were designed that the fragments could be hybridised in the second PCR step. For this, the primer 2 from trKA (ED-trKA) consisted at its 3´end sequences complementary to the 5´end sequence of TM-CPD-shrew-1 and reversely, the primer 3 from TM-CPD-shrew-1 contained sequences complimentary to the 3´end of ED-trKA. In this second amplification step, the fusion product trKA-shrew GFP or BP was linearly amplified. This fragment was purified on agarose gel and was reamplified in the third step using primers 1 and 4, the primer 4 was either from GFP tag or from BP tag. The resulting product was cloned into pEGFP-N3 vector containing a GFP tag or into pcDNA 3.1(+) vector containing a BP tag.
The other expression constructs of shrew-1 and caveolin-1 were generated by subcloning via PCR. All the clones were sent for sequencing to Sequiserve, Vaterstatten and were analysed using Immunofluorescence, Western blot or by *in vitro* translation.

2.2.1.2. RT-PCR analysis

To check for the presence of shrew-1 in different epithelial cell lines, RNA was prepared using QIA-RNA midiprep kit (QIAGEN). This RNA was stored in a precipitated form with 2 volumes absolute ethanol and 1/3 volume of 8M ammonium acetate. From this RNA, 20 µg total or 1µg poly A(+) RNA was taken and was centrifuged for 15 min at 15,000 rpm at 4ºC. The supernatant was removed and the RNA was washed with 70% ethanol for 10 min at 15,000 rpm at 4ºC. The supernatant was removed and the pellet was airdried and then dissolved in 12.5 µl water and was vortexed well for 2min. To this pellet, 5 µl Oligo (d)T-primer(3uM) was added and denatured for 10 min at 70°C and during this time vortexed 4 times. Kept on ice and to this added:

6ul 5x reverse transcriptase
3ul 0.1M DTT
1.5ul dNTP-Mix (10mM each)

This mixture was then incubated at 37°C for 2 min. To this was added 2ul M-MLV Reverse Transcriptase (200 U/µl, Gibco BRL) and incubated at 37°C for 1 hour. The enzyme was inactivated by incubation at 95°C for 5 min.
PCR amplification of this single-stranded DNA was done using primers from the sequence of shrew-1 cDNA. As an internal control to detect for any genomic contamination and to check the quality of the cDNA, primers from BiP, a housekeeping gene were used.

2.2.2. Protein Analysis

Protein analysis by SDS-polyacrylamide gel electrophoresis and Western blotting were done as described previously (Kaufmann et al., 2000). For immunoblot analysis proteins were transferred to nitrocellulose membrane (Hybond Amersham, Germany) and further processed for detection using NBT/BCIP following the manufacturers protocol (Roche). The commercial antibodies used are described above in the materials section.

2.2.2.1. Generation of antibodies against shrew-1

Monoclonal antibodies were generated against shrew-1 in mice using the peptide sequence NH_2-ACMTLQTKGFTESLDPRRRIPGGVS–amide by Nanotools, Teningen, Germany. The detection of endogenous protein in western blot was done using a 1:20 dilution and in immunohistochemistry using a dilution of 1:10
Polyclonal antibodies were generated against the cytoplasmic domain of shrew-1 in rats in collaboration with Genovac, Freiburg, Germany. The working dilution for the western blot was 1:200 and for immunofluorescence a dilution of 1:100 was used.

2.2.2.2. Protein interaction studies by *in vitro* binding assays using GST fusion proteins

1. Expression of recombinant proteins in Bacteria

Recombinant proteins were expressed in E.coli strain BL21 (DE3) pLysS as GST-fusion proteins encoded by pGEX-5X1 vector into which the cDNA of the respective protein was cloned. Expression and enrichment was done with glutathione Sepharose beads (Pharmacia) according to the following protocol:

- 20 ml of LB Amp was inoculated with the respective plasmid and kept on shaker overnight at 37ºC and 220rpm.

- Added 180ml LB-Amp the next day and incubated again for 1h under the same conditions.

- Added 0.2mM IPTG in the medium to induce *lac*-promoter of the vector. The shaking was further continued for approx. 3h.

- All the further steps were performed at 4ºC with precooled solutions.

- After the end of the incubation period, the bacteria were centrifuged at 4,000 rpm for 15 min and the pellet suspended in 5 ml NENT buffer containing 1mM DTT.

- The cells were lysed by three repeated freeze and thaw cycles with snap freezing in liquid nitrogen and thawing at 37ºC. After the last freeze and thaw cycle, 5µl of Benzonase (Merck) was added and incubated for 10min at 37ºC.

- The insoluble fraction consisting mainly of cellular debris was removed by centrifugation at 12,000 rpm for 30 min.

- The supernatant was mixed with 10% glycerol and was aliquoted into 1 ml fractions and was stored at -80ºC.

2. Purification of GST-Fusion proteins

Glutathione-Sepharose 4B Beads were washed in a 15ml falcon tube using NENT buffer at 500rpm for 5 min. The beads were then stored at 4ºC in a 1:1 slurry with NENT buffer. To a single aliquot of the bacteria extract, 20 µl of the beads were added and were rotated at 4ºC on a rotor for 1h. The Glutathione-sepharose beads bind with a very high affinity to the GST tag on the protein and formed an immobilised complex which consisted only of the GST tagged protein bound on the beads. This complex was then sedimented at 800rpm for 5 min and the supernatant was discarded. The pellet was washed 3 times with 1 ml of NENT buffer (-DTT). The washed pellet was then boiled with adequate volume of Laemmli buffer at 95ºC for 5 min and an SDS-PAGE was performed. The gel was finally stained with coomassie-blue and was controlled for the protein expression and the quality of the purified protein for further interaction studies.

3. *In vitro* Transcription and Translation

For a pull down assay, it is required that one of the proteins used for testing the interaction is purified on an immobilised substrate and the other exists in a detectable form not necessarily immobilised. For this, the *in vitro* transcription and translation of shrew-1-BP which was cloned into pcDNA 3.1(+) with a BP tag was performed. This was done using *in vitro* Transcription and Translation system (TNT®, Promega). Radioactivity was incorporated into the shrew-1 protein by using ^{35}S-methionine for easy detection of the protein using autoradiography. The procedure was performed according to the manufacturers instructions.

4. Pull down assay for testing protein-protein interaction

To detect an in vitro interaction between two proteins, Glutathione-Sepharose 4B beads coupled to the protein of interest were incubated with in vitro translated ^{35}S-methionine labelled protein-in this case shrew-1. In case of an interaction, both the proteins co-precipitated on the beads which could be detected by separating the proteins by SDS-PAGE and detecting the radioactive protein by autoradiography.

The GST fused protein was immobilised on the beads by adding the Glutathione Sepharose Beads and incubating on a rotor for 45 min in the cold room. After that, the beads were blocked for 20 min with 20% skimmed milk powder dissolved in NENT buffer. Beads were then washed twice with NENT buffer and once with TWB (+1mM DTT) incubation buffer at 800 rpm for 5 min. 100 µl of TWB (+1mM DTT) was added to the beads and to this 3-5 µl of the radioactively labelled translate was added. The mixture was then incubated for 2h at RT or overnight at 4ºC.

The beads were then washed 5 times with NENT buffer at 1000 rpm for 5 min each. The supernatant was discarded and the pellet was boiled with an adequate amount of Laemmli buffer and loaded on an SDS polyacrylamide gel. The gel was then dried and was subjected to autoradiography by which the ^{35}S labelled protein could be detected in case of an interaction occurring between the two proteins. As a negative control, the vector alone containing only the GST tag was taken.

2.2.2.3. Analysis of *in vivo* interaction by coimmunoprecipitation

It is a method to detect complexing between different proteins. Cells were washed twice with ice cold PBS and lysed for 30 min at 4°C in a buffer containing 10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 60 mM n-octyl-glucoside. Samples were precleared for 1 h at 4°C using protein G-sepharose (20 µl, 1:1) and subjected to immunoprecipitation overnight at 4°C using anti-GFP IgG (10 µl, mAb), anti-E-cadherin (5 µl, mAb 5H9), anti-Pan-cadherin (3 µl) and protein G-Sepharose (30 µl, slurry 1:1). After 4-5 washes with the immunoprecipitation buffer, samples were separated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose. Immunoblotting was performed as described.

2.2.3. Eukaryotic cell culture

2.2.3.1. Maintenance of cells

The cell lines MDCK, MCF7, Cos7, 12Z, EJ28 and RT112 were maintained in the growth medium containing DMEM (Dulbeccos modified eagle medium) with 10% FCS (Fetal calf serum) and 1% Penicillin-Streptomycin solution. The cell line PC12 was maintained in DMEM with 10% horse serum, 5% FCS and 1% penicillin-Streptomycin solution. All these cell lines were kept in an incubator with 10% CO_2 and 95-99% relative humidity at a temperature of 37°C.

2.2.3.2. Induction of PC12 and MDCK cells

The PC12 cells containing trkA were induced using Nerve Growth Factor (NGF) at a concentration of 100ng/ml. The effect was visible after 1h.

The MDCK cells were scattered using Scatter factor/Hepatocyte Growth factor (SF/HGF) at a concentration of 20 ng/ml. A visible effect could be seen after 3h of induction.

2.2.3.3. Transfection of Eukaryotic cells

Cos7, MCF7, RT112 and EJ28 and 12Z were transfected using PolyFect^{TM}. SuperFect^{TM} was used for the transfection of 12Z cells, whereas Effectene^{TM} was used for MDCK cell transfection. The procedure given in the manual from QIAGEN was followed.

2.2.3.4. Immunoflourescence of transfected cells

-The medium was removed from the cells and the cells were washed with 1x PBS.

-Cells were fixed on the cover slips using 4% Paraformaldehyde at room temperature for 10 min.

-Cells were permeabilized using 0.2% Triton X (in PBS) for 10 min at RT.

-Blocked the cells using 10% FCS and 0.05% Na-Azide at least for 50 min.

-Added the primary antibody diluted with 10% FCS in PBS with 0.05% Na-Azide and kept at RT for 1 hour.

-Washed the cells 3 times short and 3 times for 5 min with 1XPBS.

-Added the fluorescent secondary antibody diluted like the primary antibody and kept in dark at RT for 1 hour.
-Washed 3 times short with PBS and added DAPI for 5 min at RT.
-Washed 2 times for 5 min with PBS.
-Mounted the cover slips on a glass slide using flouromount.
-Dried at least for 60 min before observing under the microscope.

2.2.3.5. Immunofluorescence with frozen sections

Was performed similar to the transfected cells except the fixation/permeabilization was done with chilled methanol at -20ºC for 20 min.

2.2.3.6. DAB staining (Immunohistochemistry) of tissue sections

- Fixation was done with chilled methanol at -20ºC for 20 min
- 2x 5 min with 1XPBS
- Blocked for 30 min at RT with 10% FCS/ 0.05% azide in 1x PBS
- Washed 3x with TBS (0.05 M Tris pH 7.2-7.6 / 0.3 M NaCl/ 0.1% Tween 20)
- Blocked for 5 min with peroxidase
- Washed 3X short with TBS
- Circled the tissue area with a fat marker
- Incubated with primary antibody overnight at 4ºC
- Washed 3x short and 3x 5 min each with TBS
- Secondary antibody added for 30 min
- Washed 3x short and 3x 5 min with TBS
- Incubated with stain solution (1ml of bottle 3a+ 1 drop of bottle 3b) till stain precipitates
- Washed with ddH_2O and wiped out dry
- Counter stained with Mayer´s Haematoxylin (2 min) (Sigma)
- Washed 2x ddH_2O
- Slide was then mounted with flouromount

2.2.3.7. Generation of stable cell lines

To select for cells expressing the transfected constructs stably, Neomycin Sulfate-G418 (Gibco BRL, Germany) was used for the selection process. The transfected plasmids had neomycin resistance gene, so the cells, which had incorporated the transfected plasmid into the genome, could survive the selection pressure, which was applied by adding G418 into the medium. G418 was optimized at a concentration of 0.6 mg/ml for MDCK cells and for MCF7 cells a concentration of 0.4 mg/ml was used.

2.2.3.8. Lef-Luciferase reporter assay

MDCK cells stably expressing shrew-GFP or GFP alone were seeded in 6-well plates at 300,000 cells/well. The following day, cells were transiently transfected using the Effectene® Transfection reagent (Qiagen, Hilden, Germany). For this, 0.4 µg of reporter gene plasmid DNA (TOPFLASH, FOPFLASH: Upstate Biotechnologies, Lake Placid, USA; UAS-5x-tk-Luc: Baniahmad et al., 1995) was used. Each transfection was carried out in triplicate. Luciferase activities were measured 24 h after transfection using the Luciferase Assay System (Promega, Mannheim, Germany).

2.2.3.9. Permeablisation assay

For the antibody permeabilisation assay, MCF7 cells were transfected with shrew-1-GFP and were grown till confluency. Cells were either fixed with 4% PFA or left unfixed (living samples). IF was performed as described before.

2.2.4. Confocal laser scan microscopy (CLSM)

Visualisation by CLSM was done as described before (Kaufmann et al., 2000). Image processing of 3D-data sets was performed on a Silicon Graphics (Mountain View, CA, USA) (workstation using 'Imaris' Bitplane AG, Zurich, Switzerland) 3D multi-channel image processing software (Messerli et al., 1993). Images were further processed using Adobe Photoshop.

2.2.5. Surface biotinylation

The cell surface of confluent monolayers was labelled on ice with 0.5 µg/ml membrane-impermeable EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) in PBS, pH 9.0. After quenching (50 mM ammonium chloride in PBS, 0.1 mM CaCl2, 1 mM $MgCl_2$) the cells were lysed in 0.5 ml of RIPA buffer (150 mM NaCl,50 mM Tris pH 7.5, 0.25% sodium dodecyl sulfate, 0.1% Nonidet P-40) plus the protein inhibitor cocktail Complete (Roche) for 10 minutes of incubation at 4ºC. Protein of each lysate was used for precipitation (16h at 4ºC) with 30 µl of Neutravidin beads (Pierce). The precipitates were washed as described previously (Rajasekaran et al., 1999) and an immunoblot was performed (Kaufmann et al., 2000).

3. RESULTS

3.1. Expression analysis of shrew-1 protein

Since no homology could be detected between shrew-1 and any other known protein/domains, the characterization of this protein was started from the very beginning.

3.1.1 Endogenous expression analysis

Two different types of antibodies were generated to analyse the endogenous expression of shrew-1 at the protein level. First, custom-made mouse monoclonal antibodies were produced against a synthetic peptide deduced from the putative extracellular domain (for sequence see Materials and Methods). The resulting monoclonal antibodies were first tested in tissue sections from pancreas and uterus (Fig. 7A), since both these tissues were found to contain shrew-1 mRNA as shown by Northern blot and RT-PCR analysis (Handrow-Metzmacher, unpublished). Additionally, an immunoblot was performed with cell extracts from pancreas and uterus (Fig. 7B, lanes 1 and 2, respectively). Both tissues were found to contain a protein of approximately 45 kDa corresponding to the predicted size of the shrew-1 protein. Shrew-1 polyclonal antibodies raised in rats against the putative cytoplasmic polypeptide sequence by genetic immunization (in collaboration with Genovac, Freiburg, Germany) gave a signal of comparable size in these cell extracts (Input, Fig. 7B; lane 7).

To test for the authenticity of the shrew-1 band detected in immunoblots, immunoprecipitation (IP) was performed with monoclonal antibody and detection with the polyclonal antibody and vice-versa (Fig. 7B; lanes 4-5; 7-8). In both the cases a band of approx 45 kDa, the expected size of shrew-1 was detected. This confirmed the specificity of the antibodies since both the antibodies were generated against different peptides from the shrew-1 sequence.

But unfortunately, none of the antibodies seemed to detect endogenous shrew-1 in the cell lines. Therefore, in further experiments shrew-1 was fused to a tag against which antibodies were available.

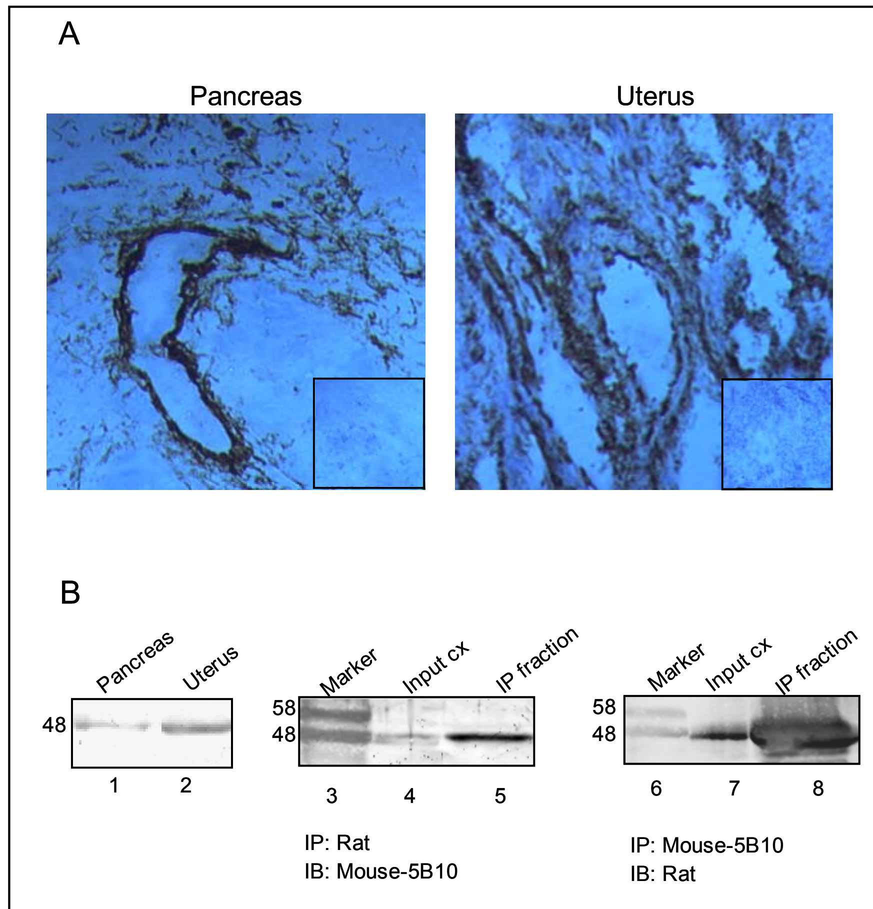


Figure 7. Shrew-1 antibody detects endogenous protein. **(A)** To detect the endogenous protein, pancreas and uterus cryosections were used and DAB staining was performed. Endogenous protein (stained in brown) could be detected in parts of these sections. The small window at the bottom right depict the secondary antibody control.**(B)** To check for the size of the protein, an immunoblot was performed with these supernatants using cell extracts from pancreas and uterus (lane 1 and 2, respectively). A protein of approximately 45 kDa was detected in both these cell extracts. Immunoprecipitation of endogenous shrew-1 from uterus cell extracts using shrew-1 polyclonal antibody and detection with the monoclonal antibody and vice versa was done to confirm the specificity of the shrew-1 band. Lane 4 depicts the input cell extract and lane 5 shows the immunoprecipitated protein detected with the monoclonal antibody. Lane 7 depicts the input cell extract and lane 8 depicts the immunoprecipitated protein detected with the polyclonal antibody. Input is 10% of the total cell extract. Lanes 3 and 6 depict the marker proteins.

3.1.2. Ectopic expression analysis

For performing experiments using ectopic shrew-1, its cDNA was cloned into two

different expression vectors containing either a 10 aa long birch profilin tag (shrew-1-BP) or a green fluorescent protein tag (shrew-1-GFP). To check whether these vectors expressed the predicted open reading frame protein of 411 aa, shrew-1-BP was translated radioactively *in vitro* using a reticulocyte lysate kit (see Materials and Methods). SDS-PAGE and autoradiography revealed that shrew-1 could indeed be translated *in vitro* to produce a protein of approximately 45 kDa (Fig. 8; lane 2). The positive control used for *in vitro* translation was luciferase cDNA supplied by the manufacturer (Fig. 8; lane 1). For detection of ectopic shrew-1, shrew-1-GFP was transfected in MCF7 cells and the cell extracts were blotted with anti-GFP antibody that detected a protein of the expected size of approximately 75 kDa in immunoblots (Fig. 8; lane 3).

Shrew-1 polyclonal antibody raised in rats against the putative cytoplasmic polypeptide sequence also gave a signal of comparable size in cell extracts of MCF7 transfected with shrew-1-GFP (Fig. 8; lane 4). The polyclonal antibody also detected ectopic shrew-1-GFP as shown in Fig. 8B. Shrew-1-GFP was transfected transiently in MCF7 cells (Fig. 8B; a-c) and subsequent immunostaining was performed with the preimmuniserum (PIS) (Fig. 8B; b) and with immune serum (IS) raised in rats against shrew-1(Fig. 8B; d). The IS specifically detected the ectopically transfected shrew-1-GFP as observed with the GFP autofluorescence.
Thus, the predicted shrew-1 aa sequence can translated in mammalian cells as confirmed by antibodies raised against putative ORFs.

3.2. Cellular localization of shrew-1

3.2.1. Shrew-1 is an integral membrane protein

Shrew-1 fused to two different tags (to rule out the possibility that cellular localization is affected by the tags) was used to determine the cellular localization of the protein. These studies were performed in epithelial cell lines 12Z, RT112, EJ28 and MCF7 transiently transfected with shrew-1-GFP and shrew-1-BP. In all cases, major pools of shrew-1 appeared to be localized at the plasma membrane, especially at the regions of cell-cell contact, irrespective of whether RT-PCR (preliminary analysis) showed that the cell lines contained endogenous shrew-1,

namely MCF7 and 12Z (Fig. 9A; a, d, e, h) or not, i.e. RT112 and EJ28 (Fig. 9A; b, c, f, g).

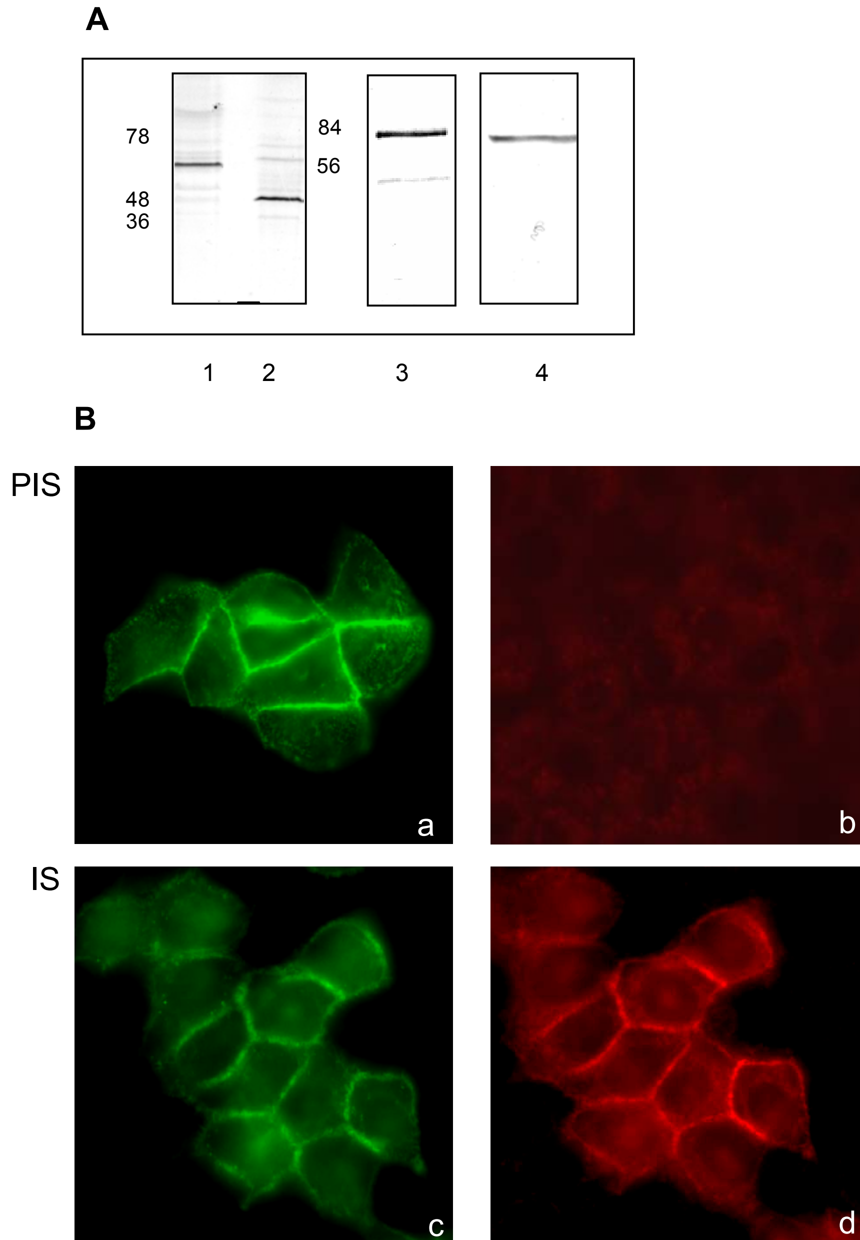


Figure 8. Detection of ectopic expression. **(A)** shrew-1 was translated *in vitro* in the presence of radioactive ^{35}S (lane 2) and was detected by autoradiography on SDS-PAGE. Lane 1 depicts the positive control-Luciferase. Lane 3 shows shrew-1-GFP detected by a monoclonal antibody against GFP and lane 4 depicts shrew-1-GFP detected by polyclonal shrew-1 antibody. Shrew-1-GFP was transfected in MCF7 cells and the immunoblots were performed with the cell extracts from these cells. **(B)** MCF7 cells transfected with shrew-1-GFP were stained with the polyclonal antibody against shrew-1. Preimmune serum (PIS) was used as a specificity control. No signal could be detected with the PIS (upper panel, b) but the immune serum (IS) detected a signal for the transfected protein (lower panel, d).

To analyse biochemically the presence of shrew-1 at the membrane, surface biotinylation experiments were done. For this, membrane impermeable surface biotin was used which binds to membrane proteins via its binding to primary amines. The strong interaction between biotin and avidin was applied to pull down the proteins bound to biotin at the membrane.

For this, neutravidin (a form of avidin) was coupled to agarose beads and these beads were then added to the cell extracts containing biotinylated proteins.

Shrew-1-GFP was transiently transfected into MCF7 cells. Surface-exposed proteins were then selectively biotinylated using membrane-impermeable biotin. After cell lysis and detergent solublization, biotinylated proteins were isolated by incubation with agarose-coupled neutravidin beads. Immunoblotting using antibodies against GFP revealed that shrew-1-GFP was present in the biotinylated protein fraction (Fig. 9B; lanes 2-6) confirming that shrew-1 is an integral component of the plasma membrane. E-cadherin, a transmembrane protein (Fig. 9B, lanes 8-11) and pyruvate kinase, a cytosolic protein (Fig. 9B, lanes 13-16) were used as positive and negative controls, respectively.

From these data it could be concluded that shrew-1 is a transmembrane protein with at least a part of it exposed at the cell surface. Further studies in this direction were done using MCF7 and MDCK cells since they are epithelial in nature and shrew-1 was isolated from an epithelial cell line and is found to be present mainly in tissues of epithelial origin. Additionally, RT-PCR done with two different set of primers, showed that shrew-1 mRNA is present in both these cell lines (Fig. 10).

3.2.2. The carboxyl terminal of shrew-1 is cytoplasmic

To test the hypothesis that the putative carboxyl terminus of shrew-1 is cytoplasmic (from computational analysis), permeabilisation studies were done. MCF7 cells were transiently transfected with shrew-1 tagged with a C-terminal GFP tag (shrew-1-GFP). One aliquot of the transfected cells was permeabilized (Fig. 11; a, b) and immunodetection was performed using GFP antibody (Fig. 11; b, d) whereas the other aliquot was not permeabilized (Fig. 11; c, d) and immunostaining was

performed on live cells using GFP antibody in the presence of Sodium Azide to prevent antibody-induced capping.

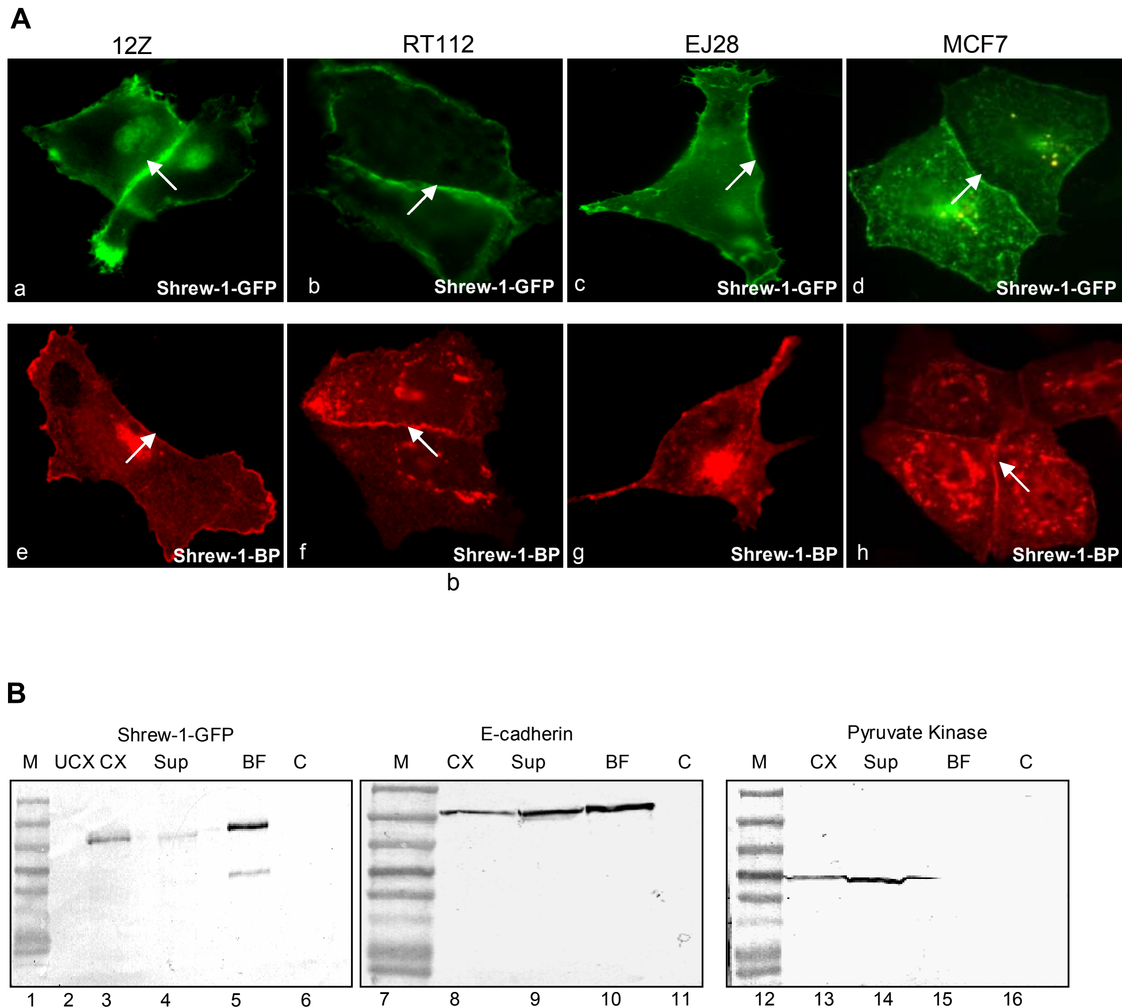


Figure 9. **(A)** Membrane localization of shrew-1. Shrew-1 tagged with GFP (shrew-1-GFP) or BP (shrew-1-BP) was expressed in the eukaryotic epithelial cells: 12Z (human invasive endometriotic cell line), RT112 (human bladder carcinoma cell line, non-invasive), EJ28 (human bladder carcinoma cell line, invasive) and MCF7 (human breast carcinoma cell line, non-invasive). a-d show shrew-1-GFP fluorescence and e-h show immunofluorescence signals using a mouse monoclonal antibody against the BP tag visualized by a mouse-specific fluorochrome-conjugated secondary antibody. The arrows indicate the expression of shrew-1 at cell-cell contacts.
(B) Cell surface biotinylation of MCF7 cells transfected with shrew-1-GFP. The biotinylated cell surface proteins were pulled down with neutravidin-coupled beads. The proteins present in various cell extract fractions were analysed by Western blots. Shrew-1-GFP was detected by anti-GFP antibody (lanes 2-6). E-cadherin, a positive control protein, was detected by a monoclonal antibody against E-cadherin (lanes 8-11) and Pyruvate kinase, a negative control cytosolic protein (lanes 13-16), was detected with a monoclonal antibody. UCX: untransfected cell extract, CX: transfected cell extract, sup: supernatant after pull-down of the biotinylated fraction, BF: pulled down biotinylated fraction, C: control of neutravidin beads bound to non-biotinylated cell extract.

The auto fluorescence from shrew-1-GFP (Fig. 11; a, c) could be seen in both cases, but antibody staining could only be seen with cells that were permeabilized. This clearly implies that the C-terminus is indeed cytoplasmic. A comparable result was obtained when a similar experiment was performed in MDCK cells (preliminary data). Unfortunately the orientation of the amino terminus could not be determined due to the lack of antibodies against the amino terminus of the protein as well as due to the fact that tagging shrew-1 at the amino terminus was not feasible since its expression as an N-terminus could not be detected.

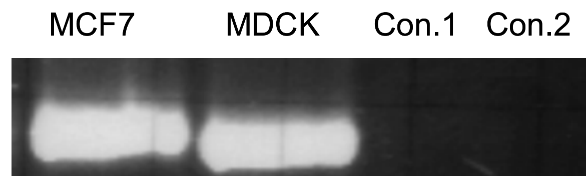


Figure 10. RT-PCR done with different primers from shrew-1 revealed the presence of shrew-1 in both MCF7 and MDCK cell lines. Con.1 and Con.2 are the negative controls performed for the two different primer sets. For primers, see Materials and Methods.

To overcome this problem, it was attempted to clone a small tag in shrew-1-GFP after the putative signal peptide which was thought to be cleaved off and to perform similar studies with this. Express tag comprising of 8 amino acids was selected for this purpose and was cloned via linear amplification after approx. the first 50 amino acids (aas), a region after the signal peptide which is composed of 43 aas. This cloning was done in shrew-1-GFP which contains a GFP tag at the C-terminus. The construct was called shrew-XP-GFP.

This construct was transfected transiently into MCF7 cells and the cells were either permeabilized in one set or were kept non-permeabilized in the other set as shown in Fig. 12. Anti-express antibody was used to detect this construct in permeabilized (Fig. 12; a, b) and non-permeabilized (Fig. 12; c, d) cells but the expression was not seen in either case. The autoflorescence from GFP could be detected in both the cases (Fig. 12; a, c) leading to the conclusion that though the construct is expressed in a similar manner as the full length shrew-1, a tag added to the ectodomain could not be detected (Fig. 12; b, d). This could mean that either the ectodomain is secreted or that the confirmation of the protein does not allow the exposure of the express tag to its antibody.

It was not possible to draw any conclusion about the orientation of the putative ectodomain form the experiments performed till now. Further attempts were then made to functionally characterise the transmembrane and cytoplasmic domains of shrew-1.

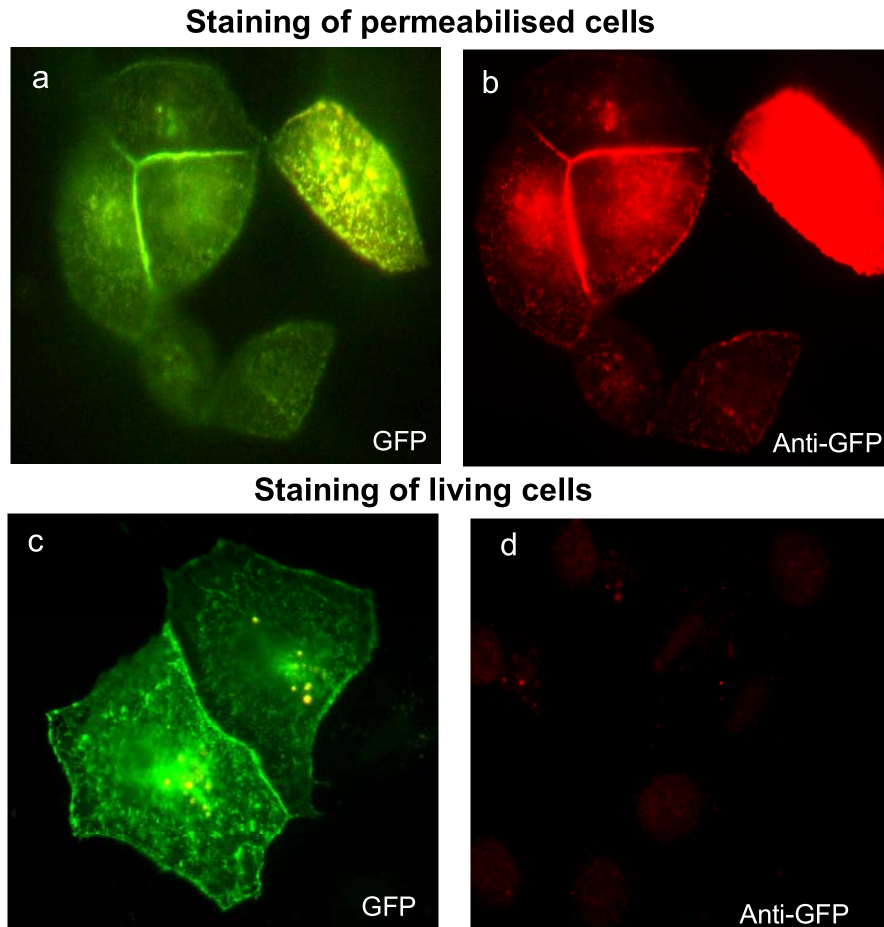


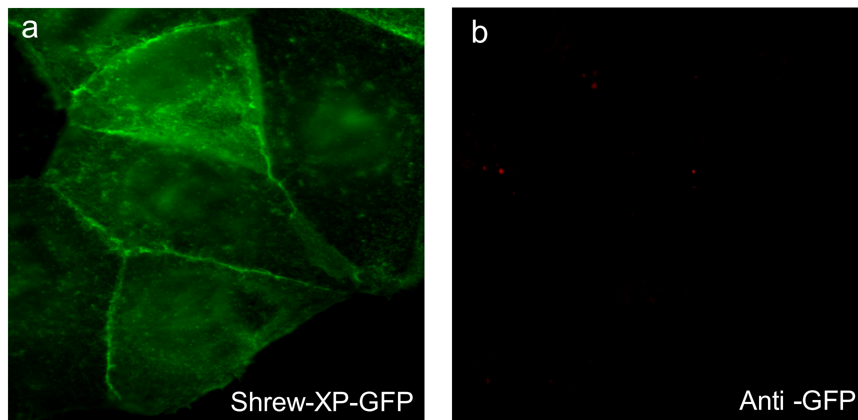
Figure 11. Carboxyl-terminus of shrew-1 is cytoplasmic. Shrew-1-GFP transfected MCF7 cells were permeabilised (a and b) or not permeabilised (c and d), as described in Material and Methods and then subjected to immunofluorescence staining with anti-GFP antibody and Alexa 594-labelled secondary goat anti-mouse antibody (b and d: red fluorescence). Intrinsic GFP fluorescence is green (a and c). Shrew-1-GFP could be detected in permeabilised cells by immunostaining with anti-GFP antibody (b) but not if the cells were not permeabilised (d).

3.3. Fusion with trkA

Shrew-1 was fused with its transmembrane domain and the cytoplasmic domain to the ectodomain of trkA. Tyrosine kinase A (trkA) is a receptor tyrosine kinase which functions as a growth receptor and is activated after binding to its ligand-nerve growth factor (NGF). After stimulation with its ligand, trkA gets phosphorylated at its tyrosine residues via the carboxyl terminal to SHC, PI3 kinase, and PLCγ

(Obermeier et al.,1994). Obermeier et al in 1996 fused the ectodomain of the tyrosine kinase growth receptor trkA with the cytoplasmic domain of some other tyrosine kinase receptors to characterise them via sending a signal for dimerisation and activation through NGF (nerve growth factor) which is a ligand for trkA. This served as a good system for studying the receptors without affecting the endogenous situation. This concept was also used for shrew-1 to get an idea about the physiological role of the cytoplasmic domain of shrew-1.

Staining of permeabilised cells



Staining of living cells

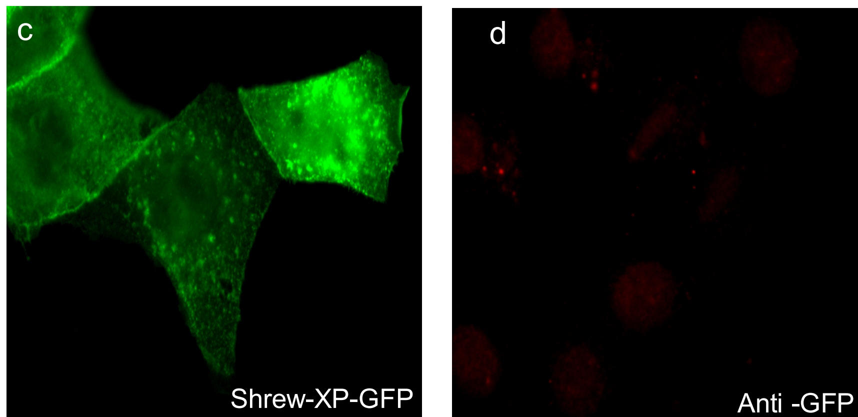


Figure12. Express tag fused between the putative signal peptide and the rest of the shrew-1-GFP could not be detected in permeabilized (upper panel, a and b) or non-permeabilized (lower panel, c and d) cells. MCF7 cells were transfected with shrew-XP-GFP (a-d). The autofluorescence of GFP could be detected in both permeabilized (a) as well as non-permeabilized (c) cells.

This chimaera of shrew-1 and trkA was fused to two different tags, i.e. BP (trk-BP) and GFP (trk-GFP) tag (Fig. 13A). The aim of these fusion constructs with different tags was to control the induction of the cytoplasmic domain of shrew-1 via NGF and look at the events and the morphological changes occurring in the cells.

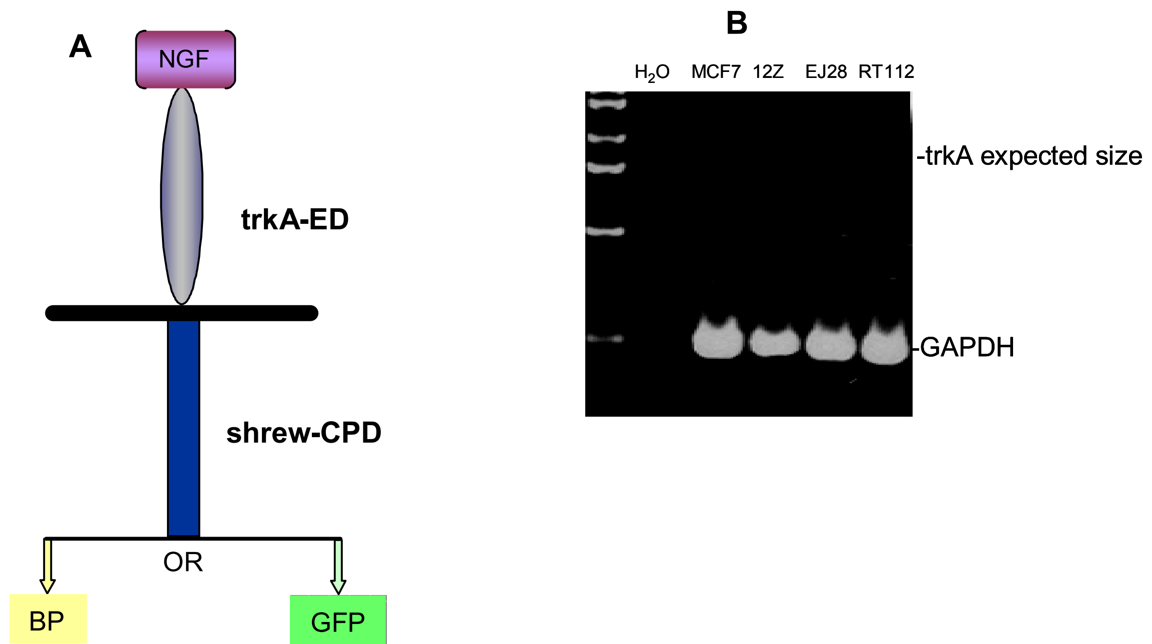


Figure 13. **(A)** Chimaera of trkA and the cytoplasmic domain of shrew-1 fused to BP or GFP tag. **(B)** RT-PCR to test for the presence of endogenous trkA in MCF7, 12Z, EJ28 and RT112 cell lines. No trkA band was detected at the expected size. GAPDH primers were used as an internal control for the cDNA used. As a negative control for the primers, water was used instead of the template in the reaction mixture.

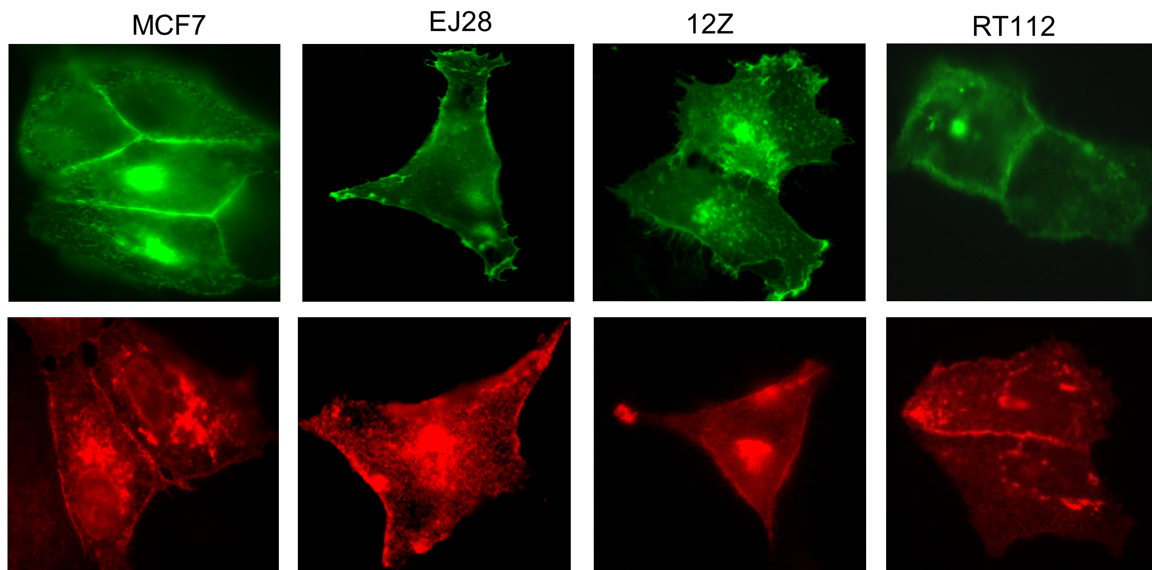


Figure 14. Expression analysis of trkA and shrew-1 fusion constructs-trk-GFP or trk-BP in MCF7, EJ28, 12 Z and RT 112 cell lines. Both trk-GFP (upper panel) and trk-BP (lower panel) localise at the membrane concentrated at the cell-cell contact sites. Trk-GFP is shown as green fluorescence and trk-BP as red fluorescence.

It would be possible to cover two different aspects of shrew-1 function: (i) In the direction of interaction partners-whether after stimulation with NGF some conformational changes might occur in the cytoplasmic domain of shrew-1 which might induce it to interact with some other proteins and (ii) If the cells behave in a similar way as shown earlier for other fusions of trkA with other tyrosine kinase growth receptors, it could imply that shrew-1 is also a member of the tyrosine kinase receptor family.

trk-BP and trk-GFP were transfected into different cell lines namely MCF7, EJ28, RT112 and 12Z. These cell lines were tested by RT-PCR (RT-PCR) for endogenous trkA expression. trkA was found to be absent in these cell lines (Fig. 13B). The expression analysis of trk-BP and trk-GFP showed their presence at the cell-cell contacts in a similar way as shrew-1 (Fig. 14). There was obviously no change in cellular localization compared with full length shrew-1.

Furthermore, NGF was added at a concentration of 100 ng/ml to induce trkA and the effect was monitored after 24 h. As expected, PC12 cells containing endogenous trkA responded to the stimulus by scattering and the cells exhibited a totally different morphology in which they became more flattened and developed protrusions. On the other hand, other epithelial cells transfected with the fusion constructs showed no change in behaviour. To rule out the possibility that this was due to low transfection efficiency, cells were stably transfected with the fusion constructs by selecting for the respective plasmid using G418. The selection pressure was applied for approximately two weeks. Only 40% of MCF7 cells were found to stably express trk-BP and trk-GFP. On inducing these cells with NGF still no effect could be seen.

Since no scattering behaviour or any other morphological effect was observed, it could be concluded that the fusion of shrew-1 with trkA had no effects on its functioning or behaviour in the cells. From these experiments the possibility that shrew-1 is a growth receptor of the tyrosine kinase family could be ruled out. Further experiments were then done in the direction of the characterisation of shrew-1 at the membrane as an integral component of the membrane.

3.4. Localisation of shrew-1 at the basolateral part of the membrane and its complexing with E-cadherin-β-catenin complexes in polarised cells

3.4.1. shrew-1 as a component of *Adherens junctions*

In most of the cases, the targeting of the membrane proteins in polarised cells is quite specific, they target either to the apical or the basolateral compartment of the membrane. To check whether shrew-1 targeted to the apical or basolateral domain of the plasma membrane, shrew-1-GFP was transfected into MDCK cells which were polarised by growing on transwell membrane filters. MDCK cells are a known model for polarised cells in which the specific sorting of the protein to the apical or basolateral compartments can easily be seen (Stegmaier et al., 2000). Confocal analysis of these cells along the xz-axis revealed shrew-1 to be present predominantly at the basolateral compartment of the membrane (Fig. 15 A, lower panel).

Since it is well-known that the basolateral part of the membrane is rich in *adherens junctions*, a marker for adherens junctions was used for testing the co-localisation of shrew-1 with another protein of these junctions. E-cadherin is a well-known protein of these junctions and plays an important role in maintaining their stability (see Introduction). Optical sectioning with confocal microscopy revealed that shrew-1 and E-cadherin colocalise at membrane especially at the regions of cell-cell contacts-observed both in xy- and xz-axis (Fig. 15; B and C).

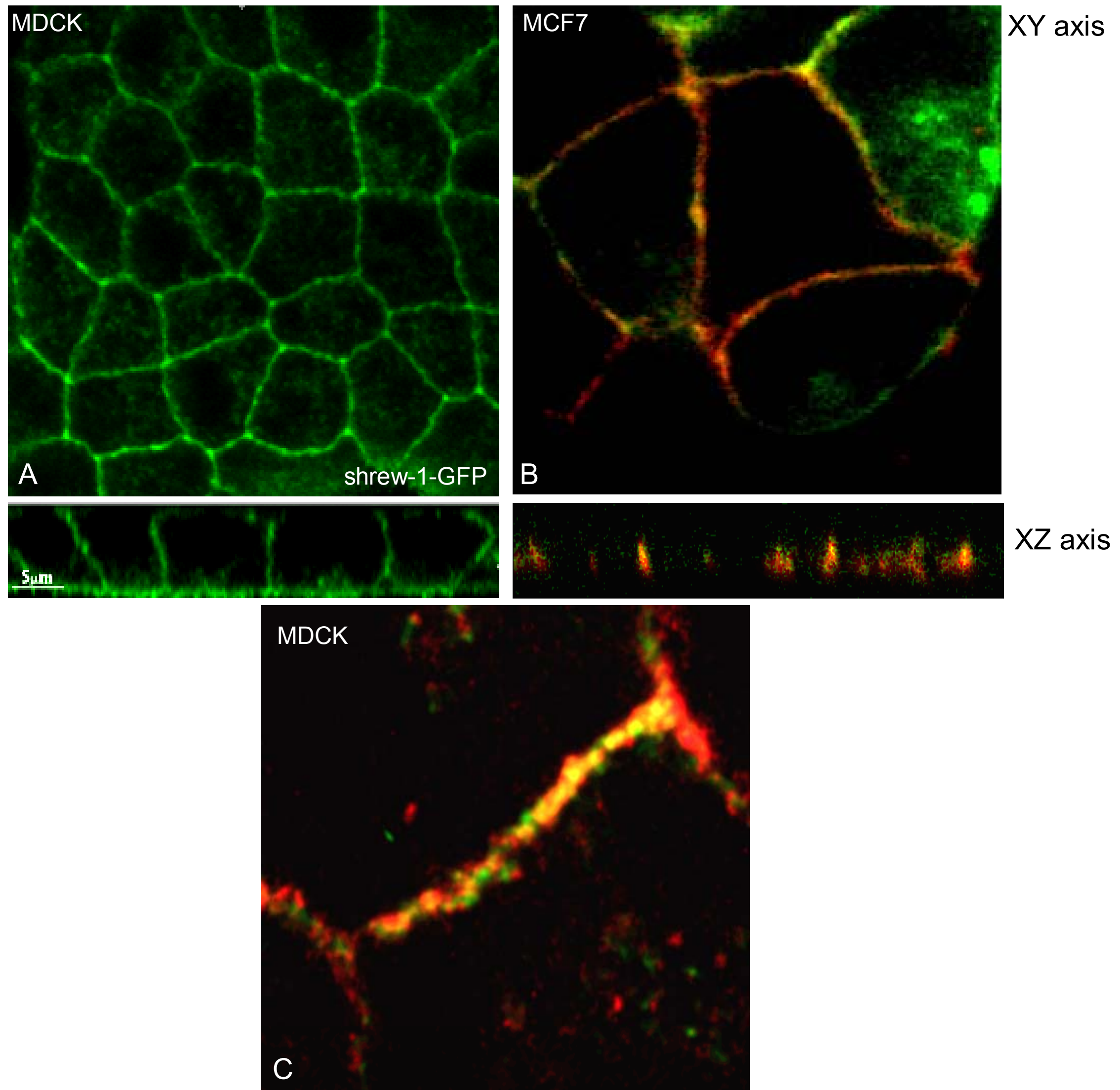


Figure 15. shrew-1 is localised to the basolateral part of the plasma membrane and colocalises with E-cadherin. **(A)** MDCK cells expressing shrew-1-GFP were grown on transwell filters. Confocal microscopy was done along the xy- and xz- axis that revealed shrew-1 to be present on the basolateral part of the membrane. **(B)** Colocalisation of shrew-1 with E-cadherin at the intercellular junctions. **(C)** Detailed analysis of physical colocalisation of shrew-1 and E-cadherin by confocal microscopy.Shrew-1-GFP is shown in green and endogenous E-cadherin staining is recorded in red.

3.4.2. shrew-1 complexes with cadherin-catenin complexes specifically in polarised cells

Whether this colocalisation was the result of shrew-1 interacting specifically with E-cadherin or just a coincidence was further investigated by doing *in vivo* interaction assays. MCF7 cells were transiently transfected with shrew-1-GFP or the vector (GFP) alone and grown to confluency.

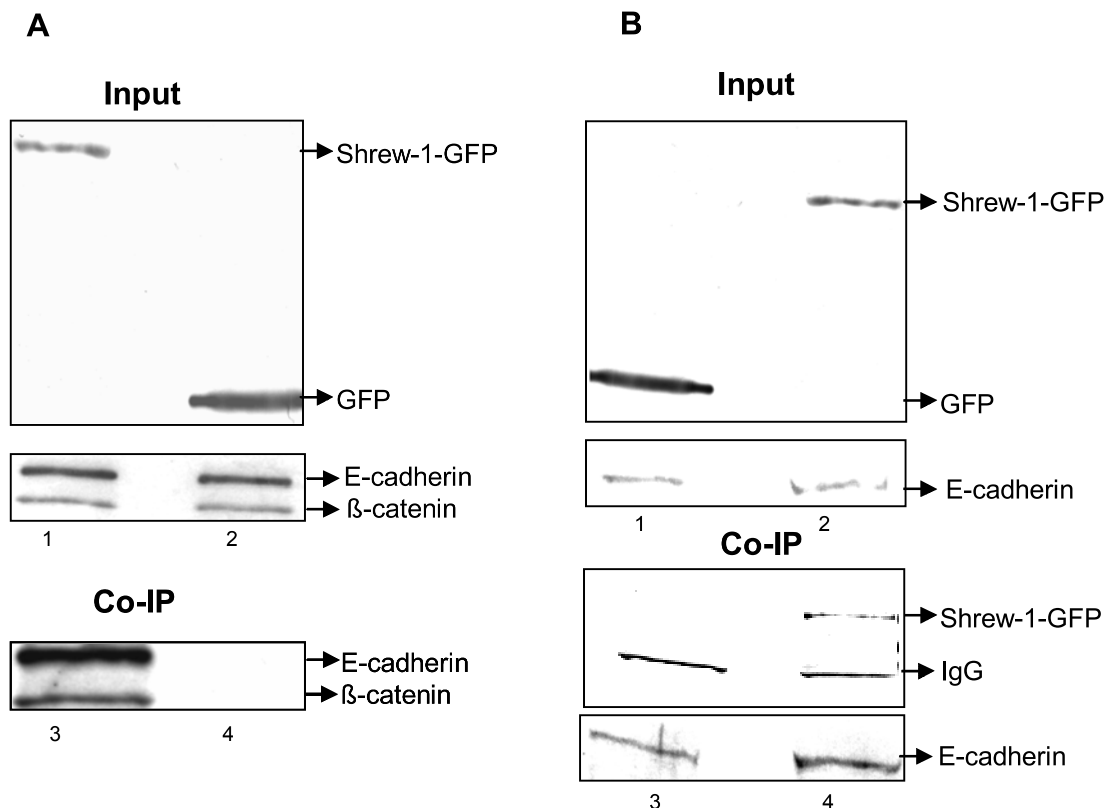


Figure 16. Interaction between shrew-1 and E-cadherin as shown by coimmunoprecipitation. **(A)** MCF7 cells transfected with shrew-1-GFP (lanes 1, 3) or with GFP (lanes 2, 4) were subjected to immunoprecipitation with anti-GFP. First, 10% of the total cell extract (Input) was immunoblotted (IB) with anti-GFP (upper panel) and anti-E-cadherin plus anti-β-catenin (middle panel) antibodies. Coimmunoprecipitations (Co-IP) were performed with anti-GFP antibody and the immunoprecipitates subjected to immunoblotting with anti-E-cadherin then anti-β-catenin antibodies (lanes 3, 4). **(B)** In the reverse experiment, the cell extracts from MCF7 cells transfected with GFP (lanes 1, 3) or shrew-1-GFP (lanes 2, 4) were subjected to immunoprecipitation (IP) with anti-E-cadherin antibody. Input panels depict 10% of the cell extracts immunoblotted with anti-GFP antibody (upper panel), or endogenous E-cadherin protein immunoblotted with anti-E-cadherin antibody (lower panel). Coimmunoprecipitations (Co-IP) were performed with E-cadherin antibody, and shrew-1 was detected by immunoblotting with anti-GFP antibody as seen in lane 4. CX denotes the total cell extract.

Cell extracts were prepared and transfection efficiencies were monitored by Immunoblotting (IB) 10% of the total cell extract using GFP antibody (Fig. 16 ; A and B; Input). The remaining cell extract was immunoprecipitated (IP) with GFP antibody, the immunoprecipitated complex was then immunoblotted using E-cadherin antibody (Fig. 16A; lanes 3, 4). E-cadherin could be detected in the immunocomplex pulled down by monoclonal anti-GFP antibody. β-catenin was also detected on reprobing the same blot with the beta-catenin antibody (Fig. 16 A; lane 3 and 4)

The reverse experiments confirmed the same results when IP was done with E-cadherin antibody and shrew-1-GFP could be detected in the same complex (Fig. 16B; lane 3 and 4).

An interesting observation made during these experiments was that no complexing between shrew-1 and E-cadherin was observed when the cells were not confluent (preliminary data). This could possibly mean that the cells need some mature junctions to stabilise this complexing (see Discussion).

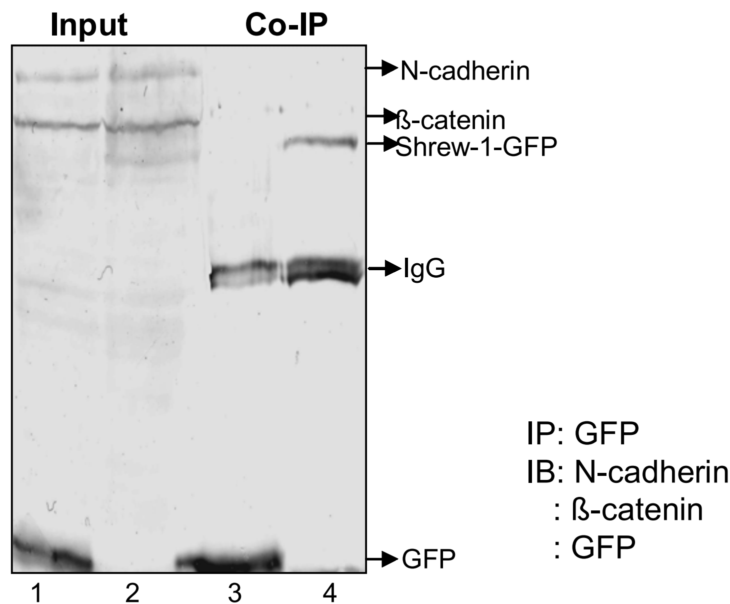


Figure 17. Coimmunoprecipitation of N-cadherin and shrew-1-GFP. EJ28 cells were transfected with GFP (lanes 1, 3) or shrew-1-GFP (lanes 2, 4). Input shows 10% of the total cell extracts (lanes 1, 2). Immunoprecipitation (Co-IP) was performed with GFP-antibody (lanes 3, 4). Immunoblotting was performed with antibodies against GFP, N-cadherin and β-catenin. No interaction of shrew-1 with N-cadherin and β-catenin was observed.

Furthermore, the ability of shrew-1 to interact with cadherins in epithelial cell lines that are unable to form adherens junctions (for example EJ28 cells, an invasive human bladder carcinoma cell line expressing N-cadherin) was analysed. EJ28 cells were transfected with shrew-1-GFP and GFP alone and the cell extracts were prepared in a suitable lysis buffer (see Materials and Methods). Monoclonal anti-GFP antibody was used for Co-IP assays performed as described above and the bound proteins were pulled down which were further subjected to SDS-PAGE and immunoblotted with anti-N-cadherin, anti-β-catenin and anti-GFP antibodies.

10% of the total cell extract prepared in the beginning was kept as input. The transfected proteins (shrew-1-GFP and GFP alone), N-cadherin and β-catenin could be detected in the input lanes (Fig. 17; lanes 1and 2) but no N-cadherin or β-catenin could be detected in the immunoprecipitated fraction (Fig.17; lanes 3 and 4). Only shrew-1-GFP and GFP alone could be seen (Fig. 17; lanes 3 and 4) which reflected that the immunoprecipitation functioned.

These data reiterate that shrew-1 can interact with cadherin-catenin complexes in junctions of polarised epithelial cells but not with cadherin-catenin complexes (here N-cadherin) in non-polarised cells.

3.4.3. Direct interaction of the cytoplasmic domain of shrew-1 with β-catenin

The results shown so far did not indicate whether the interaction between E-cadherin and shrew-1 is due to direct binding of the proteins or is caused by an intermediate protein such as a scaffolding protein in the complex (β-catenin being a candidate). *in vitro* pull-down assay between the cytoplasmic domain (CPD) of shrew-1 (used as GST fusion protein) and *in vitro* translated β-catenin was done. For this, β-catenin was translated radioactively *in vitro* (Fig. 18; lane 1) and was incubated with GST alone (lane 3) or GST fused to CPD of shrew-1 (GST-CPD-shrew-1, lane 4), purified from bacterial cell extracts (see Materials and Methods).

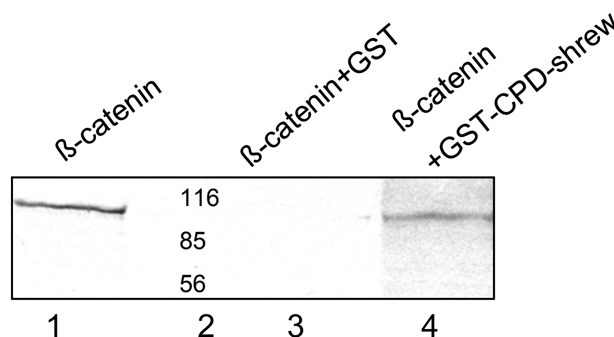


Figure 18. Direct interaction of β-catenin with the cytoplasmic domain of shrew-1 (GST-CPD-shrew) in an *in vitro* pull-down assay. Full-length β-catenin was translated *in vitro* using ^{35}S methionine. GST and GST-CPD-shrew were purified on glutathione sepharose beads, then incubated at RT for 1 h with radioactively labelled β-catenin. After washing the beads, samples prepared as described in Material and Methods were subjected to SDS-PAGE and autoradiography. Lane 1: radioactive β-catenin as input, lane 2: the marker, lane 3 : GST alone with β-catenin and lane 4: GST-CPD-shrew with β-catenin.

The beads were then washed, bound proteins were subjected to SDS-PAGE and subsequently autoradiography was done to detect the radioactively translated protein bound to GST-CPD-shrew-1.

As expected, no signal could be seen in the lane with GST alone incubated with the radioactive proteins (Fig. 18; lane 3). β-catenin could be detected in the lane with GST-CPD-shrew-1 (Fig. 18; lane 4), thus leading to the conclusion that β-catenin interacted with the cytoplasmic domain of shrew-1 *in vitro* as a direct interacting partner.

In similar studies done with GST-CPD-shrew and full length E-cadherin, no interaction could be observed between shrew-1 and E-cadherin (preliminary data).

Taken together, these data support the idea that shrew-1 interacts via its cytoplasmic domain with β-catenin but not with E-cadherin in adherens junctions. However, this does not exclude that shrew-1 binds to other as yet unidentified components of the adherens junctions.

3.5. Disruption of adherens junctions via addition of scatter factor/hepatocyte growth factor (SF/HGF) does not affect the colocalisation of shrew-1 and E-cadherin

The experiments done till now showed that E-cadherin and shrew-1 colocalise at the adherens junctions regardless of the maturity of the junctions, albeit do not complex with each other when the junctions are not mature. This was observed during the *in vivo* pull down assays when MCF7 cells were used which were not completely confluent. In continuation with this, it now became important to check whether this colocalisation persisted even after the disruption of the cellular junctions. To this aim, a physiological stimulus, scatter factor/hepatocyte growth factor (SF/HGF) was applied to the MDCK cells transiently transfected with shrew-1-GFP.

SF/HGF is a known cytokine that acts as a morphogen leading to epithelial-mesenchymal transitions. It is known to disrupt E-cadherin mediated junctions in MDCK cells through activation of its receptor c-met. During disruption, E-cadherin is

transiently transported into recycling vesicles reported to contain caveolin-1 (Akhtar and Hotchin, 2001).

MDCK cells transiently transfected with shrew-1-GFP were seeded at very low density on cover slips and were grown till the formation of islands. SF/HGF was added at a concentration of 20 ng/ml and the effect was monitored on cover slips from the same culture dish at 0h, 4h, 8h and 15h. After 8h a drastic change in the intracellular distribution of shrew-1 was observed (Fig. 19).

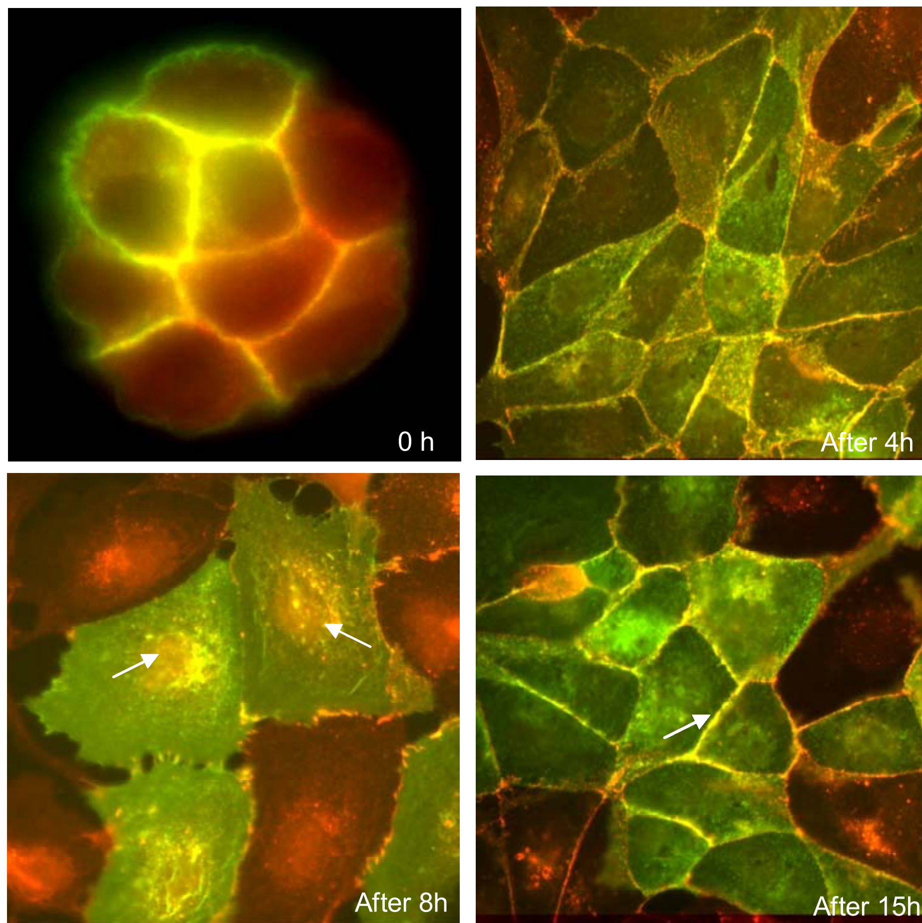


Figure 19. Effect of scatter factor (SF) on MDCK cells. MDCK cells transfected with shrew-1-GFP were seeded at very low density on cover slips and were grown till formation of small colonies. SF/HGF was added at a concentration of 20 ng/ml to these cells. The effect was monitored on cover slips from the same culture dish at 0h, 4h, 8h and 15h. After 8h, cell-cell contacts were disrupted but colocalization of shrew-1-GFP (green) and endogenous E-cadherin (red) could also be seen in cytoplasmic vesicles. After 15h shrew-1-GFP and endogenous E-cadherin colocalised again predominantly at the plasma membrane.

GFP fluorescence at the plasma membrane was reduced whereas intracellular particulate structures were labelled, which also stained for E-cadherin (marked with arrow heads). When the scattering effect of SF/HGF was gone after fifteen hours, GFP fluorescence and E-cadherin were again prominent at the plasma membrane.

These results suggest that upon disruption of junctions by a physiological stimulus shrew-1 is translocated together with E-cadherin to intracellular vesicles. Although it seems from these experiments that shrew-1 and E-cadherin have a comparable pattern of internalization, it can however not be discriminated whether this is caused by a weak interaction not detectable by coimmunoprecipitation or simply by a similar response to stimulation by SF/HGF.

3.6. Shrew-1 and caveolin-1

Taking into account the facts that i) shrew-1 is an integral component of E-cadherin-β-catenin complexes with which caveolin-1 also interacts and ii) Shrew-1 also colocalises with E-cadherin in the same vesicles coated with caveolin-1 during junctional disruption, it was worth checking the association of shrew-1 with caveolin-1.

For this, localisation studies of shrew-1-GFP and endogenous caveolin-1 were performed using CLSM. It was seen that both the proteins colocalise strongly at the membrane (Fig. 20). Whether this colocalisation also leads to any complexing of shrew-1 with caveolin-1 or not was checked by *in vivo* pull down assays. For this, shrew-1-GFP was transfected transiently in MDCK cells containing endogenous caveolin-1 and cell extracts were prepared. 10% of the total cell extract was kept as input to control the efficiency of transfection. Caveolin-1 polyclonal antibody was used to pull down caveolin-1 and the associated proteins. SDS-PAGE and subsequently an immunoblot was performed. Shrew-1-GFP could be detected in the input (Fig. 21A; lane 3) and in the immunoprecipitated fraction (Fig. 21A, lane 1). Fig. 21A, lane 2 depicts the immunoprecipitated fraction of GFP alone which is the negative control.

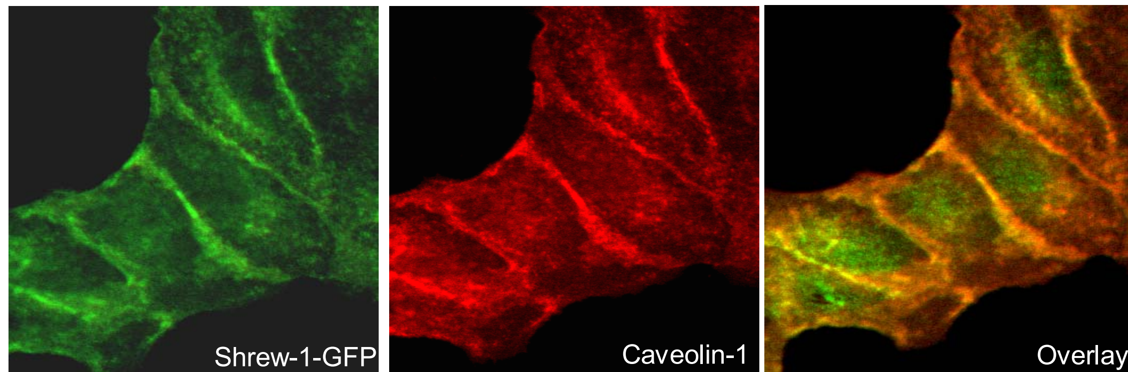


Figure 20. Colocalisation between shrew-1 and endogenous caveolin-1 in MDCK cells. shrew-1-GFP was transfected in MDCK cells which were immunostained for endogenous caveolin-1. Confocal analysis showed colocalisation of shrew-1 and endogenous caveolin-1 predominantly at the membrane. Shrew-1-GFP staining is shown as green, caveolin-1 staining is seen as red and the overlay between caveolin-1 and shrew-1 is seen as yellow

To test the direct interaction between two integral membrane proteins by a pull down assay is normally not possible since it is quite tedious to express a membrane protein in bacteria, due to the strongly hydrophobic transmembrane domain which affects the proper folding of the protein. Caveolin-1 as an exception can be expressed as a GST fusion protein in bacterial cells due to the reasons that it exists as a soluble form and secondly, both its N- and C-terminal are cytoplasmic, so it does not undergo all the modifications of integral membrane proteins (Li et al., 1996). Taking an advantage of this situation, a pull down assay was performed between radioactively labelled full length shrew-1 (Fig. 21B; lane1) and GST fused caveolin-1 (GST-cav). ^{35}S coupled shrew-1 was incubated with glutathione beads containing purified GST alone or GST-cav. SDS-PAGE and autoradiography were done with the beads after washing them a few times with suitable buffer (see Materials and Methods) to remove non-specific proteins from the complex. Radioactive shrew-1 was detected in the complex with GST-cav (Fig. 21B; lane 3) but not with GST alone (Fig. 21B; lane 2).

From this data it could be concluded that shrew-1 interacts directly with another protein of E-cadherin-β-catenin complex, i.e. caveolin-1. The interaction of shrew-1 with two key players of invasiveness suggests that it might have a role to play in this process but the mechanism is still unclear.

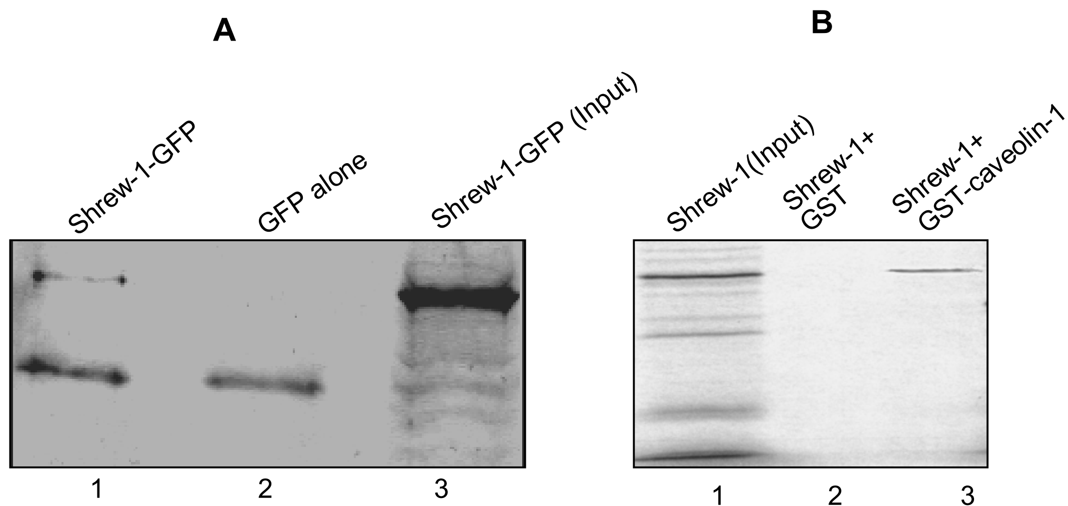


Figure 21. Shrew-1 and caveolin-1 interact *in vivo* and *in vitro*. **(A)** Caveolin-1 was immunoprecipitated using a polyclonal caveolin-1 antibody from MDCK cell extracts transfected with GFP or shrew-1-GFP. 10% of the total cell extract was kept as input. Shrew-1-GFP was detected in the input fraction (lane 3) as well as in the immunoprecipitated fraction (lane 1) when an immunoblot was done with anti-GFP antibody. The negative control containing GFP alone (lane 2) was empty. **(B)** Direct interaction between shrew-1 and caveolin-1 was shown by *in vitro* pull down assay. Shrew-1-BP was translated *in vitro* in the presence of ^{35}S. GST alone and GST-caveolin-1 were purified on Protein-G-Sepharose beads and then incubated at RT for 1 h with radioactively labelled shrew-1. After washing the beads, samples prepared as described in Materials and Methods were subjected to SDS-PAGE and autoradiography. Lane 1: radioactive shrew-1 as input, lane 2 : GST alone with shrew-1 and lane 4: GST-cav with shrew-1.

3.7. Shrew-1 overexpression studies

3.7.1. Shrew-1 overexpression confers an invasive phenotype on cells

To analyse for the function of a protein, it is necessary to downregulate it or overexpress it. In this direction, shrew-1 was overexpressed in cells by making stable cell lines. For this, shrew-1-GFP was transfected transiently into MDCK cell line which was then put under a selection with G418 for about 2 weeks. After two weeks, different clones could be seen which probably arose from a single cell. These clones were then isolated using silicon cloning rings and were further propagated and analysed. Most of the clones died in a few days, only a single clone survived. With the vector alone, there were more clones that survived this selection giving us the first hint that high concentrations of this protein might be toxic for the cells.

This single clone from shrew-1-GFP and the clones from GFP were analysed. The cells stably overexpressing shrew-1 showed a totally changed behaviour, they were very easily trypsinised and had a different morphology from normal MDCK cells. The typical honey-combed morphology of the cells was lost and the membrane had a very ruffled appearance. Immunostaining for E-cadherin and β-catenin was done in these cells. Both E-cadherin and β-catenin showed a fuzzy appearance at the membrane (Fig. 22; b and c; e and f). The confocal analysis of these cells also showed the presence of these membrane proteins partially in the cytoplasm as well, which is quite different from normal MDCK cells expressing E-cadherin and β-catenin (Fig. 22; a and d). These observations pointed towards the fact that the adherens junctions are disrupted and the cells seem to have lost the property of contact inhibition which is quite important to keep the cell in a non-invasive stage to confirm these observations, matrigel invasion assay was performed to check whether the cells are invasive or not or is it just an altered phenotype.

For the matrigel invasion assay, transwell filters coated with matrigel derived from components of the extracellular matrix were taken. Under normal conditions, it is not possible for MDCK cells to invade through this gel since they lack MMPs which are secreted only when the cells are invasive. The MMPs are essentially a feature of tumour cells where they are needed to spread and invade other tissues by digesting the extracellular matrix.

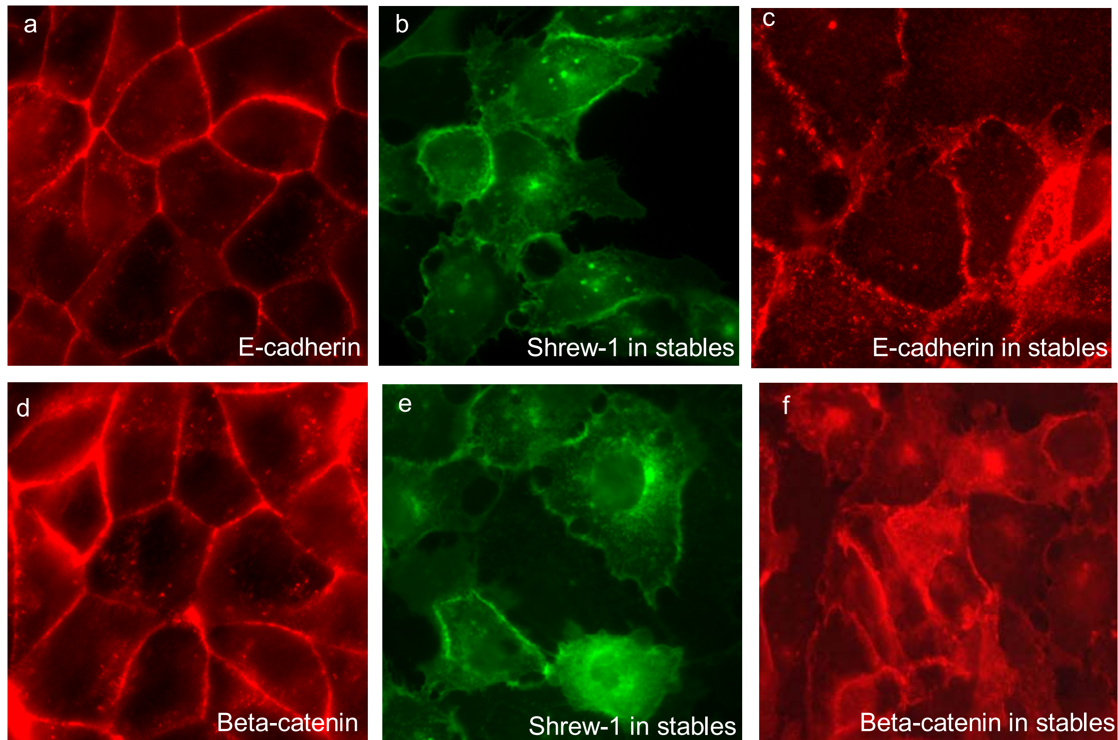


Figure 22. Shrew-1 stable cell line shows a completely altered phenotype. MDCK cells stably transfected with shrew-1-GFP showed a different staining of E-cadherin (c) and β-catenin (f). The regular pattern of endogenous E-cadherin (a) and endogenous β-catenin (d) expression seems to be completely altered. (b) and (e) show the staining of shrew-1-GFP in the stable cell line. a and d are wild type MDCK cells expressing E-cadherin and β-catenin, respectively.

As a control, transwell filters without any matrigel were taken to estimate the motility of the cells. the cells that have acquired a motile behaviour travel faster through these filters.

MDCK cells stably transfected with GFP and shrew-1-GFP were seeded on matrigel coated filters (Fig. 23A) as well as filters without any matrigel (Fig. 23B) and were grown for 72h till completely dense.
After 72h, the cells were fixed and subsequently DAPI was put on the cells to label the nuclei. The filters were then transferred to glass slides which were then viewed under the microscope. The number of cells on the lower surface of both the filters was counted in different fields. The invasive fold of the cells was finally counted as number of invasive cells/motile cells x 100. Untransfected cells were used as background control. The invasion fold was found to be increased by almost 12-fold as compared to the 3-fold increase of invasiveness in the vector control.

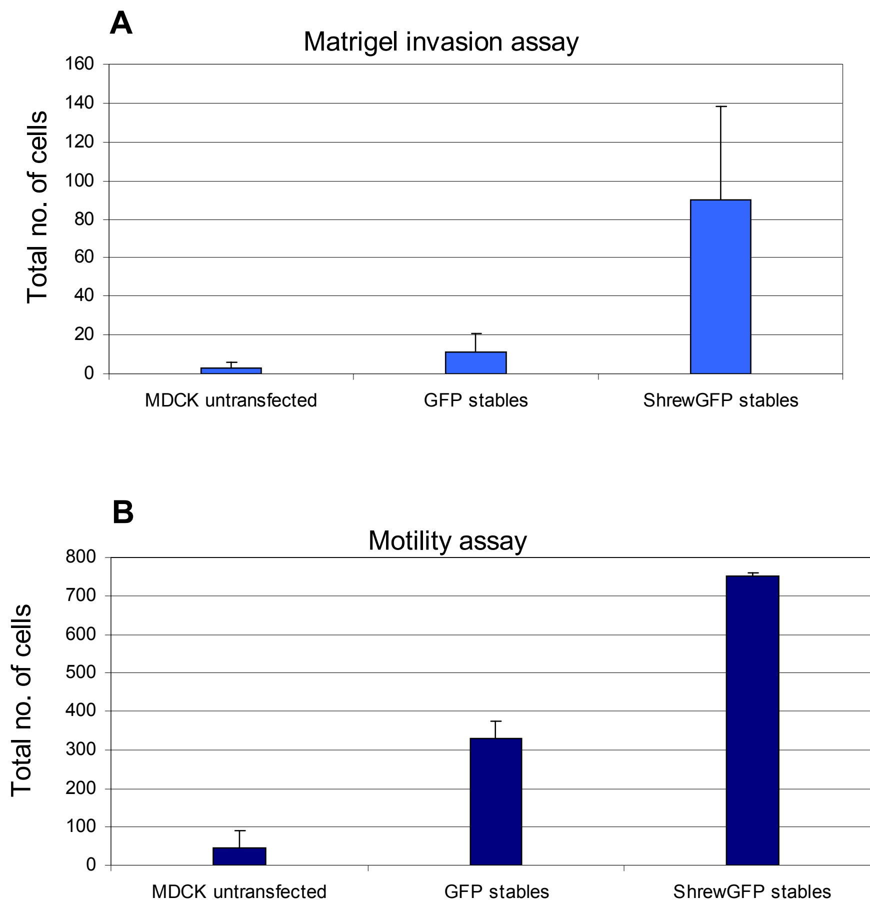


Figure 23. Shrew-1 stable cell line is invasive as shown in a matrigel invasion assay. Invasion assay was done by seeding MDCK cells stably overexpressing shrew-1-GFP, GFP and untransfected cells on transwell filters coated with matrigel (upper panel, **A**). The number of cells invading the matrigel were counted by counting the cells on the lower side of the filter after 72h. As a control, the cells were seeded in parallel on uncoated transwell filters **(B)**. The motile cells that could cross these filters were counted. This was done in a set of three independent experiments and the mean values were take. Invasion index was counted as the total number of invasive cells/ total number of motile cells x 100.

From this data, it could be concluded that stable overexpression of shrew-1 in wild type MDCK cells confers an invasive phenotype on these cells. One could hypothesise from this observation that the stoichiometry of shrew-1 is important for the maintenance of adherens junctions and hence contact inhibition of the cell and when this is disturbed, the cell becomes invasive. The deregulation of shrew-1 seems to be the key behind this mechanism. Taken together the data that shrew-1 is also present in normal epithelial tissues such as kidney and pancreas, it could be said that shrew-1 is important for the normal epithelial cells but when it is

deregulated, the epithelial cells lose their normal phenotype. Hence the regulation of shrew-1 seems to work in a spatio-temporal manner.

3.7.2. Shrew-1 overexpression leads to stabilization and activation of β-catenin in untransformed MDCK cells

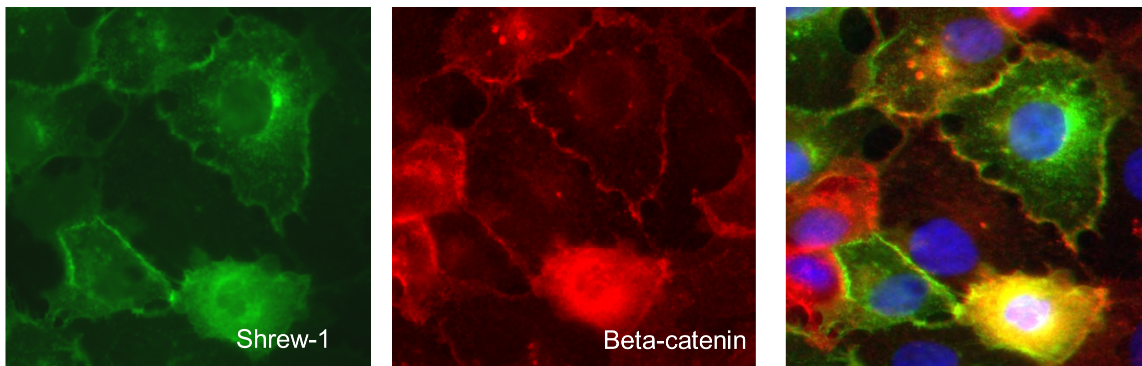
Looking closely at the costaining between shrew-1-GFP and β-catenin in the stable cell line (Fig. 24A), it could be seen that shrew-1 and β-catenin colocalize in the membrane as well as in cytoplasm which is normally not the case for either of the two proteins. This is only the case when β-catenin is stabilised in the cytoplasm, not being able to be degraded. This excess β-catenin travels to the nucleus, forms a complex with Tcf/Lef and subsequently binds to the Tcf/Lef promoter which further leads to transcription of various oncogenes such as c-myc and the cells become invasive (see Introduction).

To test this, pTOP and pFOP plasmids were used. pTOP plasmids contain the binding sites for Tcf/Lef in triplicate and when activated β-catenin comes and binds to these sites, transcription is initiated which leads to the activation of luciferase reporter which is also present in this plasmid. Hence the activation of the reporter by β-catenin can be measured in relative light units (rlu). On the other hand, pFOP plasmid has the same sequence except that the Tcf/Lef binding sites are mutated in the plasmid. It is used as a control to check non-specific activation of some other elements in this reporter which might lead to luciferase activity.

pTOP and pFOP plasmids were transfected into untransfected MDCK cells (for background), GFP stables and into shrew-1-GFP stables. The next day cell extracts were prepared in a suitable lysis buffer (see Materials and Methods) and the activity was measured in a luminometer. The pTOP Flash activity in shrew-1-GFP stable cell line was found to be increased by 150-fold as compared to GFP stable cell line (Fig. 24B). This showed that β-catenin is present in an activated form in this shrew-1 stably transfected cell line which also showed an invasive phenotype. This clearly

points to the fact that a high amount of shrew-1 somehow deregulates the stability of the adherens junctions as well as leads to activation of β-catenin in a normal untransformed cell line thus generating an invasive phenotype. Whether this activation of β-catenin also affects the other components of the Wnt signaling pathway still has to be deciphered.

A



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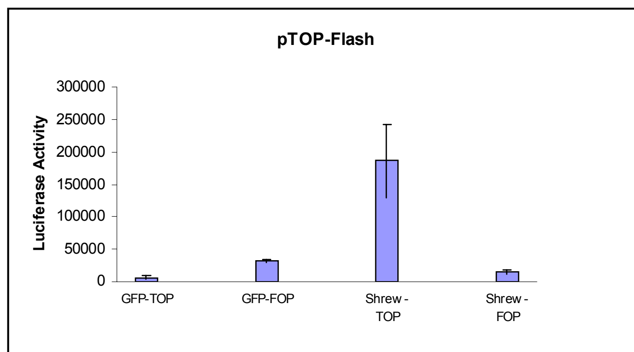


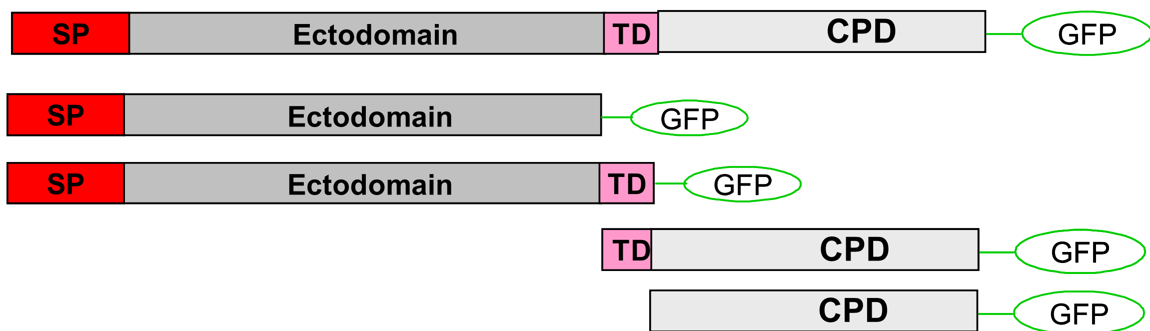
Figure 24. Overexpression of shrew-1 leads to activation of β-catenin in MDCK cells. **(A)** Altered expression of β-catenin in shrew-1 stable cell line could be seen. The presence at the membrane was not so prominent and additionally it seemed to colocalise with shrew-1 in the cytoplasm. Shrew-1 staining is shown as green, endogenous β-catenin staining is red and the overlay is seen as yellow where the nuclei are also stained with Hoechst dye. **(B)** pTOP FLASH assay with shrew-1 stable cell line. pTOP and pFOP plasmids were transfected into GFP and shrew-GFP stable cell lines and the activity of the luciferase reporter was measured. pTOP activity was approximately 150-fold higher in the shrew-1-GFP stable cell line compared to the GFP stable cell line. Luciferase activity is measured as relative light units (RLU). There was no marked change in pFOP activity.

3.8. Mapping of the interaction domain of shrew-1

3.8.1. Cellular localization of shrew-1 deletion constructs

To further characterise shrew-1, different domains of shrew-1 were cloned into the eukaryotic expression vector containing GFP tag. The ectodomain, the ectodomain with the transmembrane domain, the cytoplasmic domain and the cytoplasmic domain with the transmembrane domain were all fused with the C-terminus GFP tag (Fig. 25A). To study the cellular localisation of these domains, these plasmids were transfected transiently into MCF7 cells

A



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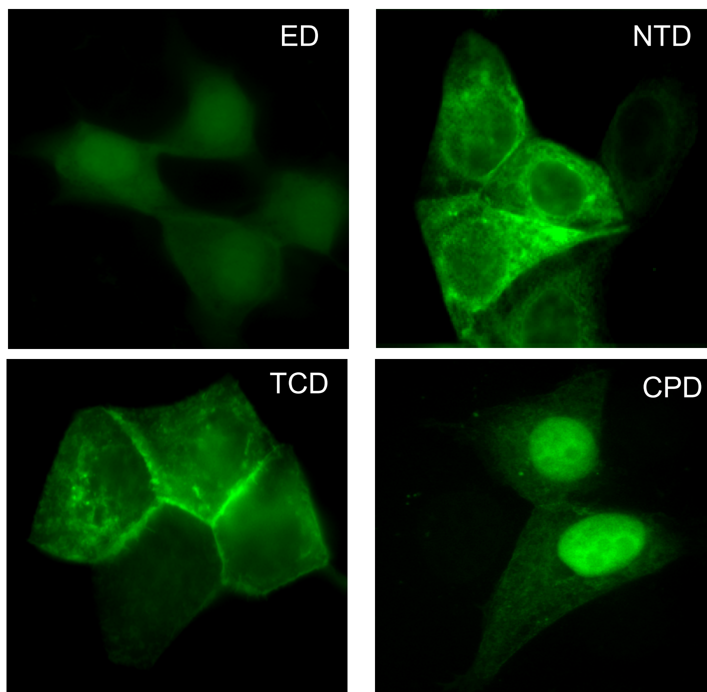

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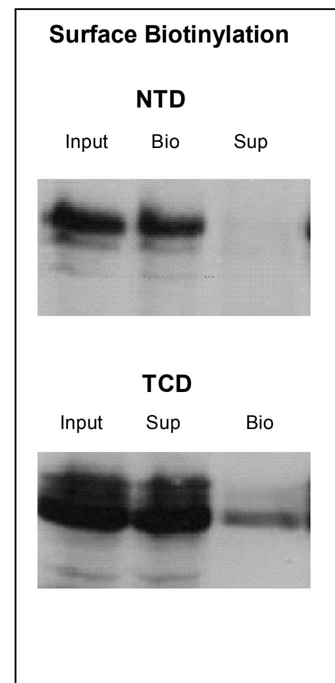

Figure 25. Deletion constructs of shrew-1. **(A)** Scheme of the deletion constructs made from different domains of shrew-1-all fused to GFP tag at the C-terminus. Ectodomain of shrew-1 (ED-GFP), ectodomain and the transmembrane domain of shrew-1 (NTD-GFP), transmembrane domain and the cytoplasmic domain of shrew-1 (TCD-GFP) and the cytoplasmic domain of shrew-1 (CPD-GFP). **(B)** Cellular localisation of shrew-1 deletion constructs –ED-GFP, NTD-GFP, TCD-GFP and CPD-GFP in MCF7 cells. **(C)** Biotinylation of TCD-GFP and NTD-GFP in MCF7 cells. Input is 10% of the total cell extract. Sup is the supernatant that contains no biotinylated protein and Bio is the biotinylated fraction bound on neutravidin beads. An immunoblot was done with the input, supernatant and the biotinylated fractions. The primary antibody used was monoclonal anti-GFP antibody and the secondary antibody was coupled to alkaline phosphatase.

and the expression was observed under the confocal microscope. It was seen that the ectodomain of shrew-1 (ED-GFP) could not be expressed alone or was degraded (as seen in Fig. 25B) which was not surprising since this effect was also seen in bacteria and could be attributed to the presence of the low complexity region in the ectodomain (discussed later).

On the addition of the transmembrane domain to the ectodomain (NTD-GFP), the construct was clearly expressed (Fig. 25B), mainly in the perinuclear space but visibly very less at the membrane. This result was quite perplexing considering the fact that both the signal peptide and the transmembrane domains which are usually responsible for targeting of the proteins to the membrane were present in this construct (discussed later).

Proceeding with the other constructs, the cytoplasmic domain (CPD-GFP) was transfected into MCF7 cells and it showed an expected expression in the cytoplasm as well as a strong expression in the nucleus (Fig. 25B) which could also perhaps be due to its small size due to which it could simply diffuse into the nucleus. The addition of the transmembrane domain to the cytoplasmic domain (TCD-GFP) lead to a drastic change in the localisation of this construct, the major part being present at the membrane similar as full length shrew-1 and some part in the perinuclear space (Fig. 25B). It could be concluded from this expression analysis that in addition to the transmembrane domain, some amino acids from the cytoplasmic domain are also responsible for the targeting of the protein to the membrane (personal communications, Gisbert Schneider).

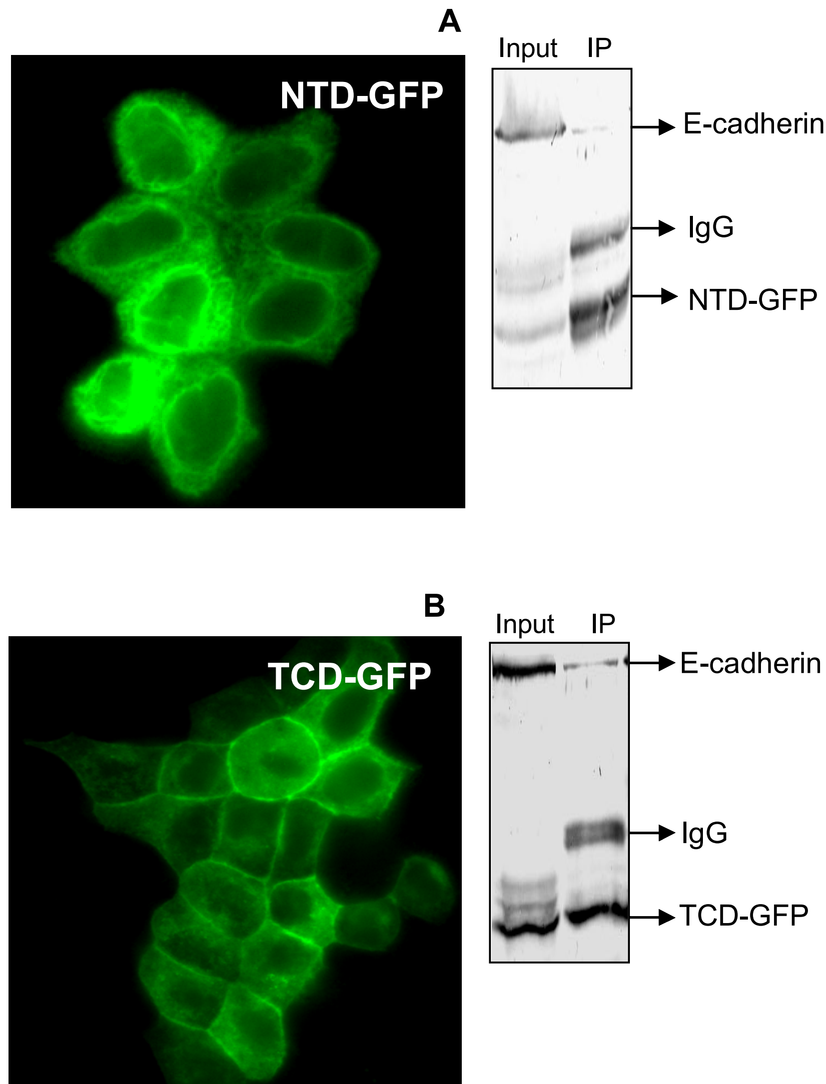

Figure 26. Characterisation of NTD-GFP and TCD-GFP. **(A)** semi-stable MDCK cell line was generated using NTD-GFP and the cellular localisation was analysed. *In vivo* interaction assay with E-cadherin was performed for which monoclonal anti-GFP antibody was used for IP and anti-E-cadherin as well as anti-GFP antibody were used for IB. 10% of the total cell extract was kept as input. The input fraction contained E-cadherin and NTD-GFP. The IP fraction contained NTD-GFP, IgG and a small amount of E-cadherin. **(B)** TCD-GFP stable line was also analysed for cellular localisation and for complexing with E-cadherin as done above. The input fraction contained E-cadherin and TCD-GFP. The immunoprecipitated fraction contained TCD-GFP, IgG and a comparably larger amount of E-cadherin.

Biotinylation studies were then performed with NTD-GFP and TCD-GFP since only these two constructs showed a clear association with the membrane, the CPD being present in the cytoplasm and the perinuclear space and the ED-GFP being degraded. For this, NTD-GFP and TCD-GFP were transfected in MCF7 cells and biotinylation was performed as described previously (also see Materials and Methods). Quite interestingly it was seen that that NTD-GFP was biotinylated very

clearly (Fig. 25C, upper panel) whereas TCD-GFP was not biotinylated as strong as NTD-GFP (Fig. 25C, lower panel), inspite of the fact that the membrane expression of TCD-GFP was much stronger as compared to NTD-GFP. The explanations behind it could be that in case of TCD-GFP, the protein is transported to the membrane, associates to it probably via its transmembrane domain and is stabilised because of the amino acid residues from the CPD but since no part is present really at the surface, there are no amino acids available for biotin to bind. In case of NTD-GFP one could speculate that there are amino acids available to bind but the membrane associated form is not very stable and perhaps is in a state of continuous equilibrium since it cannot be seen expressed at the membrane in immunofluorescence.

3.8.2. Complexing with E-cadherin

Along these lines, these constructs were expressed in polarised MDCK cells and the cells were put under a selection pressure with G418 for two weeks. Approximately, 25% of the cells were shown to stably express NTD-GFP (Fig. 26A) and TCD-GFP (Fig. 26B). The expression observed in MCF7 cells was reproduced except for the fact that a stronger membrane association of TCD-GFP was observed and the staining initially observed in the perinuclear space had disappeared.

To check for the complexing of these domains with E-cadherin, coimmunoprecipitation analysis was done with these MDCK cells. Cell extracts were prepared to which anti-GFP antibody was added to pull down the GFP tagged constructs-NTD-GFP and TCD-GFP. 10% of the total cell extract was kept as input. Protein-G-Sepharose beads were added to the extract to immobilize the antibody-protein and the other interacting proteins complex. An immunoblot was done with anti-GFP antibody to detect the GFP tagged protein in the input as well in the immunoprecipitation to detect whether the protein has been immunoprecipitated or not. The GFP antibody detected bands for both NTD-GFP (Fig. 26A) and TCD-GFP (Fig. 26B). In input as well as in IP fraction lanes. Anti-E-cadherin antibody was used to probe the blot to detect whether these deletion constructs complexed with E-cadherin or not. Both of these constructs complexed with E-cadherin but TCD-

GFP showed a more strong complexing as compared to NTD-GFP. One thing that was clear from this result was that the transmembrane domain plays an important role in this complexing but the stabilisation provided by the amino acids form the cytoplasmic domain leads to a stronger and perhaps a more stable association between shrew-1 and E-cadherin.

4. Discussion

4.1. Validation of shrew-1 mRNA and protein

Two alternatively spliced messages have been identified for shrew-1, a 4 kb message from the invasive passages of the endometriotic cell line-EEC145T and a 9 kb mRNA from the brain. Although the 4 kb isoform was detected in various other tissues such as kidney, spleen and pancreas, the 9 kb isoform was specifically present in brain in addition to the 4 kb isoform (Handrow-Metzmacher, unpublished). The longest cDNA that could be isolated was 2910 bp in size, which encodes an open reading frame (ORF) of 411 amino acids that corresponds to a protein of 45 kDa, shrew-1.

Supporting the isolation of shrew-1 from an invasive cell line, ESTS were isolated from carcinomas such as colon carcinomas that corresponded to shrew-1 cDNA sequence. Recently, another group has identified a cDNA, MOT8 that is identical in sequence to shrew-1, from an invasive fibrosarcoma cell line, encoding the same protein (Kudoh unpublished, accession number AF175409). *In situ* hybridisation studies done in our group revealed the presence of shrew-1 cDNA in carcinomas of kidney, breast, lung, liver and pancreas (Klemmt, unpublished). However, the presence of shrew-1 was also detected in normal tissues as well, such as in kidney, pancreas, spleen. This led to the idea that shrew-1 is a molecule that plays a role both in normal and invasion associated processes in higher vertebrates, since its orthologs were identified only in mouse and zebra fish.

The shrew-1 protein was predicted by computational analysis to consist of an unusually long signal peptide of 43 amino acids, an ectodomain, a transmembrane domain, and a cytoplasmic domain, which are all attributes of a transmembrane protein. Further experiments were done in the direction to prove the functional integrity of these domains. It could be shown by immunofluorescence and by biotinylation experiments that shrew-1 is indeed an integral membrane protein and a part of it is outside the cell. The orientation of at least the carboxyl terminal domain could be shown to be cytoplasmic by permeabilization studies using shrew-1-GFP tagged at the C-terminus. However, the amino-terminus could not be well-

characterised due to inability to express the protein with an N-terminal tag. Furthermore, the putative signal peptide of shrew-1 did not seem to be important for the targeting of the protein to the plasma membrane. Shrew-1 was cloned without the putative signal peptide with a C-terminus GFP tag (Δ1-43)and the cellular localization was studied (Jakob, unpublished). Δ1-43 targeted to the membrane and showed similar biotinylation pattern as full length shrew-1. Obviously the membrane targeted signal is present elsewhere, most probably in the transmembrane domain which is highly hydrophobic in nature. Taken together, it could be said that shrew-1 is an integral membrane protein with a cytoplasmic C-terminus.

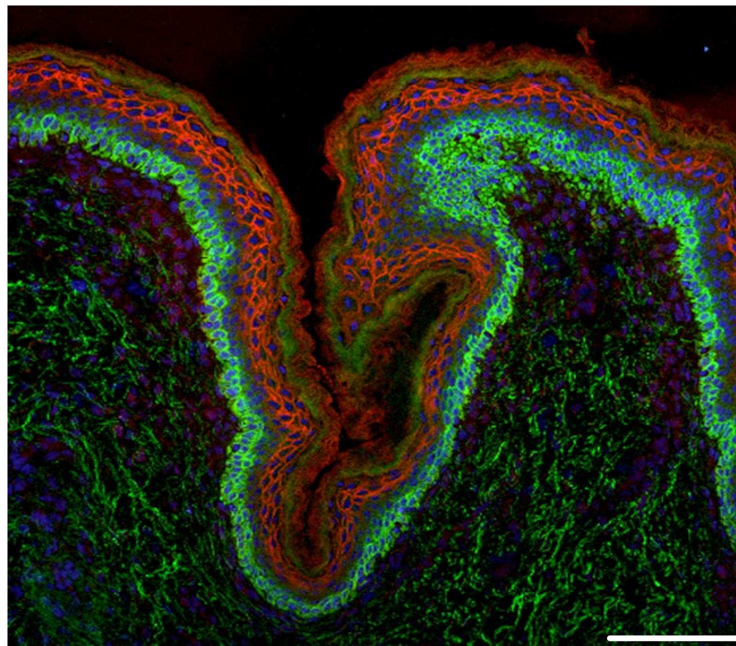

Figure 27. Specific expression of endogenous shrew-1 (green) in proliferating human keratinocytes as detected by the monoclonal antibody raised against the putative ectodomain of shrew-1 (Catherin Niemann, unpublished). Cornifin is taken as a marker for the keratinocytes and is seen as red, whereas To-Pro3, a marker for the nucleus, is seen in blue.

The endogenous expression of shrew-1 was detected by generating two different types of antibodies, raised against different shrew-1 peptides. A monoclonal antibody was raised against a peptide present in the putative ectodomain of shrew-1, the part containing the low complexity region and the polyclonal antibody was generated against carboxyl terminus peptides. Both the antibodies in an immunoblot detected a protein of about 45 kDa, the expected size of shrew-1. The authenticity of the detected shrew-1 band was confirmed by immunoprecipitating

shrew-1 with the monoclonal antibody and immunoblotting with the polyclonal antibody and vice versa, since it is extremely unlikely that both peptide sequences used as immunogen would be present in a protein unrelated to shrew-1 (Bharti et al., in press).

The presence of shrew-1 protein in carcinomas tested positive for shrew-1 mRNA was confirmed using these antibodies (preliminary data). Carcinomas of pancreas, liver, lung, kidney and endometrium tested positive for shrew-1. Interestingly, shrew-1 was found to be specifically expressed in proliferating keratinocytes in sections from skin tissue (Fig. 27) (Cathrin Niemann, Cancer research laboratories, UK).

4.2. Specific targeting of shrew-1 to the basolateral membrane

The next question that arose after observing the targeting of shrew-1 to the membrane was whether it targets specifically to any particular domain, i.e. apical or basolateral in polarised cells. It is well known that epithelial cells sort plasma membrane proteins into polarized apical and basolateral domains to perform vectorial secretory and transport functions that are critical for cellular, tissue and organism homeostasis. Shrew-1 was found to target specifically to the basolateral part of the membrane in polarised MDCK cells, as shown by confocal microscopy (Fig. 15). The basolateral targeting of shrew-1 might have important consequences for the maintenance of adherens junctions, as is known for several other basolateral targeting proteins. One example is LET-413 (similar to human ERBIN), a *C.elegans* protein that is basolaterally localised and is shown to be essential for the assembly of adherens junctions (Legouis et al., 2000). LET-413 supposedly forms a scaffold with proteins containing PDZ domains, within the basolateral domain to assemble adherens junctions.

The mutants of LET-413 are known to show defects in epithelial integrity that leads to an abnormal morphogenesis of the pharynx, intestine and pharynx-epithelia derived structures (Legouis et al., 2000). Another well-known example is E-cadherin, one of the proto-typical polarized membrane proteins in epithelia. It is delivered to the basolateral membrane and is concentrated in adherens junctions where it participates in cell-cell adhesion (Le Bivic et al., 1990., Shore and Nelson, 1991). It is indispensable for the structural and functional integrity of the adherens

junctions where it complexes with other cytoplasmic proteins and associates with the cytoskeleton, further stabilising the intercellular contacts (Gumbiner, 1996; Takeichi, 1995).

Along these lines, it would be reasonable to assume that the localisation and concentration of shrew-1 at the basolateral part of the plasma membrane might play a role in the process of cell-cell adhesion. This assumption is based on the initial line of evidence about association of shrew-1 with invasiveness. Mistargeting of shrew-1 by mutations or its deregulation could direct the cell to an invasive or abnormal phenotype.

To understand the role of shrew-1 at the basolateral part of the membrane, it would be first important to unravel the mechanism of its sorting to the basolateral membrane. The mechanisms for sorting and targeting proteins to the apical and basolateral surfaces are still only partially understood. Sorting of basolateral proteins has been shown to depend on short amino acid sequences located in the cytoplasmic tail that interact with specific adaptor proteins (Aroeti et al., 1998). This suggests that basolateral targeting is mediated by direct interaction with vesicle coat components (Puthenveedu et al., 2003).

Different classes of signals have been identified for this targeting. The most frequent and best-characterized sequences depend on a critical tyrosine residue within a consensus sequence Tyr-X-X-Φ, where Φ is a bulky hydrophobic residue (Matter et al., 1992). Most of these tyrosine based-signals may also function as signals for rapid endocytosis. Less common signals include Leu-Leu and di-hydrophobic motifs (Hunziker and Fumey, 1994) or short sequences with no apparent consensus (Hobert et al., 1997). It is quite possible that shrew-1 targets to the basolateral domain with the less common or relatively unknown signals since a scan of the shrew-1 amino acid sequence did not reveal any of the above mentioned motifs. It is not such a unique case since the basolateral targeting of neural cell adhesion molecule (N-CAM) also has been attributed to a cytoplasmic tail sequence without classic motif homology (Le Gall et al., 1997).

The approach used by Miranda et al (2001) for identification of basolateral targeting signals of E-cadherin, could also be applied to identify the basolateral targeting

signal of shrew-1. They created chimaeras of Tac (the α subunit of the IL-2 receptor) and E-cadherin containing the cytoplasmic tail of E-cadherin and alternatively some sequences from the cytoplasmic domain. Tac is a 273-amino acid protein that has been used as a reporter protein for trafficking studies (Rajasekaran et al., 1994; Mallet and Maxfield 1999). These chimaeras were efficiently targeted and trafficked to the basolateral domain of MDCK and LLC-PK_1 cells in a similar manner to that of endogenous E-cadherin or GFP-E-cadherin in either cell line. Using this model system a positive targeting signal in E-cadherin was identified that is responsible for its basolateral delivery.

4.3. Shrew-1 as an integral component of adherens junctions (AJs)

4.3.1. Shrew-1 complexes specifically with E-cadherin-β-catenin complexes

The identification of shrew-1 as an integral membrane protein targeted basolaterally raised the important question about its importance as a junctional component. Since the basolateral part is rich in junctions, attention was mainly focussed on adherens junctions (AJs) that are the most well-characterised intercellular junctions. E-cadherin, a well-studied prototype member of AJS (see Introduction) was taken as a marker protein, especially for the reason that shrew-1 is also targeted basolaterally. Colocalisation studies done with confocal microscopy showed that both of these proteins colocalise, especially in epithelial polarised cells. Interestingly, the disruption of the junctions by a physiological stimulus, scatter factor/hepatocyte growth factor (SF/HGF) elicited a similar response by shrew-1 and E-cadherin, both of them translocated intracellularly, most probably in the same structures. The removal of the stimulus led to the redistribution of these proteins on the membrane at the same time points (Fig. 19). Following these interesting observations, interaction assays were performed with shrew-1 and E-cadherin which showed, not so surprisingly though, a complexing between these two proteins. This complexing was quite specific and was limited to polarised epithelial cells since shrew-1 did not show any complexing with N-cadherin from invasive, non-polarised epithelial cells. It was established by this data that shrew-1 is a novel protein of the epithelial AJs that complexes specifically with E-cadherin.

The targeting of shrew-1 to the AJs could be mediated by a plethora of proteins, β-catenin being a potential candidate. It could indeed be shown by interaction studies that shrew-1 interacts directly with β-catenin via its cytoplasmic domain. Direct interaction between β-catenin and shrew-1 in an in vitro pull-down assay suggested that shrew-1 might be linked to the E-cadherin-mediated junctional complex via β-catenin. This interpretation also raises the possibility that shrew-1 is in fact targeted to specific cell contact sites, e.g. adherens junctions, rather than merely binding to E-cadherin *per se*. In line with such an idea is the observation that coimmunoprecipitation of shrew-1 with the E-cadherin-catenin complex was dependent on the formation of junctions and, in spite of colocalisation in immunofluorescence between shrew-1-GFP and endogenous E-cadherin, complexing could not be observed in subconfluent cells. An explanation for lack of complexing observed in subconfluent cells could be that the interaction is rather weak at this stage of cell growth and only stabilises when cellular junctions mature in confluent cells. The second explanation could be that initially E-cadherin and shrew-1 are in two independent complexes which only interact in rather mature junctions. This might be a sort of anchoring requiring additional bridging protein(s), one interesting candidate being caveolin-1, since it is now known that basolateral targeting of membrane proteins is mediated exclusively by direct interaction with vesicle coat components (Puthenveedu et al., 2003) and caveolin-1 is one of them (described later in detail).

The fact that no interaction could be found between shrew-1 and N-cadherin-β-catenin complex in non-polarised cells however favours the idea that β-catenin alone is not sufficient to target shrew-1 into adherens junctions, but other, as yet unidentified components might also be necessary. One component could be E-cadherin itself, although it was not possible to identify a direct interaction between full-length E-cadherin and the cytoplasmic domain of shrew-1 *in vitro* with the used assay conditions. The possibility that an interaction between E-cadherin and shrew-1 might be mediated via the transmembrane and/or the extracellular domains of shrew-was explored by performing in vivo interaction assays between E-cadherin and the deletion mutants of shrew-1 (Fig. 26). Both the ectodomain and the cytoplasmic domain fused to the transmembrane domain showed a complexing with E-cadherin, suggesting that the transmembrane domain is quite important for this

interaction. However, a stronger complexing was obtained with the cytoplasmic domain fused to the transmembrane domain, suggesting the presence of basolateral targeting signals in the cytoplasmic domain that target shrew-1 to the basolateral part of the membrane where shrew-1 and E-cadherin interact.

4.3.2. Probable role of shrew-1 in the regulation of *Adherens Junctions*

From the observations made till now, one could hypothesise that shrew-1 as an integral component of cadherin-catenin complexes at the AJs might play a role in the regulation of these junctions. Since an important aspect of junctional regulation is the balance of the components of the junctions, it seems that a disturbed shrew-1 expression or overexpression leads to junctional disruption and invasiveness in general. This is in agreement with the fact that shrew-1 was isolated from an epithelial endometriotic cell line, EEC145T that does not contain any E-cadherin based adherens junctions. The cells turn non-invasive and lose shrew-1 at the same time, hence supporting the hypothesis that shrew-1 plays some role in the regulation of molecules associated with invasion.

On the other hand, it was also seen that shrew-1 is present in some normal epithelial tissues such as kidney, suggesting its positive role in mediating cell-cell adhesion by interaction with E-cadherin-catenin complexes.

Taken together, this suggests that the positive regulation of shrew-1 is associated with its association with E-cadherin-catenin complexes and its negative regulation, which occurs in E-cadherin negative cells, as seen with EEC145T which is N-cadherin positive, leads to invasion. This is supported by the result that shrew-1 complexes specifically with E-cadherin-catenin complexes but not with N-cadherin-catenin complexes, hence strengthening the hypothesis that it strictly needs to associate with E-cadherin to function as a positive regulator of cell adhesion.

4.4. shrew-1 stable cell line

A direct evidence supporting the above mentioned hypothesis came from observation that a wild type MDCK cell line turned invasive when transfected stably with shrew-1. The normal honey-combed morphology that is typical of MDCK cells was lost and instead the cells had a transformed phenotype. The E-cadherin and β-catenin staining was no more regular, albeit was quite fuzzy and at times a partially

cytoplasmic staining was also seen especially for β-catenin. The cells apparently could not tolerate such high amounts of shrew-1 since they lost its expression after 4-5 passages, obviously implying that shrew-1 is toxic for the cells. Apart from the unusual E-cadherin staining that exhibited the loss of strong intercellular contacts, the cytoplasmic β-catenin staining was quite perplexing. This kind of staining is observed only when the cytoplasmic pool of β-catenin does not undergo degradation because of phosphorylation at its serine/threonine residues by GSK-3 β as a result of which it is activated and translocates to the nucleus inducing the transcription of certain oncogenes (Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998). A detailed inspection into this observation was done and activation of β-catenin was measured by TOP Flash assays. In accordance with the immunofluorescence result, a high amount of activated β-catenin was measured in shrew-1-GFP stable cell line as compared to the GFP alone or untransfected MDCK cell line. Although on one hand this result provided further evidence to strengthen the belief about the association of shrew-1 with invasiveness, there was an obvious discrepancy in this result obtained between shrew-1 cell lines transfected transiently and stably. The explanation for this discrepancy would be that the large amounts of shrew-1 present in all cells instead of a small number of cells (when transfected transiently) transmits signals to the neighbouring cells for junction disruption. It is most likely that a small number of cells overcome this effect of shrew-1 overexpression since all the cells in the neighbourhood have normal junctions and cell-cell adhesion is a process involving adjacent cells. Nevertheless, this cell line could provide a useful tool to gather more information about the mechanism behind the association of shrew-1 in invasive processes.

4.4.1. Study of β-catenin and the mechanism behind its activation in the shrew-1 stable cell line

The dissociation of catenins from cadherins in response to different stimuli is a well known phenomenon (Kinch et al., 1995). Normally the β-catenin released from E-cadherin complex is targeted for degradation by forming a complex with APC which then triggers the ubiquitin-mediated β-catenin degradation (Bienz, 1999). The β-catenin molecules that escape this degradation are targeted to nucleus where they heterodimerise with TCF/Lef transcription factor and initiate the expression of several oncogenes. In the case of shrew-1 stables, one could postulate that there is excess of β-catenin that is dissociated most probably as an after effect of disruption of cadherin-catenin complexes at the AJs or by competitive binding of excess shrew-1 to β-catenin hence decreasing the amount of β-catenin available to E-cadherin to form strong adhesion complexes at the junctions. This excess β-catenin does not form complexes with APC and GSK-3β.

To further understand this phenomenon induced by shrew-1, it would be worthwhile to test whether shrew-1 still complexes with E-cadherin and whether the interaction between E-cadherin and β-catenin still persists in this stable cell line. Lack of interaction would support the hypothesis that shrew-1 plays an important role in the maintenance of junctions via its complexing with E-cadherin.

Another level of regulation of adherens junctions is mediated by Rac1 which participates in clathrin independent endocytosis of E-cadherin in an actin-dependent manner (Akhtar and Hotchin, 2001). This group showed the involvement of Rac1 in disrupting the junctions by a physiological stimulus and observed that E-cadherin is endocytosed into intracellular vesicles that are coated with Rac1 and caveolin-1. This endocytosis was found to be clathrin-independent. The endocytosed E-cadherin is not targeted for degradation albeit for recycling which has important implications for tissue remodelling events, such as wound healing, where dynamic regulation of E-cadherin is required for the continual disruption and formation of cell-cell contacts.

Keeping in mind the observation that shrew-1 colocalises with E-cadherin in intracellular compartments on disruption of junctions by a physiological stimulus, SF/HGF, it would be quite interesting to know whether Rac1 is also involved in the endocytosis of shrew-1 and whether shrew-1 also colocalises with caveolin-1, similar to E-cadherin. Caveolin-1 is known to form complexes with E-cadherin-β-catenin complex by direct interaction with β-catenin by which it recruits β-catenin to the caveolae and inhibits Wnt signaling (Galbiati et al., 2000) .

4.5. Shrew-1 and Caveolin-1

Interestingly, it could be shown that endogenous caveolin-1 not only localises with shrew-1 but also interacts with it both in *in vivo* and *in vitro* assays. It is known from the literature and the previous findings that caveolin-1 interacts directly with β-catenin and forms a complex with E-cadherin-β-catenin complex (Galbiati et al., 2000). In additions it has been demonstrated in this study that shrew-1 also associates with the cadherin-catenin complex (Bharti et al., in press). Taken together, this data shows very clearly that shrew-1 is present in the same complex with E-cadherin-β-catenin and with caveolin-1, both being key players in the process of invasion by acting as tumor processors (see Introduction).

It can also be assumed that shrew-1 might target basolaterally via its association with caveolin-1, since it is known that basolateral targeting is mediated by interaction with adaptor proteins/vesicle coat components (Puthenveedu et al., 2003), caveolin-1 being one.

Although the functional relevance of this complexing is not known, one could hypothesise that shrew-1 itself is a coat component of the vesicles involved in the recycling of basolaterally targeted proteins during endocytosis and leads to the proper targeting of E-cadherin to the basolateral part of the membrane.

The polarized targeting of E-cadherin is of seminal importance to the maintenance of epithelial polarity and function. The accurate basolateral delivery of newly synthesized E-cadherin and of internalized and recycled E-cadherin is quite important for its incorporation into adherens junctions. In the absence or

deregulation of this mechanism, no cell-cell adhesion is possible driving the cell towards invasiveness. In the context of shrew-1 invasive cell line, it could be assumed that the deregulation of shrew-1 does not ensure the proper targeting of E-cadherin to the junctions, hence the cell turns invasive. However, this is just an assumption which requires further experimental evidence. To prove this concept, it would be important to perform electron microscopical analysis of shrew-1 localisation, whether it is present in the same endocytotic vesicles with caveolin-1 before and after disruption of the membrane via SF/HGF.

The presence of low complexity regions in shrew-1 (Peer Bork and Gisbert Schneider, personal communications) for which no structure can be predicted with the algorithms available might be significant here (Shultz et al., 1998). One general idea about the functional relevance of these low complexity regions is that they only acquire a rigid structure (e.g. an α-helix) upon interaction with another protein partner or oligomerization into a protein complex (Wright and Dyson, 1999). This feature of shrew-1 is quite reminiscent of SNAREs. SNAREs are known to be key players of vesicle docking and fusion, and only form an α-helix when they bind to their interaction partner (Fasshauer et al., 1997; Jahn and Sudhof, 1999). This similarity increases the possibility of association of shrew-1 with endocytosis. Interestingly, similar lack of structure to shrew-1 has also been seen in clathrin accessory factors such as epsin, which perform regulatory or catalytic functions at specific stages in endocytosis (Hurley and Wendland, 2002).

4.6. Future characterisation of shrew-1

In this work, shrew-1 has been identified as an integral component of the AJs that complexes on the one hand with E-cadherin and on the other hand with caveolin-1. Its association with polarisation has been shown with it specific complexing with cadherin-catenin complexes of epithelial polarised cells but not with the cadherin-catenin complexes in non-polarised, invasive cells (Bharti et al., in press).

To decipher its precise biological function in junction regulation and/or endocytosis, some further steps have to be taken and some other biological models have to be utilised. One of them would be the Identification of some yet unknown interaction partners at the membrane decipher its biological role, since shrew-1 is an integral

component of the plasma membrane. Some of the methods that can be used to identify its interaction partners at the membrane are described below:

• **Yeast spilt ubiquitin interaction analysis:** The yeast two-hybrid system is a powerful method for the *in vivo* analysis of protein-protein interactions and is also used for identifying unknown interaction partners (Bartel and Fields, 1995). However, it is not applicable for shrew-1 since it is limited to the analysis of soluble proteins or soluble domains of membrane proteins, i.e. interactions between integral membrane proteins cannot be studied. The split ubiquitin system has emerged as a valuable system to study the interactions between membrane proteins at their sites of interaction (Johnsson and Varshavsky, 1994). As a result the complete integral membrane protein can be used for interaction studies. This system basically involves one of the interaction partners to be fused to the C-terminus of ubiquitin which is tagged to a reporter protein and the other partner to the N-terminus of ubiquitin. In case of an interaction, the native ubiquitin is reconstituted and is cleaved by ubiquitin-specific proteases. This cleavage is visualised by the reporter protein attached to the C-terminus of ubiquitin. Considering the fact that shrew-1 is a mammalian membrane protein with no orthologs in yeast (Bharti et al., in press), its insertion into the yeast membrane may be different from its insertion into mammalian cells. Taking this situation into account, the split ubiquitin system is being established for use in mammalian cells (Schreiner, unpublished).

• **Bimolecular fluorescence complementation (BiFC) assay:** BiFC assay is an improvised version of fluorescence resonance energy transfer (FRET) to determine the localisation of protein interactions in living cells (Hu et al., 2002). The BiFC approach is based on complementation between two nonfluorescent fragments of the yellow fluorescent protein (YFP), when they are brought together by interactions between proteins fused to each fragment. The BiFC analysis was done to study interactions among bZIP and Rel family proteins (Hu et al., 2002) and has several advantages over other methods for the investigation of protein interactions in cells.

1. Direct visualisation of protein interactions in their normal cells.

2. The sensitivity of this method is enough to enable analysis of interactions between proteins expressed at levels comparable to many endogenous proteins.

This is essential to ensure that complex formation occurs under conditions similar to those experienced by the native proteins.

3. This system allows detection of weak and transient interactions as well, which is not possible with most other systems. Moreover, the subcellular sites of protein interactions can be determined.

This system can be exploited to determine the direct interaction between shrew-1 and E-cadherin since it was never possible to visualise this with an in vitro pull down assay due to the inability to express either of the two transmembrane proteins in bacteria. An identification of a direct interaction would provide an answer to the question whether shrew-1 is targeted to the AJs via E-cadherin or whether it is targeted *per se*.

4.7. Genetic models of shrew-1

No counterparts of shrew-1 were identified in *Drosophila*, yeast or *C.elegans* albeit in zebrafish and mouse, thus implying that shrew-1 is conserved only in vertebrates and might have a role to play in higher order tissue organisation. The shrew-1 zebrafish sequence was found to have sequence similarity with shrew-1 human sequence within the transmembrane and the cytoplasmic domain (Fig. 28). This is actually quite interesting since these domains of shrew-1 have been characterised (see Results section) for their membrane targeting and for their complexing with E-cadherin. The study of shrew-1 in zebra fish might yield some results leading to understanding of its biological role. The zebrafish *Danio rerio* is an emerging model organism for understanding vertebrate development and genetics. The similarity between humans and zebrafish, in terms of disease, extends beyond gene homology to actual similarities in the proteins involved in pathogenesis (Postlethwait et al., 2000).

```
human        1    MWIQQLLG LSSMSIRW-PGRpLGSHAWI LIAMFQLAVD LPACEALGPG
mouse        1    MWIQQLLG LSSMSIRW-PGRSLGSHAWI LIAMLQLAVD FPSCDSLGPG
zebrafish    1           SLVAWrPGGILGCRMWI LFILVHLTMD LSLCAPPGQG

human       48    PEFWLLPRSP PRPPRLW-SFRSGQPARVPA PVWSPRPPRV ERIHG--QMQ
mouse       48    PEFRLLSR-P QRPQRLW-SLRSGPPTRLPT PAWSPRAARA ERAHGpiQMQ
zebrafish   51    LTLKLLPRSV PRqqlhplSTLTGQHLRTLG VLHRVLPPPL PSVGN--HGE

human       95    MPRARRAHRP RDQAAALVPKAGLAKPPAAA KSSPSLASSS SSSSSAVAGG
mouse       96    TPRARRAHRP RDQVATLGPKGGLTKPPAAT RSSPSLA-SA TASSSIVTAG
zebrafish   99    QGGTKLLGRk srrsiqkrql ctkceltp-- -------SDK GKSVTLAEGS

human      145    APEQQALLRR GKRHLQG---DglsSFDSRG SRPTTETEFI AWGPTGDEEA
mouse      145    AAEHQGLLRR GRRHthdtef N--DFDFRG GRPTTETEFI AWGPTGDEDA
zebrafish  140    KSDVNQHLSR SRRQLKG--- D---YDSLE GRTTTVAGFI DWGPTGADEG

human      192    LESNTFPGvY GPTTVSILQT KTTVAATTT TT--TTATPM TLQTKGFTES
mouse      192    LESNTFPGgF GPTTVSILQT KTTVATTTT TTAGSTATAM TLQTKGVTES
zebrafish  184    IEeeakvtpn a--TVTSLAP TTTVTITST TTRSPQRTYA VITTahpkrh

human      240    LDPRRRIPGG VSTTEPSTSP NNGEVTQPP RILGEASGLA VHQIITITVS
mouse      242    LDPWKRTPVG VSTTEPSTSP SNGkdiQPP RILGETSGLA VHQIITITVS
zebrafish  232    s--------- ------TTQP NSRVTAKPP KPFGDTPGLA VHQIITITVS

human      290    LIMVIAALIT TLVLKNCCAQ GNTRRNSHQ RKTNQQEESC QNLTDFpsAR
mouse      292    LIMVIAALIT TLVLKNCCAP GHTRRNSHQ RKMNQQEESC QNLTDFTPAR
zebrafish  267    LIMIVAALIT TLVLKNCCAQ GNGRHSSHQ RKINQQEESC QNLTDFTPAR

human      340    VPSSLDIFTA YNETLQCSHE VRASVPVYT DETLHSTtGE YKSTFNGNR-
mouse      342    VPSSVDIFTA YNETLQCSHE VRASVPVYA DETLHST-GE YKSTFNGNR-
zebrafish  317    VSSKVDIFTA YNDSLQCSHECVRAGIPVYT DEMIQHT-PI YKTSYNGNRq

human      389    -PSSSDRHLI PVAFVSEKWF EISC
mouse      390    -TSSADRHLI PVAFVSEKWF EISC
zebrafish  366    ePSPTERQLI PVAFVSEKWF EISC
```

Figure 28. Comparison of shrew-1 protein sequences from human, mouse and zebrafish.

It is possible to design morpholinos, which are basically a stretch of nucleotides complementary to the mRNA sequence and when injected into the organism, forms a double stranded RNA as a consequence of which the whole RNA is degraded. This method is known to work most elegantly in zebra fish where the effect can be seen within 48h. By using caged morpholinos which can be activated when desired, role of shrew-1 during different stages of embryonic development can be deciphered.

More than 90% homology has been found between protein sequences of shrew-1 from mouse and human. The mouse shrew-1 protein was also found to be localised at the same position as its human counterpart because of this strong homology (Franke, unpublished). Apparently the regulation of shrew-1 is important for its role as an invasion associated protein of the adherens junctions. To study the spatio-temporal regulation of shrew-1, it would be important to study its tissue specific as

well as development stage specific regulation. For these studies, the mouse system is a beneficial tool.

Tissue specific knock outs of shrew-1 using Cre-lox system (Lakso et al., 1992) can be easily created in mouse whereby the specific regulation of shrew-1 in different tissues can be identified. Additionally, the regulation pattern of shrew-1 during different stages of development can also be studied.

5. Summary

In an attempt to search for potential candidate molecules involved in the pathogenesis of endometriosis, a novel 2910 bp cDNA encoding a putative 411 amino acid protein, shrew-1 was discovered. By computational analysis it was predicted to be an integral membrane protein with an outside-in transmembrane domain but no homology with any known protein or domain could be identified.

Antibodies raised against the putative open-reading frame peptide of shrew-1 labelled a protein of ca. 48 kDa in extracts of shrew-1 mRNA positive tissues and also detected ectopically expressed shrew-1. In the course of my PhD work, I confirmed the prediction that shrew-1 is indeed a transmembrane protein, by expressing epitope-tagged shrew-1 in epithelial cells and analysing the transfected cells by surface biotinylation and immunoblots. Additionally, I could show that shrew-1 is able to target to E-cadherin-mediated adherens junctions and interacts with the E-cadherin-catenin complex in polarised MCF7 and MDCK cells, but not with the N-cadherin-catenin complex in non-polarised epithelial cells. A direct interaction of shrew-1 with β-catenin could be shown in an *in vitro* pull-down assay. From this data, it could be assumed that shrew-1 might play a role in the function and/or regulation of the dynamics of E-cadherin-mediated junctional complexes.

In the next part of my thesis, I showed that stable overexpression of shrew-1 in normal MDCK cells. causes changes in morphology of the cells and turns them invasive. Furthermore, transcription by β-catenin was activated in these MDCK cells stably overexpressing shrew-1. It was probably the imbalance of shrew-1 protein at the adherens junctions that led to the misregulation of adherens junctions associated proteins, i.e. E-cadherin and β-catenin.

Caveolin-1 is another integral membrane protein that forms complexes with E-cadherin-β-catenin complexes and also plays a role in the endocytosis of E-cadherin during junctional disruption. By immunofluorescence and biochemical studies, caveolin-1 was identified as another interacting partner of shrew-1. However, the functional relevance of this interaction is still not clear.

In conclusion, it can be said that shrew-1 interacts with the key players of invasion and metastasis, E-cadherin and caveolin-1, suggesting its possible role in these processes and making it an interesting candidate to unravel other unknown mechanisms involved in the complex process of invasion.

6. Zusammenfassung

Auf der Suche nach Molekülen, die potentiell zur Pathogenese der Endometriose betragen könnten, wurde eine 2910 bp große cDNA identifiziert, die für ein bisher unbekanntes, hypothetisches Protein von 411 Aminosäuren codiert, das wurde shrew-1 genannt wurde. Eine Computeranalyse der Proteinsequenz ergab, dass es sich bei dem Protein um ein integrales Membranprotein handelt. Jedoch konnten keinerlei Homologien mit bekannten Proteinen oder Proteindomänen festgestellt werden.

Antikörper gegen zwei verschiedene Peptidsequenzen aus potentiellen offenen shrew-1-Leserahmen markierten in Extrakten von shrew-1 positivem Gewebe ein Protein von etwa 48 KDa. Ausserdem kann mit diesen Antikörpern ektopisch exprimiertes shrew-1 nachgewiesen werden.

Im weiteren Verlauf der Doktorarbeit konnte durch die Expression epitop-„getaggten“ shrew-1 in Epithelzellen und der Untersuchung dieser tranfizierten Zellen mittels Oberflächenbiotinylierung und Immunoblots sowie Immunfluoreszenzanalysen die Computeranalyse bestätigt werden. Es zeigte sich, dass es sich bei shrew-1 tatsächlich um ein Transmembranprotein mit einem cytoplasmatisch lokalisierten C-Terminus handelt.

Desweiteren wurde gefunden, dass shrew-1 an Adhäsionsverbindungen transportiert wird, die durch E-Cadherin vermittelt werden. Shrew-1 interagiert mit dem E-Cadherin-Catenin-Komplex polarisierter Epithelzellen (MCF7- oder MDCK-Zellen). Im Gegensatz dazu kann in nicht-polarisierten Epithelzellen (EJ28-Zellen), welche einen N-Cadherin-Catenin-Komplex enthalten, keine Interaktion nachgewiesen werden. Eine direkte Interaktion von Shrew-1 mit β-Catenin in einem in vitro „Pull-down-assay“ lässt vermuten, dass die Einbindung von shrew-1 in den E-Cadherin-Catenin Komplex zumindest teilweise über β-Catenin erfolgen kann.

Die physiologische Auflösung der E-Cadherin-vermittelten Adhäsionskomplexe durch die Aktivierung der Rezeptortyrosinkinase c-met führt, zusammen mit

E-Cadherin, zu einer Internaliserung von shrew-1 in Vesikeln, vermutlich den caveolae, in denen E-Cadherin und shrew-1 zu kolokalisieren scheinen.
Zusammmengefasst könnten diese Daten könnten darauf hinweisen, dass shrew-1 eine Rolle in der Funktion und/oder Regulation dynamischer Prozesse in E-Cadherin-vermittelten Adhäsionsverbindungen spielen könnte.

Eine stabile Überexpression von shrew-1 in MDCK-Zellen zeigte, dass shrew-1 Veränderungen in der Zellmorphologie und, damit verbunden, der Invasivität der Zellen verursacht. Ausserdem konnte in den shrew-1 transfizierten MDCK-Zellen β-Catenin vermittelte Transkriptionsaktivierung beobachtet werden. Dies ist ist möglicherweise darauf zurück zu führen, dass ein Ungleichgewicht des transfizierten shrew-1 im Adhäsionskomplex eine Deregulation der dort befindlichen Proteine, wie z.B. E-Cadherin und β-Catenin ergibt.

Caveolin-1 ist ein weiteres integrales Membranprotein, das mit E-Cadherin-β-Catenin-Komplexen direkt interagieren kann. Außerdem spielt es eine Rolle bei der Endozytose von E-Cadherin während der Auflösung von Adhäsionskomplexen zwischen zwei Zellen. Durch Immunfluoreszenz und biochemische Untersuchungen konnte Caveolin-1 in dieser Arbeit als ein weiterer Interaktionspartner von shrew-1 identifiziert werden. Eine funktionelle Relevanz dieser Interaktion ist zur Zeit allerdings noch unklar.

Zusammenfassend kann gesagt werden, dass shrew-1 mit „Key-playern“ von Invasion und Metastasierung, nämlich E-Cadherin und Caveolin-1, interagiert. Dies deutet auf eine wichtige Rolle von shrew-1 in diesen Prozessen hin und macht es zu einem interessanten Kandidaten, an dem weitere Mechanismen im komplexen Prozess der Invasivität von Zellen erforscht werden können.

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8. Curriculum vitae

Sanita Bharti (maiden name Chandra)

Born:	14.06.1974	Chandigarh, India
Education:	1993-1996	Bachelors of Science (Hons. School), Microbiology, Panjab University, Chandigarh
	1996-1999	Masters of Science, Microbiology, Panjab University, Chandigarh
	1999-2000	Diploma Thesis “Characterisation of a novel membrane protein-shrew-1 isolated from an invasive endometriotic cell line” under supervision of Prof. Dr. Anna Starzinski-Powitz.
	2000-2003	Ph.D. work in the group of Prof. Dr. Anna Starzinski-Powitz

9. Own publications

Kashyap, P., **Chandra, S**., Kaul, A. And Tewari, R. (2000). Production, purification and characterization of pectinase from a Bacillus sp. DT7. *World Journal of Microbiology and Biotechnology.* **16:** 277-282.

This paper describes the purification of pectinase from a soil isolate, Bacillus sp. DT7 that produces high amounts of an extracellular pectinase characterized as pectin lyase.

Bharti, S., Handrow-Metzmacher, H., Zickenheiner, S., Zeitvogel, A., Baumann, R., and Starzinski-Powitz, A. (in press). Novel membrane protein shrew-1 targets to cadherin-mediated junctions in polarised epithelial cells. *Molecular Biology of the Cell.*

This paper describes the work explained in this thesis, the characterisation of shrew-1, a novel integral membrane protein that targets specifically to the E-cadherin-catenin complexes in polarised cells but does not show any complexing with the cadherin-catenin complexes in non-polarised cells.

Bharti, K**.**, von Koskull-Döring, P., **Bharti, S**., Kumar, P., Tintschl-Körbitzer, A., Treuter, E., and Nover, L. Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with At-CBP/HAC1 (submitted).

This paper describes HsfB1, a heat stress inducible member of class B Hsf family, as a novel type of transcriptional coactivator in plants. The coactivator function of HsfB1 depends on a single lysine residue in the –GRGK motif in the CTD. The GRGK motif acts as a recruitment motif, and together with the other acidic activator is responsible for corecruitment of a histone acetyl transferase (HAT).

Hiermit erkläre ich eidesstattlich, dass ich die vorliegende Dissertation selbständig und nur unter Verwendung der angegebene Literatur und Hilfsmittel angefertigt habe.

Frankfurt am Main, den 14.10.2003