

Gene expression analysis in DLBCL; Identification and characterization of novel proteins.

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Helena Hauge

Oslo, February, 2008.

Errata

The following corrections (underlined> have been made in the text:

Page 8, paragraph 4, line 1: **Hauge H**, Movig T, Narvhus K...

Article IV, page 9, paragraph 2, last line: ...than control cells (Fig. 6A).

Article IV, page 10, line 2: ...cytometry (Fig. 6B).

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Abbreviations

BCR	B-cell receptor
B-NHL	B-cell non-Hodgkin's lymphoma
CDK	cyclin-dependent kinase
DLBCL	diffuse large B-cell lymphoma
ECM	extracellular matrix
Est	expressed sequence tag
FL	follicular lymphoma
GC	germinal center
HGF	hepatocyte growth factor
Ig	immunoglobulin
MAP	microtubule-associated protein
MT	microtubule
MTOC	microtubule organizing center
NHL	non-Hodgkin's lymphoma
ORF	open reading frame
PI3K	phosphatidylinositol-3 kinase
RDA	Representational Difference Analysis
RNAi	RNA interference
siRNA	short interfering RNA
+TIP	plus-end tracking protein

1. List of included publications

- (I) **Hauge H**, Patzke S, Delabie J, Aasheim HC.
Characterization of a novel immunoglobulin-like domain containing receptor.
Biochem Biophys Res Commun. 2004 Oct 22;323(3):970-8.
- (II) Patzke S, **Hauge H**, Sioud M, Finne EF, Sivertsen EA, Delabie J, Stokke T, Aasheim HC.
Identification of a novel centrosome/microtubule-associated coiled-coil protein involved in cell-cycle progression and spindle organization.
Oncogene. 2005 Feb 10;24(7):1159-73.
- (III) **Hauge H**, Patzke S, Aasheim HC.
Characterization of the FAM110 gene family.
Genomics. 2007 Jul;90(1):14-27.
- (IV) **Hauge H**, Movig T, Narvhus K, Sioud M, Aasheim HC.
FAM110C involved in the crosstalk between the cytoskeleton and Akt signaling; a role in cell spreading and migration.
Submitted.

2. Introduction

The human genome encodes ~30 000 genes, and due to alternative splicing and post-translational modifications they give rise to a much larger number of proteins with distinct functional properties. During development, pluripotent cells mature towards increasingly more committed cell lineages and the gene-expression programs become more defined and restricted. Different sets of genes are active in different cell types, tissues and organs, depending on the regulation by different combinations of transcription factors and epigenetic modifications (Reik, 2007). Because many genes and proteins have yet to be characterized, the study of genes and their protein products is important for understanding the biology of both normal and abnormal cells. During carcinogenesis, cells acquire mutations, deletions, and translocations that affect gene expression. Thus cells are selected that can escape the strict control they are normally placed under. The genetic aberrations seen in human cancers often affect cell cycle control, adhesion and migration, leading to increased proliferation, immortality and gain of invasive and metastatic potential. To identify and characterize tumor cells and improve cancer therapy, we need to define the changes in gene expression and to understand their functional effects.

2.1 Carcinogenesis

Carcinogenesis is a multi-step process driven by changes in gene expression which disrupts the normal balance between proliferation and cell death, leading to uncontrolled cell growth and tumor formation (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000). Cancer results from the accumulation of chromosomal alterations, gene mutations, and epigenetic changes that promote malignant growth. These changes include: autocrine production of growth factors, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). The targeted genes are generally classified as proto-oncogenes when involved in the promotion of cell growth and survival, and tumor-suppressor genes when involved in negative regulation of proliferation. Typically, a series of mutations in both proto-oncogenes and tumor-suppressor genes are required

before the signals for cell growth overwhelm the signals to regulate it, and a normal cell transforms into a cancer cell.

The molecular basis of cancer has been the focus of intense investigation for many years. Although many molecular consequences of cellular transformation have been identified, we are just beginning to understand the events that lead to the neoplastic phenotype.

2.2 The cell-division cycle: Growth control and cancer

Normally, the balance between proliferation and programmed cell death is maintained by tightly regulating both processes to ensure the integrity of organs and tissues. Mutations that lead to cancer disrupt these orderly processes by disrupting growth control and have led to the view of cancer as a cell cycle disease (Malumbres and Barbacid, 2001; Malumbres and Barbacid, 2007).

Briefly, the cell cycle is divided into four distinct phases: the synthetic phase (S phase) where the chromosomes are duplicated; the mitotic phase (M phase) where the cell divides; and two gap phases (G_1 and G_2 phase) where the cell has additional time for growth and prepares for the completion of S and M phases, respectively (Norbury and Nurse, 1992) (Fig. 1). The G_1 , S, and G_2 phases are collectively termed interphase, where the cell grows continuously. A mammalian cell has an average cycling time of about 24 hours, and in most cells the whole M phase takes only about an hour. When cells cease proliferation, usually in response to a lack of growth factors or nutrients, they exit the cell cycle and enter a non-dividing, quiescent state known as G_0 . Although, cells can recover from G_0 and enter the cycle in G_1 . Except for haematopoietic and epithelial cells most of the cells in the adult organism are quiescent, yet many tumor cells that undergo uncontrolled proliferation originate from such cells.

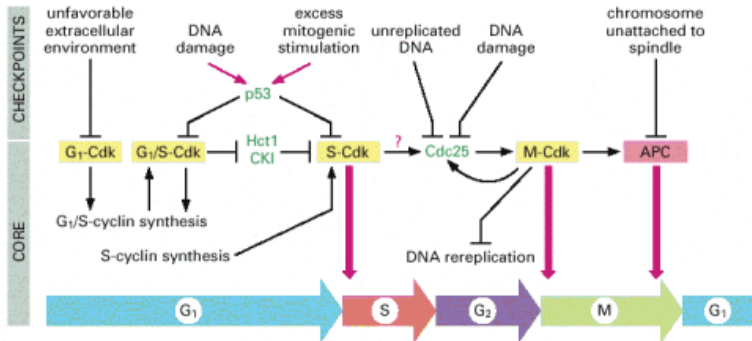


Figure 1. An overview of the cell-cycle control system. The core of the cell-cycle control system consists of a series of cyclin-Cdk complexes (yellow). The activity of each complex is also influenced by various inhibitory checkpoint mechanisms, which provide information about the extracellular environment, cell damage, and incomplete cell-cycle events (top) (Alberts et al., 2002).

To ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one (Hartwell and Weinert, 1989) (Fig. 1). These surveillance mechanisms are based on an intricate network of protein kinases known as the cyclin-dependent kinases (Cdks) and their regulatory binding partners, the cyclins (Malumbres and Barbacid, 2005). Different cyclin-Cdk complexes are assembled and activated to control the mammalian cell cycle. Cdk4/6–cyclin D and Cdk3–cyclin C complexes regulate the G_0 – G_1 transition and the early phases of G_1 by phosphorylating the retinoblastoma protein (pRb). Cdk2–cyclin E complexes have been proposed to complete phosphorylation of pRb, an event that is thought to convey mitogenic independence to dividing cells. Cdk2–cyclin E complexes have also been implicated in the G_1 –S transition by licensing DNA origins of replication. Cdk2 later associates with cyclin A during progression through S phase. Cdk1 participates in the S– G_2 and G_2 –M transitions by sequential binding to cyclin A and cyclin B (Malumbres and Barbacid, 2005). It has been well established that deregulation of Cdks is one of the most frequent alterations in human cancer, leading to abnormal cell division and genomic instability (Malumbres and Barbacid, 2007).

The centrosome orchestrates the assembly and organization of the bipolar spindle during mitosis and is therefore a prerequisite for the maintenance of genetic stability (Kramer et al., 2003; Kramer et al., 2005). This organelle has also been shown to be involved in the coordination of cell-cycle progression by serving as a scaffold for anchoring of an extensive number of regulatory proteins, including several Cdks, cyclins and other cell-cycle regulators (Doxsey et al., 2005b). Furthermore, the centrosome has been linked to the stress response mechanism and tumorigenesis (Andersen et al., 2003; Doxsey et al., 2005b; Doxsey, 2001b; Fisk et al., 2002; Lange, 2002; Rieder et al., 2001; Sluder, 2005). The microtubule organizing center (MTOC) activity of the centrosome, that forms the interphase microtubule (MT) array and cilia, will be discussed in section 2.3.2.

2.3 Adhesion and migration

Most of the cells in multicellular organisms are organized into tissues, which in turn are organized in various combinations to form organs. The cells in tissues are usually in contact with a complex network of secreted extracellular macromolecules referred to as the extracellular matrix (ECM). The ECM holds cells and tissues together, through cell-matrix adhesions, and provides an organized lattice within which cells can migrate and interact with one another by cell-cell adhesions. These adherens junctions are connected to the cytoskeleton of a cell through transmembrane linker proteins and intracellular attachment proteins that allows the cell to communicate with its surroundings. (Alberts et al., 2002) In response to internal and external signals cells rapidly alter their shape and behavior. These dynamic events require extensive remodeling of adhesive interactions as well as of the actin and MT cytoskeletons. During carcinogenesis, such remodeling can lead to loss of normal tissue architecture and proliferation control, whereby cells become motile and invasive (Yamazaki et al., 2005).

2.3.1 The actin cytoskeleton

The actin cytoskeleton is one of the major structural components of the cell and can be found in all kinds of eukaryotic cells, maintaining their shapes and motilities. It often undergoes rapid reorganization and plays crucial roles in a number of dynamic cellular processes including cell migration, cytokinesis, membrane trafficking, and

morphogenesis (Alberts et al., 2002). Actin subunits assemble into long filamentous polymers called F-actin where two parallel F-actin strands twist around each other in a helical formation, giving rise to the microfilaments of the cytoskeleton (Steinmetz et al., 1997). In contrast to MTs that are anchored at the centrosome in the cytosol, actin nucleation occurs at the plasma membrane (Condeelis, 2001). Consequently, the highest density of actin filaments in most cells is at the cell periphery where they determine the shape and movement of the cell surface. Depending on their attachments to one another and to the plasma membrane, actin structures can form different types of cell surface projections. These include spiky bundles such as microvilli or filopodia, which are radially oriented fine bundles of actin filaments, flat protrusions called lamellipodia consisting of meshworks of branched actin filaments (Biyasheva et al., 2004), and contractile stress fibers that are linear actomyosin structures comprised of actin filaments of mixed polarity and myosin II (Cramer et al., 1997).

Actin nucleation occurs through the coordinated activities of two major factors, Arp2/3 and mDia. The Arp2/3 complex binds to the sides of pre-existing filaments and generate a branched network to promote membrane protrusion, like those found in lamellipodia (Condeelis, 2001). In contrast, mDia which belongs to the formin family of proteins, binds to the barbed ends of actin filaments where it nucleates linear unbranched actin filaments found in filopodia, stress fibers and cell adhesions (Goode and Eck, 2007). Cofilin is a protein also involved in the regulation of actin dynamics that severs actin filaments, leading to an increase in uncapped barbed ends that can serve both as sites of actin polymerization and actin monomer dissociation (Condeelis, 2001). The activities of all of these proteins are regulated by intracellular signaling molecules, the Rho GTPases, at the cytosolic face of the plasma membrane (Hall, 2005), as will be discussed further in section 2.3.5.

2.3.2 The microtubule cytoskeleton

The centrosome is the major MTOC of animal cells and directs the assembly of the interphase MT cytoskeleton, the mitotic spindle, primary cilia and cytokinesis (Doxsey, 2001a). The centrosome consists of a pair of centrioles that is surrounded by the

pericentriolar material comprised of more than 300 different proteins reported so far (Doxsey et al., 2005b). Nucleated at this organelle near the cell center and in close proximity to the nucleus, the interphase MTs emanate in a star-like conformation. MT polymers of α/β -tubulin heterodimers are anchored by their minus ends, and the plus ends point outward and grow toward the cell periphery (Doxsey, 2001a). While α - and β -tubulins are the regular building blocks of MTs, another type of tubulin, called γ -tubulin form a γ -tubulin ring complex (γ -TuRC) that nucleates a MT with 13 protofilaments forming the wall of this hollow polymer (Doxsey, 2001a). During interphase, the centrosome duplicates and splits into two daughter centrosomes. These two daughter centrosomes move to opposite sides of the nucleus at the start of mitosis, forming the two poles of the mitotic spindle that directs chromosome segregation and cytokinesis (Doxsey, 2001a).

Polarized epithelial cells also have an extensive non-centrosomal MT network, including the apico-basal array extending from the apical membrane, and a cortical MT network associated with the basal membrane. These MTs could be derived by nucleation and release from the centrosome (Mogensen, 1999). However, results from experiments with plants and fission yeast indicate that γ -tubulin-mediated MT nucleation is not restricted to the centrosome, but also occur at so called secondary MTOCs (Luders and Stearns, 2007). γ -tubulin has been demonstrated to localize to the cortex of basal membrane patches, along cortical MTs and at MT branch points, and could serve to nucleate MTs at these sites (Reilein et al., 2005).

Cilia are MT-based organelles evolutionarily related to the motile flagella of lower eukaryotes that project like antennae from the surface of most cells in the body (Marshall and Nonaka, 2006). A cilium arises from a basal body, a structure that differentiates from one of the centrioles in the centrosome in non-mitotic cells and organizes the MT bundles that constitute the ciliary axoneme (Sorokin, 1962). Primary cilia act as sensors of environmental cues and are essential sites for coordination of cell signaling and development (Marshall and Nonaka, 2006).

The interphase MTs are involved in cell polarization, migration, morphogenesis, cell signaling, and intracellular trafficking. These are all highly regulated processes and MT dynamics are consequently controlled both spatially and temporally by a diversity of MT-associated proteins (Cassimeris, 1999). Several proteins have been identified which can stabilize and destabilize MTs and the balance between these accessory proteins is critical to cell fate. MTs are usually highly dynamic and undergo rapid turnover by exchange of subunits ($t_{1/2} = 5-15$ min) (Cassimeris, 1999). However, within the cytoplasmic network there also exists a stable subpopulation of MTs with increased half-life ($t_{1/2} = 1-2$ hr), which can be distinguished by a variety of posttranslational modifications (PTMs) (Verhey and Gaertig, 2007). These tubulin PTMs are suggested to dictate the recruitment of MT effectors like MT-associated proteins (MAPs), plus-end tracking proteins (+TIPs), and molecular motors, which contribute to MT stability and MT-based functions in specific cellular locations (Verhey and Gaertig, 2007).

Several actin regulatory proteins, like formins, myosins, and plakins have also been shown to be involved in the regulation of MT stability (Basu and Chang, 2007; Even-Ram et al., 2007; Wen et al., 2004). Through interaction with both actin and +TIPs such as end-binding protein 1 (EB1) and adenomatous polyposis coli (APC), actin regulatory proteins are capable of linking the dynamic plus ends of MTs to polymerizing ends of actin filaments and thereby contribute to the important interplay between the actin and MT cytoskeletons (Basu and Chang, 2007). In return, MT plus-ends have been shown to deliver formin and other actin regulatory proteins at the cell cortex to regulate actin assembly (Basu and Chang, 2007). This cross-talk between the MT and actin cytoskeletons at the cell periphery, where MTs provide directional cues for motility and help trigger local polymerization of actin networks, is necessary for proper regulation of membrane protrusion and cell migration (Palazzo and Gundersen, 2002).

By changing their dynamics in response to signaling pathways, MTs themselves may contribute to signal transduction processes. This is suggested by the multiple effects of MT-stabilizing and -destabilizing drugs on signaling pathways, and by the discovery of signaling molecules, including phosphatases and kinases, that interact with MTs

(Gundersen and Cook, 1999). Mechanisms including MT sequestering and release, MT delivery, and MT scaffolding of signaling molecules could be involved in this MT-mediated signal transduction (Gundersen and Cook, 1999).

2.3.3 Cellular polarization

Cellular polarization is a process mediated through asymmetric distribution of signaling molecules and the cytoskeleton, in addition to positioning of the endoplasmic reticulum (ER) and the Golgi apparatus, and directed membrane trafficking (Watanabe et al., 2005). Different types of cell polarity include the apical-basal polarity found in epithelial monolayers, the anterior-posterior polarity displayed by migrating cells, and T cell immunological synapse formation. However, cellular polarization is a functional aspect of almost every cell type (Nabi, 1999). In all cases the generation of cell polarity requires active remodeling of both the MT and actin cytoskeletons where actin polymerization drives membrane protrusion and generates motility and MTs are necessary for the polarization of these activities (Fig. 2).

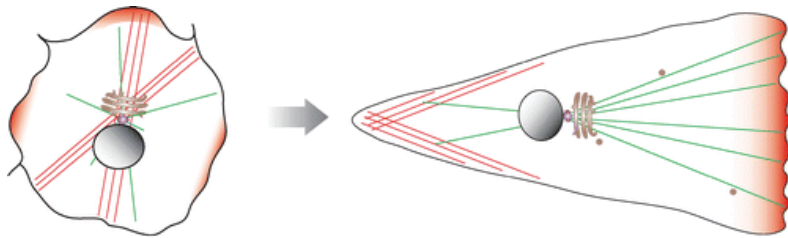


Figure 2. Cell migration requires actin-dependent protrusions at the front (red) and contractile actin:myosin filaments (red) at the rear. In addition, microtubules (green) originating from the centrosome (purple) are preferentially stabilized in the direction of migration allowing targeted vesicle trafficking from the Golgi (brown) to the leading edge (Jaffe and Hall, 2005).

An important aspect of MTs regarding cell polarization is that the filaments span the distance from the nucleus to the plasma membrane. With associated motor proteins moving back and forth, they provide a system for directional flow of information (Gundersen and Cook, 1999; Nabi, 1999). A resting cell receiving a signal from the environment to migrate reorients its MT array towards the direction of migration (Fig. 2). Here the plus ends are stabilized at the leading edge resulting in a polarized MT array

(Gundersen et al., 2004; Watanabe et al., 2005). MTs are guided along actin filaments by actin-based motor proteins, +TIPs and cytoskeletal cross-linking proteins, and are thereby targeted to cell-cell and cell-ECM adhesion sites (Fukata et al., 2002; Kodama et al., 2003; Lantz and Miller, 1998). Once MTs and their associated proteins determine the polarity site, a positive feedback loop likely initiates where cortical polarity proteins anchor MT plus ends, and allows MTs to reinforce and maintain this polarity site (Siegrist and Doe, 2007).

2.3.4 Adhesive interactions

Cadherins and integrins are the major cell–cell and cell–extracellular matrix (ECM) adhesion receptors, respectively, and represent critical determinants of tissue architecture and function (Hynes, 2002; Wheelock and Johnson, 2003).

Integrins are heterodimeric transmembrane glycoproteins composed of non-covalently linked α and β subunits, which are endowed with both structural and regulatory functions (Delon and Brown, 2007). They link the ECM to several distinct cytoplasmic proteins and the actin cytoskeleton at focal adhesion sites (Geiger et al., 2001). There they provide both outside-in and inside-out transmission of signals across the plasma membrane that control a number of critical cellular processes, including adhesion, migration, proliferation, differentiation, apoptosis, and gene expression (Hynes, 2002). Upon cell adhesion to ECM, integrin receptors become activated and cluster in the plasma membrane. The cytoplasmic domains then recruit over 50 structural and signaling proteins into a higher order complex termed focal adhesions (Miyamoto et al., 1995; Zamir and Geiger, 2001) (Fig. 3). These complexes provide the physical link between integrin receptors and the actin cytoskeleton, as well as sites of signal transduction into the cell (Zamir and Geiger, 2001). As actin polymerization drives membrane protrusion, focal adhesions are formed at the protruding edge allowing attachment of cells to the ECM and thereby generating a tractile force that cells use for locomotion (Carragher and Frame, 2004). The regulated turnover of focal adhesion complexes is also required for cell migration, since it allows release of the migrating cell to enable net forward

movement (Carragher and Frame, 2004). MT ends that target these sites have been shown to be implicated in the disassembly of focal adhesions (Ezratty et al., 2005).

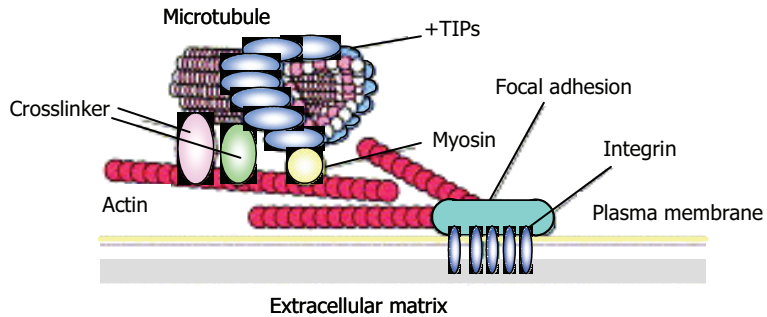


Figure 3. Upon cell adhesion to ECM, integrin receptors become activated and recruit structural and signaling proteins into a higher order complex termed focal adhesions. These complexes provide the physical link between integrin receptors and the cytoskeleton (Watanabe et al., 2005).

Cadherins are single-pass transmembrane glycoproteins that support calcium-dependent, homophilic cell–cell adhesion (Halbleib and Nelson, 2006). Together with their cytoplasmic domain interactors, α -catenin and β -catenin, they constitute the core components of adherens junctions. These specialized adhesive structures link the cadherin homophilic adhesion to the actin cytoskeleton, and are required for formation and maintenance of stable cell–cell adhesion and a differentiated phenotype in all solid tissues (Wheelock and Johnson, 2003).

2.3.5 Signaling pathways in adhesion and migration

Cell adhesion and migration are essential processes not only during development, but throughout life such as in tissue organization, wound repair, and immune surveillance. The cell must be able to respond rapidly to changes in its external environment and re-organize its actin and MT cytoskeltons to change shape and localization in response to signals from soluble factors or ECM. A huge variety of intracellular signaling molecules have been implicated in these processes, including Rho GTPases, phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinases (ERK/MAPK), protein kinase C (PKC), phospholipase C (PLC), and tyrosine kinases (Merlot and Firtel, 2003; Raftopoulou and

Hall, 2004; Zhelev and Alteraifi, 2002). Moreover, the overall signaling of adhesion and motility is additionally complicated by significant cross-talk between the different pathways.

Rho GTPases belong to the Ras superfamily of small GTPases and are central in the control of cell behavior (Hall, 2005). They regulate and coordinate signal transduction pathways that link cell surface receptors to changes in the organization of the actin and MT cytoskeletons, in vesicular trafficking and in gene transcription (Raftopoulou and Hall, 2004). Rho GTPases act as molecular switches by cycling between a GDP-bound, inactive form and a GTP-bound, active form. The Rho GTPase cycle is regulated by guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP, GTPase activating proteins (GAPs) that stimulate GTP hydrolysis, and guanine nucleotide exchange inhibitors (GDIs) that sequester the GDP-bound form from membranes (Etienne-Manneville and Hall, 2002; Raftopoulou and Hall, 2004). In their GTP-bound state, Rho GTPases interact with cellular target proteins to generate a downstream response (Bishop and Hall, 2000).

During cell spreading, a series of morphological changes transpire when studying the actin cytoskeleton; Rho induces the assembly of actin stress fibers and focal complexes at the cell periphery; Rac controls the formation of lamellipodia, and Cdc42 triggers the formation of filopodia. Both types of membrane protrusions can occur simultaneously, and both play a role in migration (Hall, 2005). Recent studies have shown that Rho GTPases also regulate the dynamics of MTs through effectors which interact with +TIPs and MAPs (Watanabe et al., 2005). In turn, MTs affect the activities of Rho GTPases, apparently through modulation of the activity of GEFs (Siegrist and Doe, 2007; Watanabe et al., 2005; Wittmann and Waterman-Storer, 2001), and thereby influence on actin polymerization.

The different organization of actin filaments in lamellipodia and filopodia suggests that these membrane protrusions are formed by different molecular mechanisms triggered by separate pathways. The paradigm has been that protrusion of lamellipodia and filopodia

is regulated by two parallel pathways: from Rac through Scar/WAVEs to lamellipodia, and from Cdc42 through N-WASP to filopodia (Faix and Rottner, 2006). Although, it has been suggested that neither Rac nor Cdc42 are indispensable as several of the 22 mammalian Rho-GTPases have been described to induce both lamellipodia and filopodia (Aspenstrom et al., 2004). Furthermore, it appears that in many of the studied systems filopodia emerge from lamellipodia, suggesting that the lamellipodium serves as a precursor structure (Biyasheva et al., 2004; Svitkina et al., 2003).

Both integrins and cadherins have been shown to activate Rho GTPases (Braga and Yap, 2005; Hall, 2005). In this way Rho GTPases could participate in the coordinated modulation of the cellular functions of these adhesion receptors which is essential to morphogenesis, tissue differentiation and renewal, wound healing, immune surveillance, and tumor progression (Retta et al., 2006).

2.4 B-cell non-Hodgkin's lymphomas

B-cell non-Hodgkin's lymphomas (B-NHL) are malignant tumors that arise from mature B lymphocytes. NHL accounts for approximately 4% of all new diagnosed cancers in Western countries, and lymphomas of B cell origin constitute about 85% of all NHLs. The remainders arise from T lymphocytes. The B lymphocytes constitute the humoral antibody response of the immune defense and secrete antibody molecules, or immunoglobulins (Igs), in the circulation that bind specifically to non-self antigens (Janeway et al., 2004). These antibodies, which are secreted forms of the membrane-bound B-cell receptor (BCR), inactivates the infectious agent by preventing the binding to receptors on host cells and marks it for destruction by other components of the immune system (Janeway et al., 2004). A hallmark of many types of B-cell lymphoma is reciprocal chromosomal translocations involving one of the Ig loci and a proto-oncogene (Kuppers, 2005). As a consequence of such translocations, the oncogene comes under the control of an active Ig locus, causing deregulated, constitutive expression of the translocated gene (Kuppers, 2005). Thus, key transforming events rely on processes that are crucial for normal B-cell development and are also the basis for determination of the

origin of the various human B-cell lymphomas (Kuppers et al., 1999; Stevenson et al., 2001).

Early B-cell development takes place in the bone marrow and concludes when a functional and non-autoreactive BCR is expressed on the surface. This development includes site-specific genetic recombination of Ig genes (V(D)J recombination) which is the basis for the huge antibody repertoire and ensures that any given non-self antigen can be recognized (Jung and Alt, 2004). These distinct gene rearrangements also equip each B cell with individual clonal markers that constitute an important basis for the analysis of B-cell lymphomas. Mature, naïve B cells leave the bone marrow and enter the circulation. Upon encounter with their specific antigen, B cells become activated and collect in the germinal centers (GCs) of secondary lymphoid organs including, lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT). In the GCs, the Ig genes of antigen-activated B cells are modified by somatic hypermutation. As a result, some B cells produce antibodies with increased affinity towards the antigen and undergo clonal expansion. Furthermore, class switch recombination alters the effector functions that the antibody can engage (Janeway et al., 2004). The process of somatic hypermutation is $\sim 10^6$ times greater than the spontaneous mutation rate in other genes. Altogether, the means by which antibody diversity is generated both in the bone marrow and the GCs, involving DNA double strand breaks, jeopardize the DNA integrity of the developing B cells and are important factors for lymphoma pathogenesis (Kuppers, 2005).

B-NHLs constitute a heterogenous group of tumors. This heterogeneity is also reflected in clinical outcome and response to treatment. In the current WHO classification, lymphomas are grouped according to morphology, immunophenotype, and genetic and clinical features (Harris et al., 2000). The aim is to create homogenous subgroups in order to optimize the treatment and improve clinical outcome. About 15 different types of B-NHL have been defined according to the WHO classification (Harris et al., 2000). When assigned to their proposed normal B-cell counterparts determined by the mutational status of the Ig genes, most lymphomas are derived from GC B cells (ongoing somatic hypermutation) or from B cells that have passed through the GC (completed

hypermutation) (Kuppers, 2005) (Fig. 4). The two largest lymphoma subtypes are follicular lymphoma (FL) (~20%) and diffuse large B-cell lymphoma (DLBCL) (~30-40%).

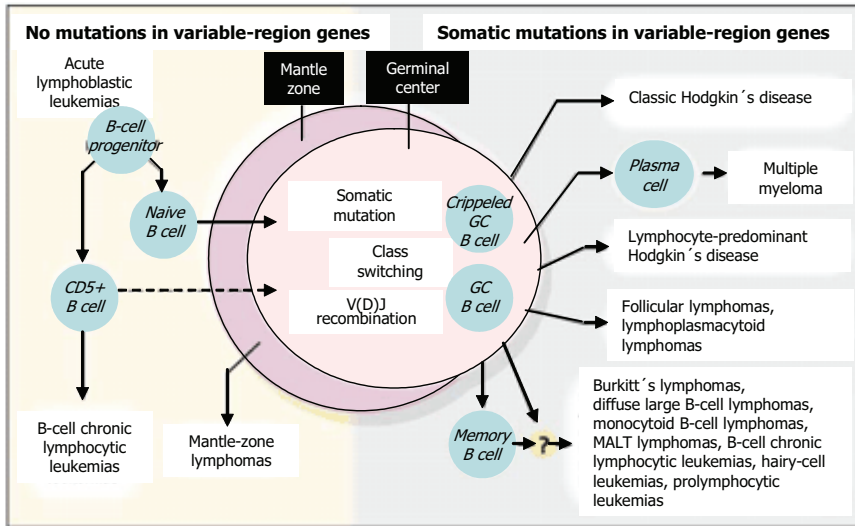


Figure 4. Assignment of human B-cell lymphomas to their normal B-cell counterparts (Kuppers et al., 1999).

2.4.1 Follicular lymphoma

Although, FL starts as a relatively indolent malignancy with a median survival of 8-9 years, they are generally incurable. 10-60% of FLs transform to the more aggressive form DLBCL, often accompanied by less than one year survival (Knutsen, 1997; Lossos and Levy, 2003; Sigal et al., 2005). Transformation is characterized by loss of the follicular histological architecture reminiscent of normal GCs, to a diffuse growth pattern (Fig. 5).

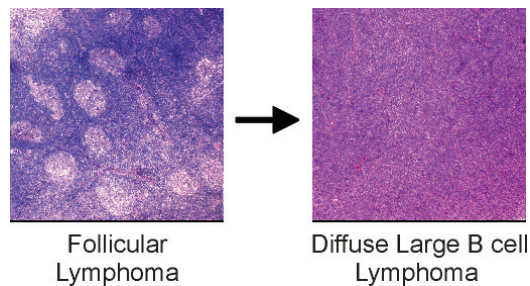


Figure 5. Patient matched biopsies of FL and histologically transformed DLBCL. FLs morphologically recapitulate normal germinal centers of secondary lymphoid follicles while DLBCLs are characterized by a diffuse proliferation of large cells with a high mitotic rate.

That FL derives from normal GC B cells is further supported by the observation that they show a pattern of ongoing somatic hypermutation and display a characteristic GC B cell gene-expression signature (Alizadeh et al., 2000; Harris et al., 1994; Shaffer et al., 2001). The initiating genetic event found in the majority of FLs is the t(14;18)(q32;q21) chromosomal translocation resulting in constitutive expression of the anti-apoptotic BCL-2 gene and accumulation of cells with prolonged survival (McDonnell et al., 1989). However, the finding that BCL-2 transgenic mice do not readily develop lymphoma (McDonnell et al., 1989; McDonnell and Korsmeyer, 1991; Strasser et al., 1993) and that B cells containing a t(14;18) can normally be present in healthy human individuals (Limpens et al., 1995; Liu et al., 1994), demonstrate that additional oncogenic mutations need to accumulate to cause FL. The exact secondary alterations leading to full FL development are still poorly defined (Bende et al., 2006).

2.4.2 Diffuse large B-cell lymphoma

Histologically, DLBCL is characterized by large transformed B cells with a diffuse growth pattern and a high proliferation fraction (Fig. 5). DLBCL comprises a highly heterogeneous group of neoplasms with different genetic abnormalities and clinical features, and several different morphological variants of DLBCL have been defined according to the WHO classification (Harris et al., 2000). DLBCL can arise as a consequence of histological transformation of primary FL and accordingly retain the t(14;18), but most commonly DLBCL occurs as *de novo* DLBCL. Genetic alterations frequently observed in DLBCL are associated to BCL-2, BCL-6, TP53, and cMYC genes, affecting regulation of apoptosis, cell cycle control and proliferation (Gascoyne et al., 1997; Ichikawa et al., 1997; Lossos et al., 2002; Ye et al., 1993). Some of these alterations correlate with clinical outcomes. However, they do not accurately reflect the heterogeneous clinical courses and responses to therapy (Fisher et al., 1993; Vose, 1998).

Improvements in molecular tumor classification have been essential to the advances seen in cancer treatment. Historically, the classification of cancer types has been primarily based upon morphological appearance of the tumor. The limitation of this approach is that tumors with similar histopathological appearance can display different clinical courses and responses to therapy (Golub et al., 1999). It is now recognized that tumors with common behavior (phenotype) has a common gene expression signature, and that classification of tumors based upon their molecular signatures is much more useful for predicting patient outcome and response to therapy than morphological characterization (Chung et al., 2002). As a consequence, DNA microarray technology and gene expression profiling has provided important insights into the biology of DLBCL. Microarray analyses have revealed that DLBCL can be divided into three subtypes based on the expression of a set of sixteen genes (Alizadeh et al., 2000; Rosenwald et al., 2002). These three subtypes include GC-like DLBCL, activated B cell (ABC) -like DLBCL, and a mixed subtype lacking high expression of either the GC- or the ABC-defining genes (type III). The ABC-like subtype is suggested to originate from post GC cells on the basis of the gene expression pattern and concluded somatic hypermutation (Rosenwald et al., 2002). The subtypes were shown to have significantly different outcomes with 60% alive

at 5 years in GC-like DLBCL compared with only 35% in ABC-like DLBCL and 39% in type III (Alizadeh et al., 2000; Rosenwald et al., 2002). The major differences between GC-like DLBCL and ABC-like DLBCL are summarized in table 1.

	GC-like DLBCL	ABC-like DLBCL
Ongoing Ig mutations	Present	Absent
Cytogenetics	t(14;18)(q32;q21) Amplification of c-rel locus on chromosome 2p 12q12 gain	Trisomy 3 3q and 18q21-22 gains
Oncogenic mechanisms	BCL-6 expression	6q21-22 loss Constitutive activation of NF-kB
Blimp-1	Low mRNA expression	High mRNA expression Lack of protein expression
Intracellular signaling	cAMP modulates pAKT and pBAD Normal IL-4-induced STAT6 signaling	PDE4B inactivates cAMP Aberrant IL-4-induced STAT6 signaling due to increase STAT6 dephosphorylation
Clinical Outcome	Favorable 60% 5-year survival	Poor 35% 5-year survival

Table 1. Differences between GC-like DLBCL and ABC-like DLBCL (Morgensztern et al., 2007).

These studies on the global gene expression of DLBCLs further highlight the heterogeneity of this type of lymphoma and the need for molecular diagnosis to design individualized and molecularly targeted therapies.

3. Aims of the present study

1. To identify and characterize novel genes differentially expressed in patient-matched biopsies of low-grade follicular and high-grade diffuse large B-cell lymphoma using cDNA Representational Difference Analysis (cDNA RDA).
2. To identify molecular mechanisms potentially associated with histological transformation and thereby increase the understanding of disease progression.
3. To consider the application of identified genes in diagnostics and therapy.

4. Summary of included publications

Article I: Characterization of a novel immunoglobulin-like domain containing receptor.

We employed cDNA RDA on a patient matched pair of clonally related FL and histological transformed diffuse large B-cell lymphoma (DLBCL) for the selection of DLBCL related transcripts. One of the identified cDNA RDA fragments was selected for further characterization due to its homology to a predicted surface receptor and hybridization to cDNA fragments of different sizes in the FL and DLBCL representations, which could indicate the expression of different alternatively spliced transcripts in the two disease states. Cloning and characterization of three splice variants revealed the existence of two membrane-spanning and one cytoplasmic isoform of the gene. An N-terminal immunoglobulin-like domain was identified in all three isoforms, and the gene was therefore denoted immunoglobulin-like domain containing receptor 1 (ILDR1). *ILDR1* expression could be detected in prostate, pancreas, kidney, testis, liver, and heart. Cellular localization showed that the two isoforms containing a predicted transmembrane domain located to the cell membrane. The third isoform lacking this domain localized to the cytosol. An ILDR1 homologue, rat lipolysis-stimulated remnant receptor (LSR), with 31% amino acid identity was identified by database search. Three isoforms of LSR are identified that heteromerize to either to a trimeric or a tetrameric plasma membrane receptor (Yen et al., 1999). LSR is proposed to bind lipoproteins and to be involved in the clearance of dietary triglycerides from the circulation. The overall resemblance in splice pattern and organization of motifs, in addition to protein analyses, suggests that ILDR1 might also form heteromers. To this end, we have not identified a ligand for ILDR1. Interestingly, we observed that the expression pattern of ILDR1 isoforms was altered after transformation in five out of six paired lymphoma biopsies examined. In addition, the cytoplasmic isoform was only detected in lymphoma samples and not in any of the normal tissues or cell lines investigated. Our results could suggest that ILDR1 expression and function is altered during tumor progression.

Article II: Identification of a novel centrosome/microtubule-associated coiled-coil protein involved in cell-cycle progression and spindle organization.

In this study we applied cDNA RDA on a patient matched pair of clonally unrelated FL and DLBCL for the selection of DLBCL related transcripts. One of the identified cDNA RDA fragments was selected for further characterization due to a reported higher gene expression of a related cDNA fragment in DLBCL than in FL (Alizadeh et al., 2000) and its induced expression in activated B cells. Cloning and characterization of the gene product of this gene, denoted centrosome/spindle pole-associated protein (*CSPP1*), revealed that CSPP is expressed in human testis in addition to all cell lines examined. This could indicate a proliferation related association. CSPP is a serine phosphorylated coiled-coil protein that localizes to centrosomes and microtubules, and induces the formation of aberrant, predominantly multipolar, spindles and aneuploidy upon over-expression. CSPP over-expression is also characterized by a block in the G₁ and M phase of the cell cycle. Interestingly, CSPP depletion by short interfering RNA impaired cell cycle progression through S phase. This phenotype was characterized by elevated levels of cyclin A, decreased levels of cyclin E, and phosphorylation of the S-phase checkpoint kinase Chk 1. Activation of Chk1 could reflect a replication stress-response due to compromised DNA replication. Finally, *in silico* examination of CSPP expression in a microarray dataset comprising 240 DLBCLs (Rosenwald et al., 2002) indicates that CSPP over-expression correlates to poor prognosis in the activated B cell lymphoma subtype of DLBCL. Altogether, this report indicates that CSPP is functionally associated with cell-cycle related and cytoskeletal processes and that deregulated CSPP expression might be related to cancer progression.

Article III: Characterization of the FAM110 gene family.

In a yeast two-hybrid screen for CSPP-interacting proteins, we identified the open reading frame (ORF) C20orf55. The corresponding protein was selected for further study due to an observed co-localization with CSPP in initial screening studies. GenBank homology search revealed that C20orf55 belongs to a not yet described gene family, consisting of three members that we set out to characterize. The ORFs of these genes were cloned and denoted family with sequence similarity 110 (FAM110), member A

(C20orf55), B, and C as suggested by the HUGO Nomenclature Committee. Except from a proline-rich stretch, no known conserved domains or homology to other characterized proteins were identified. Although, three distinct motifs highly conserved in all three protein members could represent functional motifs characteristic to this family. Expression of FAM110A was detected in several tissues, including peripheral blood leukocytes and other lymphoid tissues. In addition, FAM110A expression was induced upon stimulation of CD4⁺ lymphocytes and also increased in S and G₂/M phase of the cell cycle relative to G₁ phase. FAM110B and FAM110C expression, on the other hand was detected mainly in tissues other than lymphoid tissues. Studies in transfectants showed that the FAM110 proteins localized to centrosomes and accumulated at the microtubule organizing center (MTOC) in interphase and at spindle poles in mitosis. Interestingly, FAM110C also localized to the microtubule cytoskeleton throughout the cell cycle, and induced microtubule aberrancies upon over-expression. Furthermore, all three FAM110 proteins co-localized with CSPP at the MTOC and mitotic spindle, although yeast two-hybrid analysis only indicated binding of FAM110A to CSPP. Cell cycle analysis showed that expression of FAM110B and FAM110C impaired progression through G₁ phase. Altogether, in this study we identified and initially characterized a novel protein family where all members localize to the centrosome and spindle poles. The data presented suggest that FAM110 homologues are functionally associated to cell-cycle related processes and to CSPP, but also indicate that they are differentially regulated and have somewhat different functional properties.

Article IV: FAM110C involved in the crosstalk between the cytoskeleton and Akt signaling; a role in cell spreading and migration.

Due to the explicit phenotype induced upon FAM110C over-expression (article III), displaying an aberrant MT cytoskeleton, we decided to further investigate its function. In this manuscript we show that FAM110C is expressed mainly in adherent cells of epithelial origin. Together with our previous observation, this suggested the possibility that FAM110C is involved in events characteristic to cells with an adherent phenotype, such as cell adhesion, spreading and migration. A potential role for FAM110C in these cellular processes was therefore investigated. We present data showing that depletion of

FAM110C reduced integrin-mediated filopodia formation, HGF-induced migration, and Akt activation in the epithelial cell line HepG2. Furthermore, ectopically expressed FAM110C co-precipitated and co-localized with Akt1 and the actin-cytoskeleton organizer ezrin. In particular, co-localization and biochemical studies indicated an interaction between active Akt1 and FAM110C. We also show that FAM110C binds to and stabilizes MTs, demonstrated by increased acetylation levels and resistance towards the MT-depolymerizing drug nocodazol. These results provide the first evidence for a role of FAM110C in adhesion and migration, and suggest that FAM110C could couple the actin and MT cytoskeletons at the cell cortex promoting membrane protrusion, cell adhesion, and migration events in an Akt-dependent fashion.

5. General discussion

5.1 Methodological considerations

5.1.1 Expression analysis: General aspects

Because different sets of genes are active in different cell types, tissues, and organs an important approach to gain knowledge about biological processes or the function of genes, is to perform gene expression analyses. Traditionally, measurement of gene expression has been performed at a single target level, but the development of different high throughput technologies now allows functional genome-wide analysis. The methods used for analysis in this study comprise both strategies and include Northern blot analysis, semi-quantitative reverse-transcription PCR (RT-PCR), real-time PCR, and cDNA RDA. Northern blot analysis is a classical method for quantitative analysis of gene expression. By this method various characteristics of mRNA can be examined including mRNA abundance and mRNA size. The limitations of Northern blot analysis relates to the need for relatively large amounts of RNA and the fairly slow and labor-intensive methodology. Following the development of PCR, a number of amplification-based applications for gene expression analysis have emerged. The most common of these is RT-PCR, a method based on amplification of target sequences contained within a cDNA population. RT-PCR is rapid, extremely sensitive and specific, and requires very little template RNA. Real-time PCR has become the more frequently used version of this technique, because in addition to all the advantages of RT-PCR real-time PCR also features a quantitative output and an automated process. RT-PCR and real-time PCR are employed to assess the expression of specific genes, but are of limited value in a study aimed at gene discovery. Here differential display technologies such as cDNA RDA can be employed. This method will be discussed in detail in the next section. There are several important aspects regarding gene expression analysis that are common for all the different technologies:

- The experimental data obtained are dependent on the quality of the RNA preparations. Thus the proper handling of cells and tissues is crucial for reliable comparison of independent samples.

- The specificity of the assay relies on complementarities in the sequences of probe and target and is influenced by the stringency of the hybridization.
- The experimental data obtained may be influenced by cellular heterogeneity.
- To compare gene expression levels between different samples, the amount and quality of the RNA put into the experiment needs to be normalized. In addition, it is common to relate the expression of the genes of interest to that of a housekeeping gene such as *β-actin*, *GAPDH*, or *PGK1* that is supposedly expressed at a steady level.

5.1.2 cDNA Representational Difference analysis

The initial basis for our studies was to use cDNA Representational Difference Analysis (cDNA RDA) to detect gene expression differences between two cDNA populations, represented by different stages of disease. RDA is a method that employs subsequent rounds of PCR amplification coupled to subtraction and was originally developed to identify the differences between two complex genomes (Lisitsyn et al., 1993). The methodology was later modified for the analysis of cDNA and is based on the elimination of fragments present in both populations, leaving only the differences (Hubank and Schatz, 1994). Briefly, the procedure relies on generation of representations of cDNA fragments from two different mRNA populations by digestion with the four-cutting enzyme DpnII followed by linker ligation and PCR amplification (Fig. 6A). A restriction site theoretically present for every 256 base pairs in a random sequence ensures that the majority of cDNA species will contain at least one amplifiable fragment, which is sufficient to isolate a difference and identify the gene. As a consequence, cDNAs with less than two restriction sites are not amplified by the protocol and will not be detected by cDNA RDA even if highly differentially expressed. The amplified representation from which uniquely expressed genes are to be identified is termed “tester”, which in our study was the DLBCL samples, and the “driver” is used to subtract commonly expressed genes, which was the FL samples. The protocol utilized in this study makes use of biotinylated primers and streptavidin coated paramagnetic beads for solid phase purification (kindly provided by Dr. Joakim Lundeberg), which makes the technology suited for analysis of small tissue samples (Odeberg et al., 2000).

The generated representations are subjected to three iterative steps of subtractive cross-hybridization and selective PCR amplification of tester specific fragments. This is possible because prior to each round linkers from the preceding PCR amplification step are removed, and new linkers ligated to the tester representation only. As a consequence, driver homoduplexes are not amplified, driver/tester heteroduplexes are amplified linearly, whereas tester homoduplexes are amplified exponentially (Fig 6B). Linear amplified, single-stranded cDNA is digested by mung bean nuclease. Each round generates a difference product (DP1, DP2, and DP3) and the selective pressure is enhanced in subsequent rounds by increasing the tester: driver ratio from 1:100 (DP1), to 1:800 (DP2), and finally 1:400 000. The enrichment of tester specific cDNA fragments is visualized by a stepwise reduction in complexity of the difference products and amplification of individual bands in each successive round (Fig. 6C). Shot-gun cloning of purified cDNA fragment bands followed by sequencing shows that these bands typically contain more than one cDNA species.

One of the main advantages of cDNA RDA is that only a low amount of starting material is required due to the initial PCR amplification step. About 300 µg of driver representation and 20 µg of tester representation are required to complete three rounds of subtractive hybridization and amplification. It is critical to titrate the number of PCR amplification cycles of the digested double stranded cDNA and the amount of template in the initial PCR amplification step to avoid biased amplification of shorter fragments. There has not been reported any problems with selection of “false-positives” (non-differentially expressed genes) by cDNA RDA. Although, we and others noticed that a large portion of the identified sequences were not present in human expressed sequence tag databases (est-dbs, NCBI Blast, Ensembl) (Andersson et al., 2002; Borang et al., 2001; Frohme et al., 2000; Odeberg et al., 2000). This is probably due to two reasons: First, est-db entries are mostly cDNAs partially sequenced from either 5' or 3', whereas the selected DpnII fragments mostly represent central parts of the cDNA. Second, the high stringency (tester/driver ratio 1:400 000 in DP3) and the power of PCR amplification selects for differentially expressed low abundance transcripts. Some of the limitations of cDNA RDA are that it is unlikely to identify differences due to point

mutations, very small deletions and insertions. Fragments from the ends of transcripts or fragments which lack appropriate enzyme sites remain undetected. On the other hand it is well suited for the identification of alternatively spliced transcripts and also holds the possibility of identifying yet undescribed gene transcripts.

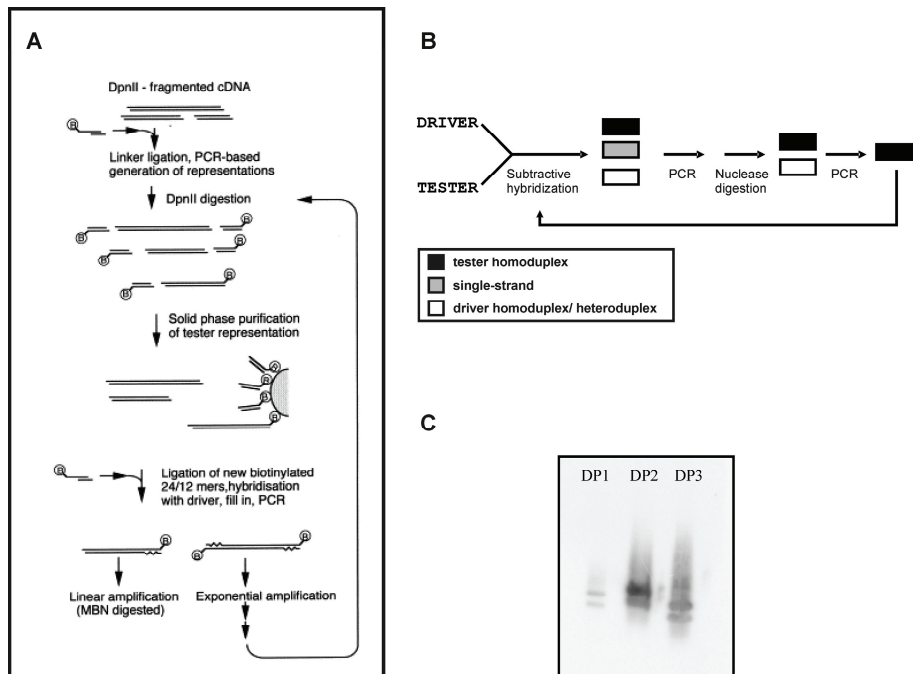


Figure 6. cDNA Representational Difference Analysis. (A) Schematic presentation of the principal steps of cDNA RDA (Odeberg et al., 2000). (B) Due to tester-specific linkers only tester homoduplexes are amplified exponentially, whereas driver/tester heteroduplexes are amplified linearly, and driver homoduplexes are not amplified. Linear amplified, single-stranded cDNA is removed by nuclease digestion. As a consequence, after several rounds of subtractive hybridization and amplification, fragments present in both tester and driver are eliminated and tester-specific genes are enriched. (C) Southern blot of difference products (DP) obtained after each round of subtractive hybridization and amplification hybridized with a DP3 probe. Tester (DLBCL) specific fragments are enriched already after the second round.

Important to note is that high-throughput array-based molecular technologies have been developed that in combination with bioinformatics-based data mining strategies enable multiple parallel experiments to be conducted through largely automated processes. This is essential in developing gene biomarkers that can be used in therapy, diagnosis, and

prognosis. cDNA RDA could be performed in combination with array analyses to assess the validity and generality of findings generated by this technique. Although less encompassing than microarray analysis, RDA is an established method to identify differentially expressed genes in similar tissue types. Unlike array-based technologies, RDA offers the advantage of being able to detect any sequence or gene, not just the ones embedded onto the arrays.

In summary, cDNA RDA has been shown to be a powerful technique for the detection of gene expression differences between two populations, also from uncharacterized genes. It requires very low amounts of starting material and due to the sensitivity cDNA RDA is able to isolate genes expressed in only a very small fraction of cells from which the tester is derived. However, because of the high sensitivity care must be taken in the generation of representations and validation of obtained difference products.

5.1.3 The cell systems

In this study cell lines have been used to study the expression, localization, and biological effects of ectopically expressed proteins (article I-IV) in addition to siRNA-mediated gene silencing (article II and IV). Furthermore, expression analyses have been performed on cells from normal tissue (article I-III) and lymphoid tumors (article I and II). The use of cell lines has four main advantages: first, the amount of cells and thus RNA and protein is not a limiting factor; second, the cell population is relatively homogenous; third, manipulation of gene expression is feasible; and fourth, there is generally a profound knowledge available about genotype, phenotype and behavior in different experimental settings. Clearly, special care should be taken when using cell lines as model systems for functional studies. After many years of *ex vivo* cultivation many of the characteristics of the *in vivo* situation may have been lost and multiple mutations may have been acquired. In addition, stable cell lines are often derived from tumor cells with genetic aberrations. The functional outcome of protein over-expression or depletion is dependent on the whole gene expression pattern of the cell, which also can be affected by the presence of serum and the lack of a natural environment during *in vitro* cultivation.

Therefore, all expression data and functional results should ultimately be confirmed by the use of primary cells or *in vivo* e.g. by the use of transgenic mice.

To summarize, it is important to have in mind the pitfalls of each of the different cell systems when evaluating the experimental data. One also needs to be careful not to extrapolate conclusions drawn from experiments in one system to another. However, functional results from cell line studies are valuable for the generation of hypotheses, which subsequently can be tested in primary cells of normal or malignant origin.

5.1.4 siRNA technology

Knowledge about the expression and regulation of a given gene is important when hypothesizing its function; however gene silencing followed by investigation of the cellular phenotype is a central tool in functional characterization. Discovery of the RNA interference (RNAi) pathway, which allows systematic suppression of gene expression in mammalian cells, has greatly facilitated loss of function studies (Elbashir et al., 2001). RNAi was originally identified in plants, fungi, and worms where it protects the genome from viruses and other insertable genetic elements and regulates gene expression during development (Fire et al., 1998; Jorgensen, 1990; Romano and Macino, 1992).

In this study we have utilized chemically synthesized double stranded short interference RNAs (siRNAs). Once introduced within cells, the siRNAs are unwound by a multiprotein RNA-inducing silencing complex (RISC) in an ATP-dependent process, and the antisense strand directs recognition of the target mRNA sequences (Fig. 7) (Hannon and Rossi, 2004). The mRNA homologous to the introduced siRNA is cleaved at a single site and then subsequently degraded, resulting in a loss of gene function (Hannon and Rossi, 2004). The small size of the molecules (21-23 nucleotides) enables robust and consistent transfection, although the transfection requirement limits this technology to transfectable cell lines. A drawback is also that gene suppression will occur transiently, limiting assay phenotypes to those that occur over the course of a few days. Furthermore, siRNAs are relatively stable molecules but cell division will gradually dilute siRNAs. Although, with the advent of vector-mediated siRNA delivery methods it is now possible

to make transgenic animals that can silence gene expression stably without the production of knockout mice through homologous recombination (Dykxhoorn et al., 2003). An additional factor that can influence siRNA-mediated silencing is the half-life of the protein. To promote efficient gene silencing consideration of the target sequence is a crucial but empirical process, as the rules that govern efficient siRNA directed silencing are still largely unknown (Dykxhoorn et al., 2003). As a consequence of this, siRNAs targeting different sites in the gene of interest should be synthesized and validated (Dykxhoorn et al., 2003). To avoid off-target effects, a BLAST search of the selected sequence should be performed against sequence databases such as est-dbs, NCBI Blast, or Ensembl. If the same phenotype is observed after introduction of two different siRNAs, then the off-target effects should also be minimized.

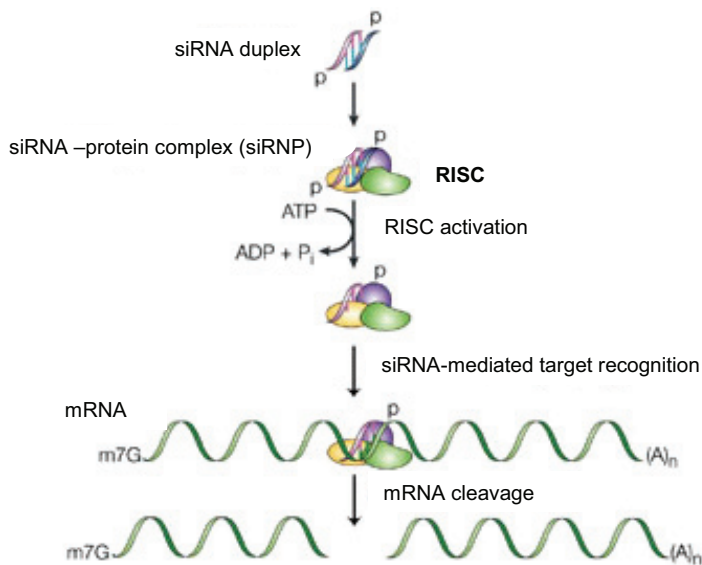


Figure 7. The short interfering RNA (siRNA) pathway. siRNAs are incorporated into the RNA-inducing silencing complex (RISC) and unwound in an ATP-dependent process. The single-stranded antisense strand guides RISC to mRNA that has a complementary sequence, which results in the cleavage of the target mRNA (Dykxhoorn et al., 2003).

In summary, gene silencing using RNAi has become an effective and frequently employed approach for the analysis and understanding of gene function, which also holds the promise for the development of therapeutic gene silencing (Sioud, 2004).

5.2 cDNA RDA on lymphoma biopsies

Our experimental setup was to enrich cDNAs preferentially expressed in DLBCL using cDNA RDA by subtraction between DLBCL and FL. cDNA RDA was performed on two sets of patient matched biopsies of FL and DLBCL. Only tissue that was left over from diagnosis was used for research. Patient I was initially diagnosed with FL carrying the t(14;18) BCL-2 translocation and two years later diagnosed with clonally related DLBCL characterized by structural abnormalities involving chromosome 5 and 9 in addition to the original t(14;18) translocation. Patient II was initially diagnosed with FL carrying the BCL-2 translocation and five years later diagnosed with clonally unrelated DLBCL with a deletion on chromosome 6q, but no t(14;18) translocation (secondary *de novo* DLBCL). Del 6q has been related to aggressive DLBCL with short survival times (Whang-Peng et al., 1995). These two patients thus represent two independent ways of progression.

Total RNA was isolated from twenty 50 µm tissue sections. In the case of FL, malignant B cell containing follicles accounted for approximately 30% of the tissue, whereas DLBCL almost exclusively consisted of malignant B cells. DLBCL specific genes should therefore be representatives of genes whose expression is associated with disease progression but not with the initial disease development. 95 gene fragments were identified and 80% were evaluated by Southern blot analyses to be differentially expressed in the initial FL and DLBCL representations, that is expressed in the DLBCL, and not, or to a lesser degree in the FL. The fact that no gene fragments were identified in both transformed and secondary *de novo* DLBCL could indicate that for the most part different genes are involved in the transformation process of clonally related versus clonally unrelated transformed follicular lymphoma. This is also suggested after comparison of the gene-expression patterns of 12 transformed DLBCLs with those of 11 *de novo* DLBCL specimens (Lossos et al., 2002), and further underlines that similar phenotypical appearance can be reflected through different gene programs.

Several of the identified genes have previously been related to cancer development, such as H-prune (Marino and Zollo, 2007), cytochrome c oxidase I (COXI) (Tarantul and Hunsmann, 2001), arylsulphatase A (Omer et al., 1994; Turkmen et al., 2001), thymosin

$\beta 4$ (Chen et al., 2005a), ATP2A2 (Chung et al., 2006; Korosec et al., 2006), and thymidine kinase (Broët et al., 2001; Di Raimondo et al., 2001; Hallek et al., 1992). The human homologue of the *Drosophila* gene PRUNE has been correlated with cancer metastasis and cell motility and has been shown to be amplified (1q21) in a variety of human cancers including DLBCL (Martinez-Climent et al., 2003; Palanisamy et al., 2002). H-prune would therefore be an interesting candidate to study further in histologically transformed lymphomas. In addition, a large number of the sequences identified were poorly characterized genes that should be the subject of further studies.

To investigate if any of the identified gene fragments could be related to disease progression in general, the intention was to array the identified cDNA clones, together with other known lymphoma-related cDNAs, on glass-slides and perform microarray-based profiling of an extended series of paired biopsy samples. This could not be performed due to difficulties in slide preparation at that time. In addition, several laboratories reported on gene expression analyses of lymphoma biopsies (Alizadeh et al., 2000; Lossos et al., 2002; Rosenwald et al., 2002). We therefore decided to perform candidate gene studies. Thus two gene fragments were selected for further investigation: the first from patient I (later denoted *ILDRI* (article I)) and the second from patient II (later denoted *CSPP1* (article II)).

In summary, cDNA RDA has proved to be a robust technique for identification of differently expressed genes in lymphoma biopsies as evaluated by Southern blot analysis. Among these, there are several potentially interesting genes related to carcinogenesis but also a large number of uncharacterized genes that should be further explored.

5.3 Identification and expression of ILDRI

In article I, we characterized a novel gene denoted *ILDRI*. Three transcribed splice variants of this gene were identified. Sequence analyses and cellular localization data indicated that two of the variants encode cell membrane-spanning isoforms, and the third variant a cytosolic isoform. Western blot analysis and immunofluorescence microscopy of ectopically expressed isoforms, together with the sequence homology to a rat lipolysis-

stimulated remnant receptor (LSR) (Yen et al., 1999), suggest that these proteins hold an intrinsic ability of forming heteromeric complexes. This would be possible to further investigate by co-precipitation analyses.

The membrane-associated isoforms are predicted to expose an immunoglobulin-like (Ig-like) domain on the cell surface. Ig-like domain containing proteins form one of the largest superfamilies in vertebrates and are involved in many different kinds of interactions (Barclay, 2003) where the Ig-like domain appears to be involved in binding functions. The overall structural resemblance with LSR prompted the investigation of lipoproteins as ligands for ILDR1 (data not shown). Due to the lack of an appropriate positive control, our experiments could not conclude on this.

Semi-quantitative RT-PCR analysis did not show association for any of the isoforms to histological transformation which was the initial aim of the study, but interestingly, the samples investigated suggested that the cytoplasmic isoform might be a FL/DLBCL-specific variant. The expression of this isoform could be involved in modulating extracellular ligand binding. Alternative splicing of pre-mRNA is a process leading to the production of distinct protein isoforms, which may have entirely novel functional properties or even dominant negative or antagonistic functions. The two main mechanisms that lead to splicing defects in cancer are mutations in cis-splicing regulatory elements and changes in trans-splicing factors affecting the splicing machinery (Faustino and Cooper, 2003). Normally, inappropriately spliced forms are eliminated by the nonsense-mediated mRNA decay (NMD) pathway (Culbertson, 1999), but in some pathological conditions aberrant mRNAs escape this quality control mechanism. For many genes, dramatic changes in alternative splicing patterns are associated with neoplasia and metastasis (Nissim-Rafinia and Kerem, 2002; Pajares et al., 2007; Philips and Cooper, 2000). The gene products are often regulators of apoptosis, hormones, and receptors mediating cell-cell and cell-matrix interactions, and confer a selective advantage to cells housing the changes. Altered splicing patterns can also provide diagnostic and prognostic information and serve as markers for disease even when they are not in the primary pathway of the disease mechanism (Pettigrew and Brown, 2008).

Importantly, due to the limited number of samples investigated for expression of ILDR1 isoforms so far, a larger panel of FL/DLBCL biopsies, cell lines and tissues should be explored. To evaluate the potential as a tumor marker, other neoplasms should also be investigated. In addition, its functional role should be determined.

5.4 Identification and expression of CSPP1

In article II, a novel coiled-coil protein denoted centrosome/spindle pole-associated protein (CSPP) was characterized. Initial *in silico* expression analysis suggested a correlation of *CSPP1* expression and DLBCL, while expression was low in FL (Alizadeh et al., 2000). This expression pattern could simply reflect the high proliferation rate in high-grade disease or, alternatively, contribute to disease progression. The observed aneuploidy upon prolonged CSPP over-expression could be in favor of the last assumption and suggests that CSPP expression could partially account for abnormal mitosis, and chromosome and centrosome aberrations reported in DLBCL (Bloomfield et al., 1983; Johansson et al., 1995; Knutsen, 1997; Kramer et al., 2003; Kramer et al., 2005; Tilly et al., 1994).

We show that CSPP is associated with centrosomes and MTs and may play a role in G₁/S-phase progression and spindle assembly. Subcellular localization data and cell-cycle analyses are based on ectopic expression of CSPP in transfectants. Upon evaluation of experimental data from over-expression studies, it is important to keep in mind that the protein of interest will be expressed throughout the cell cycle. This may force protein activity at a stage where it is normally inactivated or absent, or in a context where regulatory mechanisms might be different from the normal situation. In addition, over-expression can lead to artificial localization and sequestration of interacting proteins. Because ectopic CSPP forms aggregates at high expression levels, as also reported for other coiled-coil domain containing proteins (Andersen et al., 2003), the analysis of cells showing low ectopic expression levels should be emphasized. However, even experimental results at aberrant expression levels are valuable for the generation of

hypotheses, which subsequently can be tested by siRNA-mediated protein depletion or antibodies to characterize endogenous proteins.

Cell-cycle analyses showed that ectopic expression of CSPP blocked cell-cycle progression in G₁ phase and in mitosis, even at relatively low expression levels. Furthermore, CSPP-depletion induced cell-cycle arrest/delay in S phase. A relevant question raised is how a protein can affect progression through three different cell cycle stages? Firstly, during interphase, CSPP associates with MTs and mainly accumulates at and around centrosomes, while in mitosis localization is mainly conferred to the spindle poles of aberrant mitotic spindles and their kinetochore MTs. The impaired mitotic progression was characterized by the appearance of multipolar spindles in ~70% of CSPP expressing mitotic cells. This might be explained by a CSPP-associated (minus-end) MT bundling activity which could be mediated by the coiled-coil mid domain. Supporting this are data showing that CSPP-L, a larger isoform of CSPP, which mainly induces the formation of monopolar and bipolar spindles, has a 51 amino acids insertion in this domain that might abrogate MT bundling (Patzke et al., 2006). Indeed, the deletion of this insertion from CSPP-L increases the percentage of multipolar spindles, and expression of isolated MT binding/organizing domains of CSPP and CSPP-L differently affect MT organization. Strikingly, among CSPP-expressing mitotic cells normal metaphase plate alignment of chromosomes were rarely seen. The predominantly observed phenotype was characterized by condensed chromosomes aligned in ring-like structures around multiple spindle poles. The spindle checkpoint monitors the status of kinetochore-MT attachments and prevents cells from progressing to anaphase until all kinetochores are properly attached to MTs (Musacchio and Salmon, 2007). Based on the assumption that CSPP over-expression disturbs normal spindle assembly, the observed mitotic arrest could be due to activation of the spindle checkpoint. Other studies have also shown that ectopic expression of MT-associated proteins leads to MT-bundling and mitotic cell-cycle arrest. TMAP/CKAP2 is a protein frequently up-regulated in various malignancies and expression of a nondegradable mutant increase the occurrence of spindle defects and cytokinesis failure (Hong et al., 2007). NuSAP is another example of

a protein that induce MT bundling, spindle deficiency, and mitotic arrest upon over-expression (Li et al., 2007).

Secondly, the hypothesis based on our results is that CSPP is involved in a G₁/S checkpoint preventing premature entry into S phase. Then, lack of CSPP could allow premature entry into S-phase, which might lead to activation of the DNA replication checkpoint shown by the activation of Chk1 (Machida and Dutta, 2005). This might also explain the G₁ block observed upon over-expression that prevents S-phase entry. Several studies have established a role for the centrosome organelle in the regulation of G₁-to-S-phase progression (Doxsey et al., 2005a; Doxsey et al., 2005b; Mikule et al., 2007; Sluder, 2005; Srsen et al., 2006). Indeed, depletion of centrosomal proteins in p53 deficient cell lines induces mitotic aberrancies similar to what is observed upon CSPP over-expression (Mikule et al., 2007). Thus, sequestration of such proteins by CSPP could contribute to the observed phenotype. Further support of a role for CSPP in G₁-phase checkpoint control comes from a report showing that upon knockdown of the transcription factor Miz1, a protein involved in the control of G₁ arrest in response to DNA damage, cells failed to up-regulate *CSPP1* expression (Wanzel et al., 2005).

Importantly, characterization of the CSPP-L isoform (Patzke et al., 2006) showed that the siRNA used to deplete CSPP in article II also targets CSPP-L. We can therefore not conclude if the observed defect in S-phase progression is due to CSPP or CSPP-L depletion, or both. This paper also shows that CSPP expression peaks in the G₁ phase of the cell cycle (Patzke et al., 2006) and suggests that the observed CSPP-induced mitotic block upon over-expression could be due to forced protein expression at a stage where it is normally present at a reduced level. A CSPP1-specific antibody recognizing both isoforms has been developed and characterization of endogenous CSPP has been initiated (Patzke, S., personal communication). So far, preliminary data confirm the centrosomal and midbody localization (Patzke et al., 2006) of CSPP1 isoforms. Furthermore, localization to the basal body and parts of the MT axoneme of non-motile primary cilia has been unraveled (Patzke, S., personal communication). This is the subject of further studies.

5.5 Identification and expression of a new gene family, FAM110

In article III and IV we describe the characterization of the family with sequence similarity 110 (FAM110), member A, B, and C. As discussed in the previous section, precaution when evaluating experimental data from over-expression studies has to be taken. This study was initiated to explore potential CSPP-interaction partners with the purpose of adding further insight to the role of CSPP. In addition, the FAM110 family of proteins was subjected to further characterization due to the specific subcellular localization patterns and phenotypes induced both upon over-expression and depletion.

A cDNA clone corresponding to FAM110A was first identified in a yeast two-hybrid analysis where the first 360 amino acids (aa) of CSPP were used as bait to screen a peripheral blood lymphocyte (PBL) library. Further, homology search identified FAM110B and FAM110C, harboring 31% and 26% aa identity to FAM110A, respectively. In article III expression analyses showed that FAM110A is broadly expressed in human tissues, including lymphoid tissues and further analyses in article IV indicated that FAM110C is expressed foremost in adherent cell lines of epithelial origin. The FAM110 family displays no homology to other previously characterized proteins or protein domains. However, three sequence elements in addition to a proline-rich region are highly conserved among the members, which could indicate functional motifs characteristic to the FAM110 family. These motifs should be dissected further to understand the role of and identify interaction partners for these proteins. Despite of the structural similarities, expression patterns suggests that the FAM110 homologues are differentially regulated which could indicate that they harbor somewhat different functional properties.

A liquid β -Gal assay in yeast indicated that FAM110A and CSPP are able to interact, which was further supported by co-localization in transiently transfected HEK293T cells. Although co-localization could be observed also with FAM110B and FAM110C, these homologues did not show any significant binding to CSPP in the liquid assay. FAM110A contains a unique proline-rich region, and such sequence elements are often involved in protein-protein interactions. To explore the contribution of this motif in the potential

CSPP-interaction, two FAM110A deletion constructs were generated. In the first, the proline-rich region was removed (aa 136-146, GenBank Accession No. DQ431181) and in the second, the first sequence element, the SAVERLEAD-motif, common to all homologues was deleted (aa 41-51). A liquid β -Gal assay showed that none of the FAM110A mutants longer bound to CSPP (data not shown). This could either indicate that the globular structure of the protein was obstructed and a potential conformational epitope disintegrated, or that both motifs are important for the interaction. In any case, this did not elucidate the potential FAM110A-CSPP interaction any further. However, it would be interesting to study the subcellular localization of the mutant lacking the SAVERLEAD-motif to explore whether this sequence element could be involved in the centrosome/spindle pole localization of FAM110 members. Furthermore, an immunoprecipitation-approach of tagged proteins from lysate of co-transfectants could not detect a putative FAM110A-CSPP interaction (data not shown). Since studies on co-transfected HEK293T cells showed that FAM110A and CSPP co-localized only at the MTOC, we speculate that there could be a conformational change induced in one or both of the proteins at this site that allows binding and that this interaction “drowns” in the excess amounts of protein produced in the cell. We can of course not rule out that these proteins do not interact directly but are linked through a common protein complex. This might be elucidated when antibodies are available to study endogenous proteins.

To identify potential FAM110A-interacting proteins, a further yeast two-hybrid analysis was performed. RGS14 was one of three clones identified (data not shown) that might be of particular interest because it is a MT-associated protein and a component of the mitotic spindle that may regulate MT polymerization and spindle organization (Martin-McCaffrey et al., 2005). RGS14 is thereby involved in processes that CSPP and FAM110 proteins also seem to be implicated in.

In many aspects FAM110C has more features in common with CSPP than FAM110A. Both are expressed in testis, localize to centrosomes and MTs, and induce G₁-phase arrest upon ectopic expression. Like CSPP, exposure of transiently transfected HEK293T cells to the MT depolymerizing drug nocodazole indicates MT-independent association of

FAM110C with centrosomes (data not shown). Proteins that localize to centrosomes independently of a nucleated MT array, like γ -tubulin and pericentrin, are generally considered as “core” centrosomal components (Oegema et al., 1995).

The mechanism causing the FAM110C induced G₁-phase arrest could be the result of bundling of interphase MTs which could abrogate the rapid turnover of MTs necessary for mitotic entry (Cassimeris, 1999). It has been shown that a critical balance between MT stabilizers and MT destabilizers is necessary for assembly of the mitotic spindle (Cassimeris, 1999) and this balance might be pushed upon over-expression of FAM110C. Furthermore, several other MT-associated proteins than FAM110C and CSPP have been shown to affect G₁-phase transition like CDC14B, Ckap2, RASSF1A, and RGS14 (Cho et al., 2005; Liu et al., 2003; Martin-McCaffrey et al., 2005; Rong et al., 2004; Shivakumar et al., 2002; Tsuchihara et al., 2005). We also observed that adherent HEK293T cells expressing high levels of ectopic FAM110C rounded up and lost their natural morphology. Integrin-mediated adhesion has been demonstrated to be critical to cell-cycle progression through G₁-phase (Assoian and Schwartz, 2001; Danen and Yamada, 2001; Schwartz and Assoian, 2001), so disruption of these contacts could be involved in the observed arrest as well. It should be investigated if also the expression of FAM110C is cell-cycle phase dependent, like it is for FAM110A and CSPP, which could give indications on when the protein is most essential to the cell. To further characterize the endogenous protein a FAM110C-specific polyclonal antibody has been developed and studies have been initiated.

5.6 FAM110C in cell adhesion and migration

Due to the explicit phenotype induced upon FAM110C over-expression, we decided to pursue the characterization of this particular member of the FAM110 family of proteins in article IV. Our data indicate that FAM110C is a protein expressed predominantly in cells of epithelial origin. We show that filopodial formation and cell spreading is abrogated upon FAM110C-knockdown and that FAM110C depletion alters the migratory potential of the epithelial cell line HepG2. Furthermore, the activation of Akt upon integrin-mediated adhesion is almost completely abolished compared to controls. Akt has

been demonstrated in several studies to be central in filopodia formation and cell motility (Enomoto et al., 2005; Higuchi et al., 2001; Kim et al., 2001; Qian et al., 2004), suggesting that FAM110C promotes membrane protrusion through activation of Akt. Additionally supportive for this is that FAM110C co-precipitates and co-localizes with Akt1. These experiments also suggested that FAM110C interacts with active Akt1. A predicted Akt substrate recognition motif in FAM110C suggested that Akt in turn could exert influence on FAM110C. Indeed, immunoprecipitation data indicated that mutation of the putative threonine phosphorylation target affects the interaction between Akt1 and FAM110C. To establish whether FAM110C is a true target of Akt phosphorylation and to identify the functional implications of this modification will be important in the further characterization of FAM110C. Phosphorylation could potentially affect the MT-binding properties of FAM110C, as well as its subcellular localization and interaction with other proteins as suggested for other MT-associated proteins (Berti et al., 2004; Cassimeris, 1999; Rong et al., 2004).

In addition, the results indicate that ectopically expressed FAM110C co-localizes with acetylated MTs, a marker of stabilized MTs, and also increase the level of stabilized MTs compared to controls. However, the exact mechanism by which FAM110C enhance MT stability is not clear. Notably, there are studies showing that MT-stabilization is important for membrane protrusion and migration (Even-Ram et al., 2007; Mikhailov and Gundersen, 1998; Palazzo et al., 2004; Wen et al., 2004) and MT acetylation in particular has been proposed to have a role in cell motility. A recent report suggested that the reduced migratory potential of cells where the tubulin deacetylase HDAC6 was inhibited could be due to decreased MT-dynamics and focal adhesion turnover (Tran et al., 2007). In that respect it is interesting to note that CSPP-L contains a putative HDAC interaction domain in its N-terminus and co-precipitates with HDAC6 (Patzke, S., personal communication). It is thereby tempting to speculate that FAM110C and CSPP are part of a protein complex that could regulate the activity of HDAC6 and thereby affect MT-stability. Further it has been shown that Akt can promote MT-stabilization in migrating cells (Onishi et al., 2007) and that HDAC6 inhibitors and HDAC6 depletion suppress Akt

phosphorylation and induce MT acetylation (Bali et al., 2005; Chen et al., 2005b; Fiskus et al., 2007).

The data presented indicate that FAM110C harbors several properties to mediate filopodia formation: first, the ability to affect MT stabilization; second, to facilitate activation or recruit activated Akt; and third, to crosslink the actin and MT cytoskeletons at the cell membrane via ezrin. In summary, FAM110C could serve to bring together structural and signaling components important for cell adhesion and migration.

Interestingly, in the panel of carcinoma cell lines investigated the level of FAM110C expression is low or absent in HeLa and SKBR-3 that are E-cadherin negative cells. Cadherins are transmembrane proteins that mediate cell-cell adhesion through homotypic interaction and linkage to the actin cytoskeleton via intracellular catenins (Provost and Rimm, 1999). Down-modulation of E-cadherin expression and reduction of cell contacts is a key event during carcinoma progression leading to increased invasive and metastatic potential (Birchmeier and Behrens, 1994; Vleminckx et al., 1991). Provided that there is a coordinated downmodulation of E-cadherin and FAM110C expression in invasive carcinomas to escape from their original tissues, a potential function for FAM110C in cell-cell adhesion should be investigated.

The results indicate that FAM110C is involved in filopodia formation and migration. To further elucidate the biological function of FAM110C and the mechanisms involved, a microarray study and real-time PCR analysis has been performed on FAM110C-depleted HepG2 cells. Notably, a significant number of the genes affected are involved in cytoskeleton organization, cell adhesion, and cell motility. Furthermore, two proteins that can negatively regulate the PI3K-pathway (p85alpha and PIPP) (Geering et al., 2007; Ooms et al., 2006) are upregulated in the expression analyses. Considering the observed lack of activated Akt in FAM110C-knockdown cells upon integrin-mediated adhesion, this should be investigated further.

6. Conclusions

- The use of cDNA RDA on patient matched biopsies of FL and DLBCL has proven valuable for the identification of novel genes whose expression is potentially associated with DLBCL. Two previously undescribed genes have been identified and characterized during the course of our study: the immunoglobulin-like domain containing receptor 1 gene (*ILDR1*) and the centrosome/spindle pole-associated protein 1 gene (*CSPP1*).
- The *ILDR1* gene encodes three isoforms, of which two are cell-membrane spanning and one is cytosolic, and might assemble into a multimeric receptor. The cytosolic isoform was found to be expressed only in the lymphoma samples and not in normal tissue or cell lines investigated. This might be due to aberrant splicing in lymphoma cells which could modulate extracellular ligand binding and could potentially serve as a marker for disease. Both the receptor ligand and biological function remains at present elusive.
- The *CSPP1* gene encodes a centrosome/microtubule associated protein that appears to be temporally expressed through the cell cycle and affect cell cycle progression and MT organization in mitosis. Over-expression of CSPP has been correlated to high-grade DLBCL by gene expression analysis (Alizadeh et al., 2000). An oncogenic role of CSPP is further supported by that over-expression of CSPP disturbs normal spindle assembly and promotes multipolar spindles.
- The characterization of *CSPP1* led to the discovery of three undescribed homologous genes constituting the family with sequence identity 110 (*FAM110*), member A, B, and C. The three members are to some extent expressed in the same tissues but *FAM110A* is expressed also in lymphoid tissues and peripheral blood leukocytes, while *FAM110C* is expressed predominantly in epithelial tissues. *FAM110* proteins localize at the MTOC in interphase and at spindle poles in mitosis, where they also co-localize with CSPP. In addition, *FAM110C* localize to the MT-cytoskeleton throughout the cell cycle and induce MT aberrancies upon over-expression, which may also induce the observed G₁-phase arrest. *FAM110C* depletion indicates that this homologue is involved in Akt-dependent filopodia

formation and MT stabilization in epithelial cells, and supports a role for FAM110C in cell adhesion and migration.

7. Perspectives

The importance of ILDR1 expression should be further explored with main focus on the identification of a potential ligand and the cell type specific expression of the individual isoforms.

A CSPP-specific monoclonal antibody has been developed and characterization of endogenous CSPP has been initiated. In addition to confirm a centrosomal localization, preliminary data has shown that CSPP localize to the basal body of non-motile primary cilia (Patzke, S., personal communication). This should also be investigated in relation to FAM110 homologues. Experiments to explore the nature of the cell cycle arrests and the role of CSPP in MT organization and mitosis are also initiated. The identification of protein interaction partners and kinases involved in CSPP phosphorylation will be of importance.

A FAM110C-specific polyclonal antibody has been developed and the characterization of endogenous FAM110C expression in different cell types has been initiated. Our focus will be to investigate the cellular localization of FAM110C and participation in filopodia-formation. In relation to this, the potential phosphorylation by Akt will be important to examine. We will validate the binding to Akt and the cytoskeleton-membrane organizer ezrin, and also seek to identify additional protein interaction partners to further explore the function of FAM110C. Furthermore, microarray- and real-time PCR data indicating an effect of FAM110C-depletion on the expression of several proteins involved in cytoskeletal organization and membrane ruffling should be further examined. Identification of the mechanism behind the MT-stabilizing effect of FAM110C will be pursued and a potential link to the deacetylase HDAC6 will be investigated. It should also be considered if targeting FAM110C expression could have therapeutic applications in treatment of invasive and metastatic tumors.

To develop antibodies recognizing FAM110A and FAM110B, and further characterize these homologues will be a subject of future studies. The potential binding of FAM110A to the MT-associated protein RGS14 identified in a yeast two-hybrid screen will be

pursued. The expression of FAM110A in leukocytes and lymphoid tissues could potentially be interesting with regard to cytoskeletal organization and polarization events taking place during leukocyte activation, signaling, and migration, especially in light of the observed increased FAM110A expression upon stimulation of resting CD4⁺ lymphocytes.

8. References

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