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MYCN and microRNAs in neuroblastoma

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Buechner J, Henriksen JR, Haug BH, Tomte E, Flaegstad T, Einvik C: **Inhibition of *mir-21*, which is up-regulated during *MYCN* knockdown-mediated differentiation, does not prevent differentiation of neuroblastoma cells.** *Differentiation* 2011, **81:**25-34.

Paper II:

Buechner J, Tømte E, Haug BH, Henriksen JR, Løkke C, Flægstad T, Einvik C: **Tumour-suppressor microRNAs *let-7* and *mir-101* target the proto-oncogene *MYCN* and inhibit cell proliferation in *MYCN*-amplified neuroblastoma.** *Br J Cancer* 2011, **105:**296-303.

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Haug BH, Henriksen JR, Buechner J, Geerts D, Tomte E, Kogner P, Martinsson T, Flaegstad T, Sveinbjornsson B, Einvik C: ***MYCN*-regulated *miRNA-92* Inhibits Secretion of the Tumor Suppressor DICKKOPF-3 (DKK3) in Neuroblastoma.** *Carcinogenesis* 2011, **32:**1005-1012.

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2 Introduction

Cancer in children is rare. In industrialized countries, only 0.5-1 % of all cancers occur in children below the age of 15 years [1]. The annual incidence in Europe was 139 per million children for the period 1988-1997 [2]; in Norway, approximately 160 children develop cancer each year [3]. The overall chance to survive childhood cancer has increased substantially during the last decades, from 54 % for cases diagnosed between 1978-1982, to 75 % for those diagnosed 1993-1997 [4]. Highest survival rates in Europe are reported from Western Europe and the Nordic countries [5, 6].

In contrast to adult cancer, which comprises mainly carcinomas in the respiratory, gastrointestinal and reproductive organs, cancer in children is rarely manifested in these localizations. In addition, the histopathology of pediatric malignancies is remarkably different and mainly characterized by immature or embryonal cells from different developmental stages – rapidly proliferating and embarrassed to mature [7].

Leukemia is the most common pediatric cancer (35-45 % of cancer cases), followed by tumors of the central nervous system (around 30 %). The majority of other cases belong to the groups of lymphomas, sarcomas, or embryonal tumors like nephroblastoma, hepatoblastoma, and neuroblastoma [1].

2.1 Neuroblastoma

With an annual incidence of 7-12 new cases per million children [1, 8, 9], neuroblastoma is the most frequently diagnosed extra-cranial solid tumor in childhood, accounting for 7-8 % of all pediatric malignancies and 15 % of childhood cancer deaths [10, 11]. For patients with high-risk tumors, long-term survival rates are still below 40 %, making treatment of neuroblastoma to one of the major challenges in pediatric oncology [12-14].

Neuroblastoma comprises a group of biologically distinct tumors with extremely heterogeneous behavior. On the one hand, localized tumors, which are often asymptomatic, but even metastasized neuroblastomas in infants can regress completely or differentiate spontaneously into benign histological variants without any treatment at all [15-17]. On the other hand, metastatic neuroblastomas in children older than 18 months at diagnosis cause severe systemic illness and are associated with poor prognosis, despite all modern and aggressive multi-modal treatment efforts [18].

In 2003, Garrett Brodeur addressed the "enigmatic" nature of neuroblastoma in a state-of-the-art review and noted that "few tumors have engendered as much fascination and frustration for clinical and laboratory investigators as neuroblastoma" [19]. One way to improve treatment and hopefully increase survival is to unravel the molecular basis of neuroblastoma tumorigenesis. The identification of genes and their regulators, like miRNAs and signaling pathways, responsible for the malignant transformation of neuroblastoma cells, will help to define patient risk groups on a molecular basis [20-23] and aid to develop new therapeutic strategies based on directly targeting these biological pathways [14, 24].

2.1.1 Neuroblastoma – a disorder of normal development

Neuroblastoma belongs to the group of embryonal tumors, neoplasms in which the cell of origin physiologically participates in the organogenesis during embryonal development. Normal development is characterized by precisely controlled stages of proliferation and differentiation, and is dependent upon communication between distinct populations of precursor cells. The failure of precursor cells to exit from a proliferative phase and enter the differentiation process is a mainstay of embryonal neoplasms [25]. Simplified, embryonal tumors can be regarded as a disorder of normal development.

Neuroblastoma originates from precursor cells of the sympathetic nervous system. During neurulation, a developmental step in the embryonic formation of the central nervous system, the neural plate, or neuroepithelium, folds in on itself to form the neural tube, a structure that will later develop into the brain and the spinal cord (Figure 1). This process of folding brings the two outer edges of the plate together to create the transient embryonal structure called neural crest. As a consequence, precursor cells from the neural crest then build the dorsal part of the neural tube. From this position, a subset of neural crest cells are initiated to undergo an epithelial-to-mesenchymal transition (EMT), a process in which cell-cell contacts are abrogated, the cytoskeleton is reorganized and cells subsequently acquire a motile phenotype [26]. These cells start to migrate along stereotypical migratory pathways, and form a variety of diverse cell types, including peripheral neurons, Schwann cells, craniofacial bones, melanocytes in the skin, and heart valves, depending on their axial level of origin. Migratory neural crest cells from the trunk populate the primordia of the sympathetic ganglia and the adrenal gland, and finally differentiate into the sympathoadrenal lineage of sympathetic neurons and adrenal chromaffin cells [27, 28].

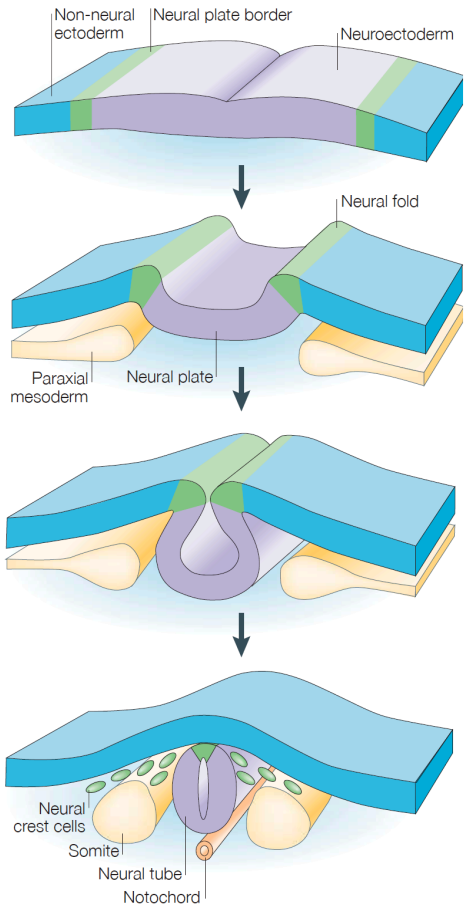

Figure 1: Border induction and neurulation (from [29], with permission)

A variety of extracellular signals from the microenvironment (cell-cell and cell-matrix interactions) as well as intracellular signaling events induce the complex process of neural crest formation, guide the migratory neural crest cells along the migration routes and control the sequential process of proliferation and differentiation into the sympathoadrenal lineage (reviewed in [29, 30]).

For example, cells from the non-neural ectoderm or mesoderm secrete Wnt proteins that activate Wnt signaling in adjacent cells at the border of the neural plate to initiate neural crest formation [31]. In addition, secretion of bone morphogenic protein (BMP), Sonic hedgehog (Shh) and fibroblast growth factors (FGFs) are involved in neural crest induction [29, 32]. These inductive signals activate genes in neural crest cells known as neural crest specifiers, including *FoxD3*, *Snail (Snai1), Slug (Snai2)*, *Sox9* and *Sox10* [29]. Neural crest specifiers, in turn, are involved in the initiation of the migratory process, e.g. through the repression of adhesion molecules like E-cadherin (by Snail [33] and Slug [34]) and the induction of Cadherin-7 (by FoxD3 [35]), as well as in the differentiation of post-migratory cells (by Sox9

and Sox10 [36]). Differentiation into the sympathoadrenal lineage requires expression of BMP family proteins in the neural crest-derived cells [37, 38].

A strong intracellular stimulus for the ventral migration of neural crest cells is expression of the proto-oncogene *MYCN*. N-myc, the protein encoded by *MYCN*, is found in moderate levels in the nuclei of all trunk neural crest cells before and during migration. After migration, cells committed to differentiate into neurons retain high N-myc expression. During neuronal maturation, N-myc promotes sympathetic neuronal differentiation in cooperation with other intracellular factors (like Mash1 and Phox2b) and extracellular signals (like BMP proteins)[30, 39, 40].

In summary, neural crest cells represent a proliferative cell population that maintains self-renewal capacity during the migratory course [41]. Disturbances in the spatiotemporally precisely controlled processes with consequent inhibition of cell-cycle exit and normal differentiation, maintaining proliferation at time points when differentiation is required, can initiate the transformation of neural crest cells and, for example, give rise to neuroblastic tumors [25].

2.1.2 Localization and classification of primary neuroblastic tumors

The embryonic migration of trunk neural crest cells explains why neuroblastic tumors can arise anywhere along the defined migratory routes. The most common sites for neuroblastoma are the adrenal medulla (35%), the paravertebral sympathetic ganglia in the abdomen (30-35%) and mediastinum (20%). Less common sites are the pelvis (3-5%) and the neck (1-5%) [42].

Histologically, the group of neuroblastic tumors consists of several categories that are defined according to the maturation degree of the neuroblastic cells and the presence or absence of Schwann cell-stromal components in the tumor. The histopathology of neuroblastic tumors has been classified in the International Neuroblastoma Pathology Classification (INPC) [43, 44]. The INPC distinguishes four basic morphologic categories, in which the degree of Schwannian stromal development is noted in parenthesis:

Neuroblastoma (Schwannian stroma-poor) is the most immature variant of neuroblastic tumors and consists of small blue round cells with little, if any, features of differentiation towards maturing ganglion cells. Neuroblastomas can be further subdivided into undifferentiated, poorly differentiated and differentiating tumors. *Undifferentiated* neuroblastomas, in which background neuropil (thin neurite processes) are completely absent,

have to be separated by immunohistochemistry from other small blue round cell tumors, like Ewing sarcoma/primitive neuroectodermal tumors (PNET), rhabdomyosarcoma, Wilms tumors or myeloid sarcoma. The subtype of *poorly differentiated* neuroblastoma is diagnosed when neuropil background is present, but the percentage of cells with ganglion differentiation does not exceed 5 %. The subtype of *differentiating* neuroblastoma is defined as a tumor with abundant background neuropil and 5 – 50% of the neuroblasts showing differentiation towards ganglion cells.

On the opposite side of the differentiation scale, the most differentiated category of neuroblastic tumors is a ganglioneuroma (Schwannian stroma-dominant). These tumors are composed of a dominating Schwann cells stroma with a minor component of ganglion cells of different maturation degree. In its completely maturated form, ganglioneuroma lacks any neuroblastic component.

Between the immature neuroblastomas and mature ganglioneuromas, the INPC classifies two categories composed of both ganglioneuromatous ("*ganglio*-") and neuroblastic components ("-*neuroblastoma*") and therefore referred to as ganglioneuroblastoma. Neuropil is generally abundant. The category of intermixed ganglioneuroblastoma (Schwannian stroma-rich) contains well-defined microscopic nests of "embryonal residues" with neuroblastic cells in different maturation stages. The category of nodular ganglioneuroblastoma (composite Schwannian stroma-rich/stroma-dominant and stroma-poor) consists of a more heterogeneous cell population ("composite"). The neuroblastic cells form nodules in the tumor that are often macroscopically apparent and hemorrhagic and contain aggressive malignant cell clones due to acquired genetic aberrations or the persistence of malignant clones [43].

In a review of 224 neuroblastic tumors [44], the distribution of the INPC categories was as following: 190 neuroblastomas (85%), 19 nodular ganglioneuroblastomas (8%), 5 intermixed ganglioneuroblastomas (2%), one ganglioneuroma (<1%) and 9 tumors not classifiable (4%).

2.1.3 Differentiation of neuroblastoma cells

Although impaired in their physiological differentiation program, most neuroblastoma cells retain the capacity to differentiate – spontaneously *in vivo*, and upon stimulation with various agents and growth factors *in vitro* and *in vivo* (reviewed in [45]). Spontaneous differentiation and regression of neuroblastoma tumors is a peculiar phenomenon in mainly localized tumors [15]. These biologically favorable tumors express high levels of the neurotrophic tropomyosin kinase receptor A (TrkA), the receptor for nerve growth factor (NGF) [46, 47]. Expression of

TrkA appears to mediate either differentiation or apoptosis of neuroblastoma cells, depending on the presence or absence of NGF, respectively [48, 49], suggesting that the NGF/TrkA pathway is responsible for the differentiation and regression of favorable neuroblastomas [48]. Retinoids, like the naturally occurring all-*trans*-retinoic acid (ATRA) and 9-*cis*-retinoic acid (RA) or the synthetic 13-*cis*-RA, induce neuronal differentiation and growth arrest of neuroblastoma cells both *in vitro* and *in vivo* [45]. 13-*cis*-RA is therapeutically used in neuroblastoma treatment ([50], and chapter 2.1.6). Stimulation of the neuroblastoma cell line SH-SY-5Y with 12-O-tetradecanoyl phorbol-13-acetate (TPA) induces a strong differentiation process, and is used as a model system for mechanistic studies of human sympathetic neuronal differentiation. In addition, combinations of growth factors with physiologically roles during development of the sympathetic nervous system, like basic FGF and insulin-like growth factor 1 (IGF1), induce neuronal differentiation of neuroblastoma cells *in vitro* [45]. Neuroblastoma cell lines generally lack Trk receptors, however, pre-treatment with retinoids induce Trk expression and sensitize the receptors for their neurotrophin ligands (TrkA for NGF; TrkB for brain-derived neurotrophic factor (BDNF) and neurotrophin (NTF) -4; TrkC for NTF-3) (reviewed in [45, 49]). Interestingly, the capacity to response to exogenous differentiation stimuli is retained even under high *MYCN* expression [51]. Knockdown of *MYCN* in *MYCN*-amplified neuroblastoma cells induces neuronal differentiation [52].

Taken together, the potential of neuroblastoma cells to differentiate, either spontaneously or upon distinct triggers, is one of the major neuroblastoma research foci due to fact that knowledge about these processes may have direct clinical implication for tumor differentiation therapy.

2.1.4 Molecular aspects in neuroblastoma

Neuroblastoma is a complex genetic disorder [19, 53, 54]. Acquired somatic aberrations, either through segmental chromosomal aberrations like translocations and gene amplification, or numerical chromosomal changes, substantially influence tumor biology and clinical behavior (see also chapters 2.1.5.3 and 2.1.5.4, and [55]). In addition, several pre-disposing germline aberrations have been identified (reviewed in [54]). The following sections will review molecular aspects in neuroblastoma, focused on relevance for the papers in this thesis.

2.1.4.1 MYCN amplification (MNA)

Amplification of the human proto-oncogene *MYCN* is found in approximately 20% of neuroblastoma tumors [53]. Due to its profound effect on clinical outcome (see chapter 2.1.5.3), *MYCN*-amplification (MNA) is routinely used as a biomarker for treatment stratification. The amplification is cytogenetically detectable as autonomously replicating double minute chromosomes (dmins), or intrachromosomal homogenously staining regions (hsr). The transcription factor N-myc, which is encoded by *MYCN* on chromosome 2p24, belongs to the Myc-family of DNA binding basic region/helix-loop-helix/leucine zipper (bHLHZip) proteins, in which c-Myc, L-Myc and N-myc are the best characterized members [56]. The genomic sequences of *MYCN* and *c-MYC* share wide structural homology. Both genes consist of three exons, where the first exon is untranslated and exon 2 and 3 encode the translated regions [57]. N-myc and c-Myc proteins are of similar sizes (464 and 454 amino acids, respectively). However, the *MYCN* mRNA is longer, mainly due to a larger 3'-untranslated region (3'UTR). In addition to structural and sequence homologies within the Myc-family, the functions of these proteins are closely related. Myc-proteins heterodimerize with the bHLHZip-protein Max to a transcription factor complex that binds to specific E-box DNA motifs (5'-CA*NN*TG-3') and activates transcription of genes involved in diverse cellular functions, including cell growth and proliferation, metabolism, apoptosis and differentiation [58-60]. N-myc preferentially binds to the E-box motifs CATGTG and CAACTG. Under MNA conditions, however, N-myc becomes less specific and binds additionally to CATTTG and CATCTG [61]. In addition to Myc, Max also dimerizes with the bHLHZip-proteins Mad/Mnt. These complexes also bind to E-box elements, but repress transcription through the recruitment of co-repressors [62]. Through interaction with Sp1 and Miz-1 at promoters, N-myc has been shown to silence gene expression by recruitment of the histone deacetylase HDAC1 [63, 64].

Dysregulation of Myc activity is an oncogenic hallmark in many human malignancies. Over-activation of Myc proteins is mainly caused by gene translocations or amplifications, or enhanced protein translation or stability, leading to overexpression of a structural normal protein [65, 66].

Given the fundamental role of Myc proteins on cellular processes, their activity in normal cells needs to be spatially and timely controlled. While c-Myc is expressed during all developmental stages and in a distinct pattern throughout the cell cycle of dividing cells, *MYCN* expression is restricted mainly to the peripheral and central nervous system, kidney,

lung and spleen during particular embryonal stages [67]. Expression is controlled at multiple levels, including gene transcription through upstream regulators, mRNA turnover, and protein activation or decay upon phosphorylation of specific protein residues [56]. In addition, *MYCN* is regulated by microRNAs (see chapter 2.3.10 and **paper II**).

The reason why MNA is associated with aggressive tumor biology is still not completely understood. Usually, MNA results in overexpression of N-myc protein. Several direct and indirect N-myc-targets have been identified, including genes involved in cell cycle regulation (e.g. *MDM2*, *MCM7*, *ODC*, *and ID2*), apoptosis (e.g. *TP53*), differentiation (e.g. *CDC42*, *PAX3*), drug resistance (e.g. *MRP1*) and *MYCN* stability (*Aurora kinase A*) (reviewed in [58]). In addition, N-myc regulates miRNAs with both oncogenic and tumor suppressor functions (see chapter 2.3 and **paper I**).

2.1.4.2 Numerical and segmental chromosomal aberrations

Neuroblastoma tumors harbor numerical chromosomal changes (gains and losses of whole chromosomes), segmental aberrations (translocations, amplifications), or combinations of both. In a recent study, 224 neuroblastoma tumors were genetically classified according to array-CGH profiles to carry either numerical or segmental aberrations alone, in combination, or a MNA with or without numerical aberrations (5 groups). Survival dichotomized into 2 groups: excellent outcome, regardless of stage and age, was found for tumors with solely numerical aberrations; segmental aberrations, in contrast, resulted in poor survival. Most common segmental alterations were deletions at 1p, 3p and 11q, as well as gains of 1q, 2p and 17q. Notably, the presence of segmental alterations, whatever their type, was the strongest predictor for relapse, regardless of the concomitant *MYCN* status [55]. The existence of tumors with both numerical and segmental changes is indicative for a tumor model in which low-grade tumors with numerical changes can secondarily acquire segmental aberrations and evolve into aggressive tumors [54]. The fact that the age at diagnosis of mixed profile tumors is higher than numerical-only cases, supports the idea of transition, and may be a rationale for neuroblastoma mass screening at later ages (see chapter 2.1.7 and [68]).

2.1.4.3 ALK mutations

Although chromosomal aberrations are frequent in neuroblastoma, only few mutations in typical cancer genes have been reported. In 2008, activating somatic mutations in the *anaplastic lymphoma kinase* (*ALK*) gene were identified in approximately 6-11% of sporadic neuroblastoma tumors [69-73], making *ALK* to the second major neuroblastoma gene. The

oncogenic nature of *ALK* mutants has been demonstrated in several neuroblastoma *in vitro* systems. Currently, extensive research is ongoing to develop, and clinically evaluate, small molecule *ALK* inhibitors, selectively targeting *ALK*-mutant neuroblastoma cells (reviewed in [74]).

2.1.4.4 Wnts, the Wnt pathway and DKK3 in neuroblastoma

The human Wnts are a family of 19 secreted proteins with broad implication in central nervous system development [75], including neural crest-derived structures [32, 76], as well as many other cellular functions like proliferation, apoptosis, cell adhesion and differentiation (reviewed in [77]). Wnt proteins act primarily through the canonical Wnt signaling pathway, which finally results in stabilization of the intracellular proto-oncoprotein β-catenin and activation of target gene transcription. In detail, but still simplified, Wnt ligands bind to a cell surface receptor complex consisting of the proteins frizzled and LRP5 and -6. Binding leads to phosphorylation of LRP5/6, which in turn recruits Dishevelled (DVL) proteins to interact with frizzled. As a consequence, a destruction complex consisting of APC (adenomatous polyposis coli) and axins, which otherwise rapidly degrades β-catenin, is inactivated by phosphorylated LRP5/6 and DVL and releases stabilized β-catenin that translocates to the nucleus. Here, β-catenin forms a complex with the LEF/TCF transcription factors to activate transcriptional targets (reviewed in [78]).

The identification of proto-oncogenes like c-Myc [79] and N-myc [80] as targets for activated Wnt-β-catenin signaling, as well as the detection of mutations in components of the Wnt pathway in cancer, broadly connects the Wnt pathway to cancer development (review [81]).

Mutations in inhibitors of the Wnt signaling pathway are another mechanisms for abnormal Wnt signaling contributing to oncogenic transformation. Dickkopf proteins (DKK1-4 and soggy) are secreted modulators of the Wnt pathway [82]. DKK1 and -2 antagonize Wnt signaling through binding to LRP5/6, which in turn interferes with the receptor´s ability to interact with Wnt-bound frizzled. Alternatively, DKKs can interact with the receptor Kremen (Krm), leading to endocytosis and destruction of the DKK-Krm-LRP5/6 complex [82].

The function of DKK3 is more obscure and seems to be dependent on the cell type. DKK3 acts as an inhibitor of Wnt signaling by blocking β-catenin translocation to the nucleus in several cell models, including cancer cell lines [83-85]. In contrast, it has also been shown that DKK3 does not interact with LRP5/6, but with Krm. Through internalization of the Krm-DKK3 complex, the Wnt pathway stays intact and Wnt signaling can be increased [86].

DKK3 is a tumor suppressor frequently inactivated by promoter methylation in cancers of the cervix [85, 87], liver [88, 89], breast [90], lung [91], mouth [92], and gastrointestinal tract [93, 94]. In pancreatic carcinoma, high DKK3 expression in tumor vessels was associated with favorable outcome and response to cytotoxic treatment [95]. In neuroblastoma, similarly, *DKK3* expression was higher in prognostic favorable tumors [96]. DKK3 did not seem to affect Wnt/β-catenin signaling in neuroblastoma, suggesting other still unknown tumor suppressor functions of DKK3 [96].

Previous reports have demonstrated that the expression of *DKK3* mRNA in neuroblastic tumors and neuroblastoma cell lines is inversely correlated to the expression of N-myc [96, 97]. By modifying *MYCN* expression with *MYCN* siRNA or induced *MYCN* expression, the reverse correlation between N-myc and *DKK3* mRNA expression was confirmed in several neuroblastoma cell lines. However, no direct binding to the *DKK3* promoter was identified, indicating an indirect regulatory mechanism [96].

2.1.4.5 Familial neuroblastoma and germline susceptibility genes

A family history of neuroblastoma can be detected in 1-2% of cases [98]. The majority of cases (90%) can be attributed to germ line mutations in either the paired-like homeobox *PHOX2B* gene [99] or the anaplastic lymphoma kinase (*ALK)* gene [73]. Patients with sporadic or familial neuroblastoma in conjunction with other diseases of neural-crest origin, like Hirschsprung's disease and congenital central hypoventilation syndrome, typically have *PHOX2B* mutations. *ALK* mutations were also found in 5-15% of sporadic neuroblastoma cases [71, 73], making *ALK* to the major neuroblastoma-predisposing gene (see also chapter 2.1.4.3 and [24]). Genetic testing for *ALK* or *PHOX2B* mutations is recommended in patients with a family history of neuroblastoma or other disorders of the neural crest to identify unaffected siblings with germline mutations who may profit from neuroblastoma screening and early diagnosis [53].

In addition to mutations in *ALK* and *PHOX2B*, several heritable genetic variations (single nucleotide polymorphisms, SNPs) in the human genome, identified by genome-wide association studies, predispose for the development of sporadic neuroblastomas [24, 53, 100].

2.1.5 From histopathology to risk stratification

The degree of differentiation in neuroblastic tumors, but also other pathological and clinical markers like mitosis-karyorrhexis-index (MKI), mitosis rate, stage and age at diagnosis, are surrogates for underlying tumor burden and biology [11, 19, 24]. Risk group classification

systems incorporating these and other markers attempt to predict the aggressiveness and clinical course of an individual tumor. Given the clinical heterogeneity of neuroblastic tumors, it is highly relevant for treating physicians to be able to estimate tumor behavior, classify patients into risk-groups, and tailor therapy by treatment stratification. In 2009, an International Neuroblastoma Risk Group (INRG) Classification System was proposed based on a retrospective analysis of 8.800 neuroblastoma patients from North America, Europe and Japan [18]. The INRG Classification System (INRGCS) allows assessing risk prior to any treatment. The next section will briefly summarize the criteria incorporated in the INRGCS. Histological categories and grade of tumor differentiation, both integral parts of the INRGCS, have already been discussed in chapter 2.1.2.

2.1.5.1 Tumor stage

In 1988 and 1993, an International Neuroblastoma Staging System (INSS) was established [101, 102]. The system separated localized (stage 1 and 2) from locoregional (stage 3) and metastatic tumors (stage 4). Tumors in infants younger than 1 year with metastases limited to the skin, liver or bone marrow, were classified as stage 4S, attributing the fact that these tumors have a distinct biology and excellent prognosis with minimal or no treatment due to spontaneous regression or maturation (Figure 2). The INSS system is still in use in many countries.

Panel: **INSS staging system**

1 Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour could be positive)

2A Localised tumour with incomplete gross excision; representative ipsilateral non-adherent lymph nodes negative for tumour microscopically

2B Localised tumour with or without complete gross excision, with ipsilateral non-adherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes should be negative microscopically

3 Unresectable unilateral tumour infiltrating across the midline, with or without regional lymph node involvement; or localised unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement

4 Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, or other organs (except as defined by stage 4S)

4S Localised primary tumour in infants younger than 1 year (as defined for stage 1, 2A, or 2B), with dissemination limited to skin, liver, or bone marrow (<10% malignant cells)

Figure 2: The International Neuroblastoma Staging System (INSS)

However, a disadvantage of the INSS is that staging is dependent on surgical species, and the degree of excision and number of involved lymph nodes may be dependent on the surgeon's expertise and abundance of resected nodes. Therefore, a new staging system (International Risk Group Staging System, INRGSS) has recently been proposed [103].

The INRGSS is based on pre-surgical, radiological characteristics of the primary tumor. Through the definition of a set of image-defined risk factors (IDRF), tumors are separated in groups with or without IDRFs. Image modalities include CT/MRI and metaiodobenzyl-guanidine (MIBG) scintigraphy. In addition, bone marrow involvement has to be assessed. By these investigations, four disease stages were delineated (Figure 3).

Stage	Description
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment
L2	Locoregional tumor with presence of one or more image-defined risk factors
M	Distant metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow

Figure 3: The International Neuroblastoma Risk Group (INRG) staging system

2.1.5.2 Age at diagnosis

Age is a strong independent prognostic factor in neuroblastoma. Basically, young children, especially under the age of 1 year, have often localized tumors with favorable tumor biology and superior prognosis, while the chance for disseminated disease, unfavorable biological features and death from refractory disease is continuously increasing in older children [11]. Traditionally, the age cutoff for risk classifications in clinical trials has been 12 months. However, retrospective data from larger trials revealed that age, as a risk factor, is rather a continuum than a binary variable. From statistical and biological standpoints, the optimal discriminatory cutoff has been determined to be somewhere between 15 and 19 months [104]. The INRG propose 18 months as age cutoff for future risk classifications [18].

2.1.5.3 Genetic markers

The cytogenetic aberration most consistently associated with poor prognosis in neuroblastoma is genomic amplification of the proto-oncogene *MYCN* (see chapter 2.1.4.1). *MYCN*-amplification (MNA) in neuroblastoma and its impact on prognosis has been known since 1984 [105, 106]. MNA correlates strongly with advanced disease and treatment failure [107]. Hemizygous loss of large segments on chromosome 11q defines another major genetic

subtype of high-risk neuroblastoma, and unbalanced deletions of 11q- material are independently prognostic for outcome [108]. MNA and loss of 11q are strong inversely correlated and can be found in about 70 % of metastatic tumors. Typically, both genetic subtypes occur with additional genetic alteration. Loss of chromosome 1p is frequently found in MNA tumors, while 11q-loss is significantly associated with gain of 7q and 3p and 4p-loss. Gain of 17q-material is frequent in both 11q- and MNA tumors, most often caused by unbalanced t(11q;17q) and t(1p;17q) translocations, respectively [54, 109, 110]. Gain of 17q is a strong indicator of adverse outcome [111].

The *MYCN*-status and absence/presence of 11q-deletions are risk criteria in the INRGCS [18]. The 17q-status was not implemented in the INRG system due to lack of data in the INRG study patient cohort [18]. The prognostic value of 1p-deletions is still controversial. Although Attiyeh *et al*. [108] reported 1p-deletions to be independently associated with decreased survival in low- and intermediate-risk patients, the INRG data revealed superior overall rates at least in low-risk patients regardless of the 1p-status [18]. In the INRGCS, 1p-status is therefore not included as a prognostic discriminator.

2.1.5.4 DNA ploidy

In general, two main categories of cellular DNA content can be separated in neuroblastoma: near-diploid content, or hyperdiploid (often near-triploid) content [112, 113]. DNA content (or DNA index) has long been known as a predictor for chemotherapy response for neuroblastoma patients < 2 years of age. While hyperdiploidy or near-triploidy was associated with long-term survival in these patients, near-diploidy (or tetraploidy) predicted early treatment failure [114-116]. Genetic tumor models have suggested that less aggressive neuroblastoma cells have mitosis defects, resulting in gains and losses of whole chromosomes (numerical chromosomal aberrations) and near-triploid cells. In contrast, aggressive tumors are characterized by genomic instability causing unbalanced translocations and chromosomal rearrangements (segmental copy number alterations, see also chapter 2.1.4.2) [11, 54, 55, 112]. The DNA index has been included in the risk assessment of disseminated neuroblastoma without MNA in children under 18 months [18].

2.1.5.5 The International Neuroblastoma Risk Group Classification System (INRGCS)

In summary, current prognostic factors applied in clinical risk assessment are stage, age at diagnosis, histological category and differentiation grade, *MYCN*- and 11p-status as well as tumor DNA ploidy.

The pre-treatment risk classification system proposed by the INRG is based on these factors and allows the definition of 4 risk groups: very low risk, low risk, intermediate risk and high-risk (Figure 4). The groups were defined by event-free survival cut-offs (>85% EFS; >75% to ≤85%; ≥ 50% to ≤75%, or <50%, respectively).

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		Hyperdiploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

Figure 4: The International Neuroblastoma Risk Group Classification System (INRGCS). Blank field = "any"; diploid (DNA index ≤1.0); hyperdiploid (DNA index >1.0, includes near-triploid and near tetraploid tumors); GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified. Modified from [18].

The purpose of the INRG classification system is to enable that children diagnosed with neuroblastoma in any country of the world can be stratified into homogenous pretreatment groups. This will facilitate the comparison of risk-based clinical trials conducted in different regions of the world [18].

2.1.6 Risk-adapted treatment and prognosis

The definition of risk-groups made it possible to conduct cooperative clinical trials with the aim to establish, evaluate and optimize risk-adapted treatment of neuroblastoma patients. Norway actively participates in the clinical trials designed by the International Society of Pediatric Oncology European Neuroblastoma (SIOPEN) research group. Briefly and simplified, patients are stratified according to the INRGCS into three treatment protocols: Low-risk patients (L1) without MNA are treated according to the LNESG2 protocol by surgery alone. L1-patients with MNA, L2- and MS-patients without MNA, and M-patients younger than 18 months without MNA were treated according to the LINES protocol. The

LINES protocol contains several treatment subgroups in which intensity gradually increases from observation alone to combinations of chemotherapy, surgery and irradiation. High-risk patients, except those with localized disease, were treated according to the HR-NBL-1 protocol, one of the most aggressive treatment protocols in pediatric oncology. It combines rapid multidrug chemotherapy cycles, tumor surgery, irradiation, high-dose chemotherapy with autologous hematopoietic stem cell rescue, and differentiation-inducing treatment with 13-*cis* retinoic acid ([50], and chapter 2.1.3). Very recently, immunological targeting of residual tumor cells by a combination of subcutaneously injected monoclonal antibodies against a neuroblastoma surface antigen (anti-GD2 antibody) and concomitant stimulation of the patient's immune system by interleukin-2 [117] has been implemented into the protocol treatment. Immunotherapy against resistant tumor cells either by antibody-based strategies [13, 117-120] or haploidentical stem cell transplantation [121] has shown promising results for refractory and relapsed disease.

The 5-year event-free survival (EFS) and overall survival (OS) rates for the complete INRG study population of 8.800 neuroblastoma patients was 63% and 70%, respectively [18]. Adrenal primary tumor site had significantly worse EFS than other primary sites. Survival rates varied substantially between patients of INSS stages 1,2,3,4S (OS 91% ± 1%) and stage 4 (OS 42% ± 1%). Disseminated disease with MNA in the tumors showed worst prognosis, with OS rates between 22-29%, regardless of age [18]. In contrast, disseminated neuroblastomas without MNA in children below the age of 12-18 months have a superior prognosis with OS rates exceeding 90 % [18, 122].

2.1.7 Mass screening

The dichotomized prognosis of neuroblastoma, with superior survival rates for young patients with localized disease contrasted by the poor prognosis for older children with often metastasized disease and unfavorable biological features, initiated mass screening studies in the 1980-1990s to clarify if long-term survival of high-risk patients can be improved when tumors were detected earlier in the course of disease [15, 16, 123, 124]. Mass screening was performed by the investigation of urine samples for tumor-secreted catecholamines [123]. In fact, screening detected primarily tumors with biological favorable near-triploid DNA content that are prone to spontaneous differentiation or can be cured with minimal therapeutic interventions [125]. Mass screening at the age of 6 or 12 months increased the incidence of neuroblastoma [124], but not long-term survival [16, 17, 126], indicating that aggressive tumors either develop *de novo* at later ages, or have aggressive courses even when detected

early. The results from these mass screening studies support the model that neuroblastoma is separated into two biological subgroups: favorable tumors in younger children, and tumors with unfavorable biology in older children, where the former rarely evolves into aggressive types [19]. However, the occurrence of tumors with both numerical and segmental aberrations may indicate the possibility for tumor transition (see chapter 2.1.4.2). By now, there is no clear indication from prospective studies for population-based mass screening; however, screening at the age of 18 months may prove beneficial in prospective studies [68].

2.2 MicroRNAs

MicroRNAs (or miRNAs) are an abundant class of genome-encoded, endogenous, small non-protein-coding RNA molecules that negatively regulate protein expression in cells [127]. The first miRNA, *lin-4*, was discovered in 1993 in the nematode *C.elegans* [128, 129]. Since that time, miRNAs have been discovered in nearly every organism, from plants and simple multicellular organisms to flies, vertebrates and humans. MiRNAs are annotated and catalogued in the public-accessible web-based database miRBase (www.mirbase.org)[130-134], which was founded at the Sanger Institute in England and is now managed by the University of Manchester. The current miRBase release 17 (april 2011) annotates 19724 mature miRNAs in 153 species, including 1719 mature human miRNAs. In humans, the total number of annotated mature miRNA sequences has thereby increased by 43% compared to the previous release (2010), emphasizing that the complete mapping of all human miRNAs is still in progress.

The biogenesis of miRNAs is a complex multi-step process that starts in the nucleus and ends in the cytoplasm of cells (Figure 5; reviewed in [135-138]). Most miRNAs are transcribed as long monocistronic or polycistronic primary transcription units (primary miRNA or pri-miRNA) by RNA polymerase II. Typically, a pri-miRNA is characterized by a hairpin structure, containing a double-stranded (ds) RNA stem of ~33 base pairs (bp), a terminal loop, and single-stranded (ss) RNA flanking regions. The stem-loop structure contains the miRNA in the 5' or 3' half of the stem. The pri-miRNA is cleaved in the nucleus by a protein complex (the "microprocessor complex") consisting of several proteins including the RNase III enzyme Drosha and its co-factor DGCR8. DGCR8 functions as a molecular anchor and defines the binding site for the microprocessor, while Drosha cleaves the RNA approximately 11 bp from the ss-dsRNA junction, producing the shorter, ~ 65-70-nucleotide long hairpin pre-miRNA.

Following completion of this nuclear processing step, the pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5. Here, the pre-miRNA is cleaved by another RNAse III enzyme called Dicer. Dicer cleaves ~22 nt from the pre-existing end of the pre-miRNA, producing ~22 nt double-stranded RNA molecules. One of the two strands (the guide strand or mature miRNA) is, selected upon thermodynamic properties, loaded on an Argonaute (Ago) protein, the main constituent of the RNA-Induced Silencing Complex (RISC). The other strand (passenger strand) is degraded. The mature miRNA sequence guides the RISC complex to recognize and target partial complementary mRNA sequences, primarily within the 3'-untranslated region (3'UTR)[136-138].

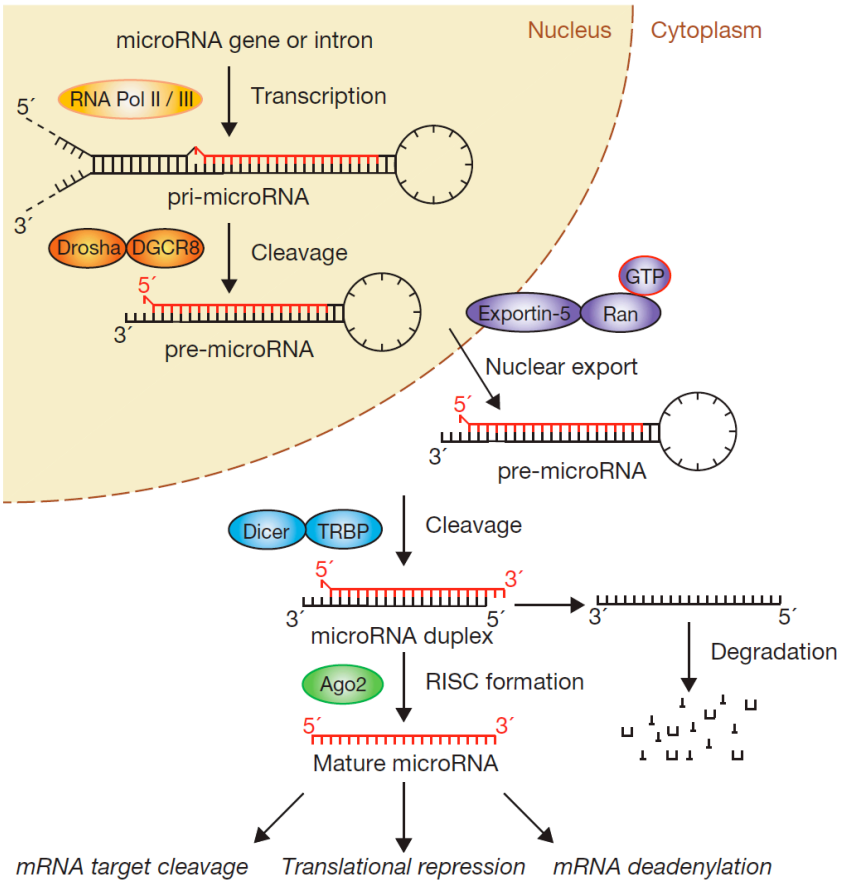

Figure 5: Schematic overview over miRNA biogenesis (from [135], with permission)

Expressional changes of even single miRNAs have profound effects on the protein composition in cells [139, 140]. The degree of complementarity between the mature miRNA sequence and the target mRNAs determines the mechanism responsible for blocking gene expression. Near-perfect pairing, as it is mainly found in plants, causes mRNA destruction through Ago-catalyzed mRNA cleavage [141, 142]. In vertebrates, miRNA-mRNA interactions are most often through imperfect base pairing [127]. Here, the precise

mechanisms behind miRNA-mediated gene silencing is still scientifically debated [143]. Destabilization of the mRNA by de-adenylation, de-capping and rapid degradation through standard mRNA-turnover processes ("mRNA-destabilization scenario") seems to be the mainstay of miRNA-mediated protein repression [144]. In addition, translational repression through blocking translation initiation, or a combination of both mechanisms, is an established mechanism to block protein expression [140, 143].

Different types of miRNA target sites have been identified [145]. In general, target sites are characterized by a varying degree of complementarity to the miRNA sequence, and often evolutionary conserved between different species (reviewed in [127]). As shown by studies where single nucleotides in miRNA sequences were systematically mutated, sites with as little a seven base-pairs of complementarity at the 5'end of the miRNA were sufficient to repress the predicted mRNA target *in vivo* [145]. This complementary sequence at the 5'-position 2-7 of the miRNA is called "seed", and is the key determinant for miRNA specificity [146, 147]. Four variants of the 6-nt-seed have been identified (Figure 6): the 7mer-m8 site, which comprises the seed match supplemented by a match to miRNA nucleotide 8; the 7mer-A1 site, which comprises the seed match supplemented by an A across miRNA nucleotide 1; the 8mer site, which comprises the seed match supplemented by both the m8 and the A1; and a site with only 6 nt perfectly matching (6mer site) [147], resulting in a hierarchy of site efficacy: 8mer > 7mer-m8 > 7mer-A1 > 6mer [148].

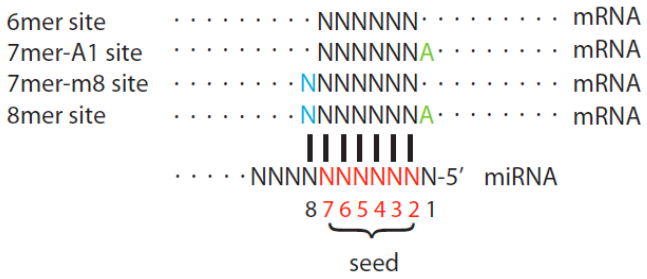

Figure 6: Types of miRNA target sites (adapted from [127, 149])

In addition to the seed sequence, complementarity at the 3'-end can both compensate for a single mismatch in the seed (3'-compensatory site), as well as increase affinity of the miRNA (3'-supplementary sites)[148].

The miRNA nomenclature is managed by miRBase and has been slightly changed with up-coming releases of the database. In general, miRNA names start with a 3-4-letter prefix to designate the species (e.g. *hsa-* for homo sapiens miRNAs). They are further assigned by a

three-letter prefix, such as miR- or let-, followed by a sequential number (e.g., miR-1). By definition, the mature miRNA is labeled “miR” [132], while the precursor is labeled “mir”; however, this discrimination is not stringently used in the literature, and it has been recommended to use “mature” or “precursor” when a clear distinction is necessary. Identical miRNAs transcribed from different genes are given a numeric suffix, e.g. miR-1–1 and miR-1–2. Very similar miRNAs (paralogous miRNAs), often sharing the same seed sequence, are designated as a “miRNA family” (e.g. mir-29 family) and discriminated by numeric and letter suffixes (e.g. mir-29a, mir-29b, mir-29c) [150]. In some cases, two mature miRNAs are processed from the same stem-loop precursor, one from each arm, and are accordingly designated by an additional suffix “-5p” (for that released from the 5’-arm) and “-3p” (for that released from the 3’-arm); e.g., miR-199a-5p and miR-199a-3p. The star-forms (miR*), previously used for minor forms, have been “retired” according to the latest nomenclature convention [134].

MiRNA clusters are polycistronic transcription units consisting of several miRNAs located in close proximity. In the human genome, more than 85 % of all miRNAs are located within intronic regions, while the rest are located within exons [151].

As miRNAs tend to target many different mRNAs, and each mRNA may contain several to hundreds of different miRNA binding sites, it is obvious that the miRNA-mRNA regulatory network is extremely complex. It has been estimated that 30-60 % of all human genes are regulated by miRNAs [147, 149]; others suggest that small RNAs, including miRNAs, will have the potential to regulate all human genes [137]. A plethora of more than 2.000 review articles on miRNA and 20.000 original research papers indexed in PubMed illustrate the broad implication of miRNAs in more or less all aspects of cellular function. Established roles for miRNAs are their involvement in the development of organisms and organs, in cellular processes like proliferation, differentiation, signal transduction and apoptosis, in cell fate decisions and immunological defense of viral attacks (reviewed in [138, 152]). As a consequence of this broad function, miRNA biogenesis has to be tightly controlled. Deregulated miRNA expression has been associated with a diversity of diseases, including cancer; a fact attributed in the term “oncomirs” for cancer-related miRNAs. MiRNA transcription is regulated by several transcription factors, including oncogenes like *c-MYC* [153, 154] and *MYCN* (see chapter 2.3), and tumor suppressors like p53 [155].

2.3 MicroRNAs, N-myc and neuroblastoma

2.3.1 Studying N-myc and miRNA expression – general aspects

The two very first studies investigating the role of N-myc on miRNA expression in neuroblastoma tumors were published by Chen and Stallings in 2007 [156] and Schulte *et al*. in 2008 [157]. Both studies profiled the miRNA expression in a smaller set of primary tumors (18 and 24 tumors including 6 and 7 with MNA, respectively) to define differentially expressed miRNAs between the MNA and non-amplified groups. Using miRNA-specific real-time PCR, Chen and Stallings profiled 157 known miRNAs and reported mainly down-regulation of miRNA expression in MNA tumors (26 out of 31 differentially expressed miRNAs). In contrast, Schulte et al. used a microarray approach, supplemented by real-time PCR validation, to profile 384 miRNAs and found exclusively up-regulation of miRNA expression (14 miRNAs) in their MNA tumor samples. Among these were several members of the oncogenic mir-17-92 cluster as well as four of the five up-regulated miRNAs reported by Chen and Stallings.

The effect of N-myc on miRNA expression was in both studies further investigated by altering *MYCN* expression in neuroblastoma cell lines. Chen and Stallings used anti-*MYCN* siRNA technology to repress *MYCN* expression in MNA Kelly cells and reported mainly up-regulation of miRNA expression. The in vitro experimental system used by Schulte *et al.* is based on ectopic over-expression of *MYCN* cDNA in non-amplified SH-EP cells (SH-EP MYCN-ER). Here, they observed up-regulation of 11 miRNAs, 7 of which were also up-regulated in their MNA tumor samples. Surprisingly, two miRNAs, *mir-92* and *let-7b*, both differentially expressed in the experimental cell line models, were correlated to *MYCN* expression in opposite ways in these studies.

The discrepancies between these two pioneer studies illustrate general methodological challenges when studying miRNA expression in neuroblastoma:

(1) *Tumor sample size*. The heterogeneous genetic background of neuroblastoma tumors requires large tumor sets to delineate miRNA expression signatures for complex genetic subgroups. In one of the largest miRNA profiling studies in neuroblastoma so far, Bray *et al.* profiled 430 miRNAs in a total of 145 primary neuroblastoma tumors, including 36 with MNA [158]. They found both up- and down-regulated miRNAs (14 and 23, respectively) when MNA tumors were compared to non-amplified tumors. Importantly, they also determined large-scale genomic gains and losses in each tumor by array-CGH and correlated

the genomic localization of differentially expressed miRNAs to chromosomal gains and losses. About 15 % of all detectable miRNAs changed expression as a result of chromosomal imbalances in the tumors, highlighting that gains or losses of miRNA encoding regions contribute significantly to miRNA dysregulation in neuroblastoma, in addition to N-myc overexpression.

(2) *MYCN expression*. Experimental systems using *MYCN* induction or knockdown do not reflect two sides of the same coin, but initiate two distinct biological processes, where the former results in cell cycle progression and proliferation, while the latter in differentiation and apoptosis.

(3) *Profiling platforms*. The nature of miRNAs (small size and base-paired structure) poses a challenge for miRNA detection techniques [159]. Different technical platforms, like northern blotting, high-throughput real-time PCR-techniques, microarray analyses or next-generation sequencing, may therefore generate partially diverging expression profiles, mandating confirmation between the platforms.

(4) *Number of miRNAs.* The number of investigated individual miRNAs varies between studies, especially over time, not least because the overall number of identified miRNAs (and other small RNA molecules) in the human genome is still increasing. Profiling studies based on ultra-deep next-generation sequencing of the total small RNA transcriptome in neuroblastoma [160] have the potential to provide ultra-specific and absolute miRNA expression data in future studies.

(5) *Functional confirmation*. Differential miRNA expression data should be supported by functional studies *in vitro* and *in vivo* to prove biological relevance of each individual miRNA.

2.3.2 N-myc induces miRNA expression – the mir-17-92 cluster

In 2008, Fontana *et al.* published the first comprehensive functional study on a *MYCN*-regulated miRNA cluster – the mir-17-92 cluster [161]. It is transcribed as a polycistronic unit from chromosome 13, and comprises 7 individual miRNAs (*mir-17*, *mir-18a*, *mir-19a*, *mir-19b*, *mir-20a*, *mir-92a*). Fontana *et al.* confirmed the observation made by Schulte *et al.* [157], showing that miRNAs of the mir-17-92 cluster are higher expressed in tumors and neuroblastoma cell lines with high N-myc expression. By the use of chromatin immunoprecipitation (ChIP), they validated direct binding of N-myc to several E-box motifs in the mir-17-92 promoter and demonstrated transcriptional activation in luciferase reporter gene assays. Moreover, Fontana *et al.* shed light on the functional consequences of mir-17-92

over-expression in MNA neuroblastoma cells: the tumor suppressor p21 (*CDKN1A*) was shown to be targeted by *mir-17*, and over-expression of *mir-17* in non-amplified cells increased proliferation, colony-formation and *in vivo* tumor growth. *Vice versa*, inhibition of *mir-17* by antagomirs in MNA cells decreased proliferation and tumorigenesis, and increased p21 expression. Surprisingly, antagomir-17 increased apoptosis in neuroblastoma cells; an effect not attributable to increased p21. Instead, *mir-17* was found to additionally target *BIM* (*BCL2 Interacting Mediator of cell death*, or *BCL2L11*), a pro-apoptotic BH3-only member of the BCL2 (B-Cell Lymphoma 2) family. In conclusion, Fontana *et al.* proposed that *mir-17* functions as a major effector of *MYCN*-mediated tumorigenesis, by targeting p21 while at the same time protecting MNA cells from N-myc induced apoptosis through translational inhibition of BIM.

Other studies have confirmed direct binding of N-myc to the mir-17-92 promoter [162, 163], as well as a positive correlation between expression of *MYCN* and members of the mir-17-92 cluster in primary tumors and/or neuroblastoma cell lines [22, 158, 160, 162, 164-168]. As miRNAs simultaneously target a variety of different mRNAs, it became clear that activation of the mir-17-92 cluster enables N-myc to turn multiple cellular processes towards malignant transformation. In 2009, Beveridge *et al.* showed that *mir-17* and *mir-20a* target three differentiation-associated genes in neuroblastoma cells; *BCL2*, *MEF2D* (*Myocyte Enhancer Factor-2D*) and *MAP3K12* [169]. Another differentiation-associated protein, the estrogen receptor-α (ESR1), was also recently reported to be a target for miRNAs of the mir-17-92 cluster [162]. ESR1 is expressed in fetal sympathetic ganglia during human neuronal development and has been shown to be inversely correlated to *MYCN* expression in neuroblastoma tumors [162]. Loven *et al.* demonstrated that *mir-18a* and *-19a* target ESR1, providing a mechanism on how N-myc regulates ESR1 expression [162]. Notably, Loven *et al.* showed that N-myc also binds to E-boxes of the mir-17-92 paralogous miRNA clusters mir-106b-25 (chromosome 7) and mir-106a-363 (chromosome X), enabling a concerted action of N-myc-activated miRNAs to synergize N-myc functions.

In a genome-wide proteome analysis, Mestdagh *et al.* used a tetracycline-inducible mir-17-92 expression system in non-amplified neuroblastoma cells (SHEP-TR-miR-17-92) to show that 144 proteins were down-regulated upon mir-17-92 induction, including multiple key effectors along the TGF-β signaling cascade [170]. Both *TGF-beta receptor type II* (*TGFBR2*) and *Smad2/Smad4* were shown to be direct targets of *mir-17/20* and *mir-18a*, respectively.

Interestingly, TGF- β responsive genes include *p21* and *BIM* in gastric cancer [171], both targets of mir-17-92 in neuroblastoma [161].

These studies illustrate how N-myc is able to regulate multiple steps of oncogenic processes through the activation of the mir-17-92 cluster (Figure 7).

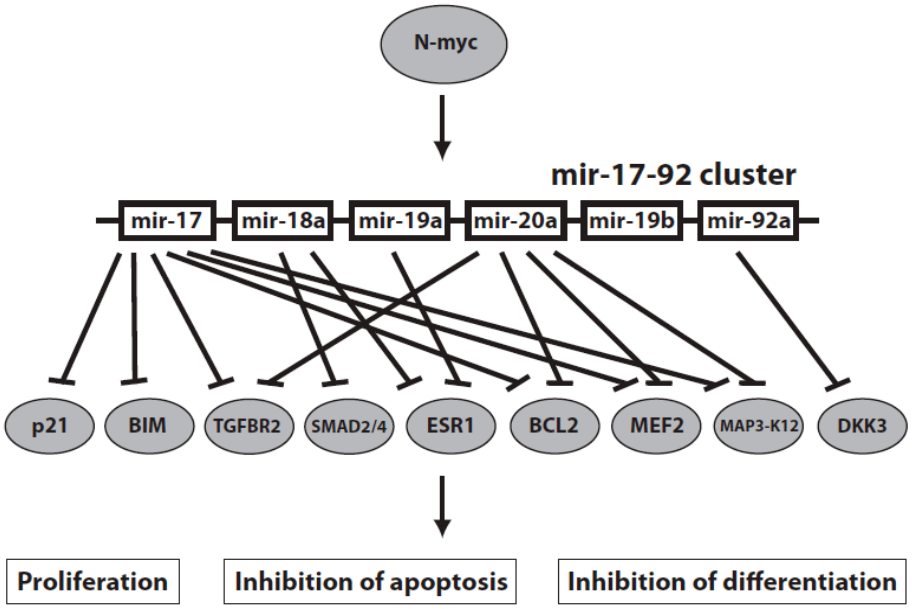

Figure 7: N-myc induces expression of the mir-17-92 cluster. Several miRNAs in the cluster have been confirmed to target genes involved in proliferation, inhibition of apoptosis and inhibition of differentiation.

2.3.3 N-myc induces miRNA expression – mir-9

Another functionally characterized miRNA positively correlated to *MYCN* expression is *mir-9*. This miRNA is highly expressed in the brain and other neural tissues and coordinates the proliferation and migration of human neural progenitor cells [172]. Recently, Ma *et al.* used an inducible *MYCN* expression system and genome-wide ChIP-on-chip analyses to confirm that *mir-9* (at the mir-9-3 locus) is directly activated by N-myc and that *mir-9* targets the tumor suppressor E-cadherin (*CDH1*) [165]. E-cadherin is a ubiquitously expressed transmembrane glycoprotein on the surface of epithelial cells, with a pivotal role for cell-cell adhesion of adjacent cells. E-cadherin function is frequently lost in epithelial cancers and associated with invasion and metastasis. In neural crest development, during the process of neurulation, down-regulation of E-cadherin allows the neural crest cell to detach from the neural tube and migrate along the migratory pathway [27]. Ma *et al.* found that *mir-9* was significantly higher expressed in 23 metastasized neuroblastoma tumors (stage 4, all MNA), compared to 22 non-amplified tumors without metastases. They demonstrated that *mir-9*

promotes cancer cell motility and invasiveness through the suppression of E-cadherin. Moreover, the decrease in E-cadherin increased expression of the pro-angiogenic factor VEGFA through activated β-catenin signaling in the cells. The study by Ma *et al.* propose for the first time a model on how N-myc might be able to contribute to metastasis formation through the activation of a single microRNA (Figure 8).

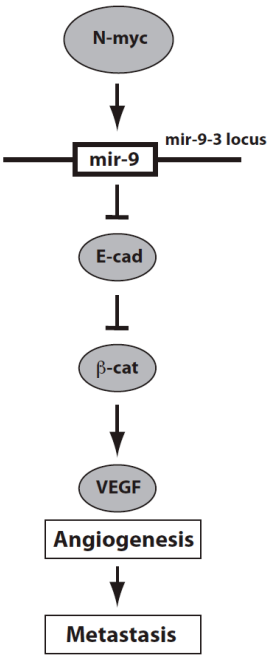

Figure 8: Model for N-myc – *mir-9* – E-cadherin pathway involved in neuroblastoma metastasis.

2.3.4 N-myc induces miRNA expression – mir-421

In 2010, a link between disturbed double-strand break (DSB)-induced DNA damage response and an N-myc-activated miRNA was reported by Hu *et al.* [173]. The authors found increased expression of *mir-421* co-varied with reduced levels of ATM (ataxia-telangiectasia mutated kinase) in MNA neuroblastoma cell lines. With the use of a luciferase reporter assay, *mir-421* was shown to directly target the 3'UTR sequence of *ATM*. They further demonstrated that N-myc binds to the promoter region of *mir-421* to enhance its expression. This establishes a linear signaling pathway (N-myc – *mir-421* – ATM) explaining how N-myc negatively regulates *ATM* expression. ATM is a tumor suppressor that transduces the DSB damage signals to down-stream effectors of the DNA repair machinery during cell cycle checkpoints at G1-S and intra-S phase. Impaired ATM activity leads, most often through gene mutations, to genomic instability and predisposes for cancer transformation, especially after radiation exposure [174]. In conclusion, this study showed a new mechanism for ATM dysregulation related to neuroblastoma tumorigenesis.

2.3.5 N-myc is predominantly a repressor of miRNA expression

Although several miRNAs have been documented to positively correlate with *MYCN* expression, there is now growing evidence that N-myc predominantly acts repressive on the overall miRNA composition in MNA neuroblastoma cells [22, 156, 158, 167, 168, 175] and upon N-myc induction in non-amplified neuroblastoma cells [162]. Lin *et al.* profiled the expression of 162 miRNAs in 66 primary neuroblastoma tumors (including 13 with *MYCN*-amplification) and found a nearly global down-regulation of miRNAs in high-risk tumors, especially in those with *MYCN*-amplification [175]. The authors hypothesized that dysregulation in Dicer and/or Drosha, key enzymes in the miRNA processing pathway, may contribute to the widespread miRNA down-regulation. Indeed, both Dicer and Drosha were lower expressed in stage 4 tumors compared to other stages, with the most strikingly differential expression between stage 4 and stage 4S. This suggests that repression of miRNAs may be involved in tumor progression.

2.3.6 N-myc-regulated tumor suppressor miRNAs in neuroblastoma

Chen and Stallings found that *mir-184* was significantly down-regulated in MNA tumors and up-regulated upon *MYCN*-knockdown in a MNA neuroblastoma cell line [156]. Overexpression of *mir-184* reduced cell viability of both MNA and non-amplified cell lines through the induction of apoptosis and G1 cell cycle arrest. A follow-up study by Foley *et al.* confirmed the inverse correlation between N-myc and *mir-184* in primary tumors and showed that inhibition of *mir-184* by antagomir treatment increased proliferation of neuroblastoma cells [176]. Moreover, they demonstrated that mir-184 directly targets *AKT2* (*Protein kinase B beta*). AKT2 is a down-stream effector of the phosphatidylinositol 3-kinase (PI3K) pathway, one of the most potent pro-survival pathways in cancer. Activation of AKT is associated with poor prognosis in neuroblastoma [177]. Finally, Tivnan et al. used an *in vivo* murine xenograft model where *mir-184*-transfected MNA or non-MNA neuroblastoma cells were orthotopically injected into CB-17/SCID mice [178]. Tumors arising from *mir-184*-transfected cells were smaller than the controls, and mice survived longer. In summary, these comprehensive studies clearly established *MYCN*–regulated *mir-184* as a tumor suppressor in neuroblastoma.

Another tumor suppressor miRNA repressed by N-myc is *mir-542-5p*. Several studies have shown an inverse correlation between *mir-542-5p* and *MYCN*-amplification in primary tumors [22, 158, 160, 179]. In a large-scale profiling study of 430 miRNAs in 69 primary tumors, Schulte *et al.* found increased expression of 4 miRNAs in MNA tumors while 35 miRNAs

were repressed, including *mir-542-5p* [22]. *Mir-542-5p* expression was found to be predictive for outcome, with a significantly higher expression in patients with event-free survival compared to relapsed patients. Bray *et al.* profiled the expression of 449 miRNAs in 145 neuroblastoma tumors and correlated *mir-542-5p* expression to clinical data [179]. Expression of *mir-542-5p* was non-randomly distributed among tumor genetic subtypes, with lowest expression in MNA tumors (77 % completely lacking expression) and highest expression in stage 1,2,3 and 4S tumors. Patients with tumors lacking *mir-542-5p* expression had the poorest prognosis, independently of the *MYCN* status in the tumors [22, 179]. Bray *et al.* further demonstrated that *mir-542-5p* overexpression in MNA and non-MNA neuroblastoma cells reduced invasiveness *in vitro*, and restricted tumor growth and metastasis *in vivo* when cells were orthotopically injected into mice.

2.3.7 Genome-wide analysis of N-myc-regulated miRNAs

So far, only two studies have used a next-generation sequencing approach to analyze activation or repression of miRNAs by N-myc on a genome-wide basis [160, 168]. Schulte *et al.* used ultra-deep SOLiD sequencing to compare the total small RNA transcriptome in 5 unfavorable MNA tumors with 5 favorable non-MNA tumors [160]. Analyzing the absolute number of miRNA reads, there was a trend toward a higher proportion of mature miRNAs in the favorable patient group, indicating a possible global suppression of miRNA transcription in MNA tumors. Expression data of 204 miRNAs were validated by RT-qPCR with good correlation between the technical platforms. The SOLiD sequencing data confirmed previously data on differential expression in MNA versus non-MNA tumors, including the mir-17-92 cluster and *mir-181* (positive N-myc-correlation) and *mir-542-5p* (nearly absent in MNA tumors). In total, 76 miRNAs were differentially expressed between MNA and non-MNA tumors (43 up-regulated and 33 down-regulated). Next-generation sequencing allowed the discovery of several new miRNAs in neuroblastoma and revealed insight into miRNA editing and distribution of mir-5p/-3p and mir* forms. In addition, cluster analysis was able to exactly separate the two clinical outcome groups based on their differential miRNA expression, indicating that the miRNA transcriptome reflects tumor aggressiveness [160].

Very recently, Shohet *et al.* performed a genome-wide study of N-myc binding sites in promoters driving miRNA expression in neuroblastoma [168]. Using a combination of ChIP and Massively Parallel Sequencing (ChIP-seq) in a neuroblastoma cell line with inducible N-myc expression, they identified 20 gene promoters, hosting a total of 30 miRNAs, to which N-myc specifically bound to E-box motifs. The majority of host genes that were correlated

with survival were down-regulated by high N-myc levels, suggesting a tumor suppressor function for these host genes as well as the co-expressed intronic miRNAs. However, functional studies of two *MYCN*-regulated intronic miRNAs (*mir-591* and *mir-558*) identified tumor suppressor functions for *mir-591* as expected, while *mir-558* was reported to function as an oncomir. These data are supportive for the hypothesis that N-myc has to restrain growth as well as to promote it during the processes of tumor initiation and tumor transformation from neural crest cells to undifferentiated malignant tumor cells [168].

2.3.8 C-myc/N-myc-induced miRNAs repress gene networks

In a large-scale miRNA expression study, Mestdagh *et al.* profiled the expression of 430 miRNAs in 95 neuroblastoma tumors and delineated a signature of 50 unique miRNAs differentially expressed between MNA and *MYCN* single-copy tumors (16 up-regulated and 34 down-regulated miRNAs) [167]. Interestingly, the miRNA signature further delineated 2 distinct tumor sub-groups within the *MYCN* single-copy group: tumors with high or low c-myc expression. The three tumor groups defined by the 50-miRNA signature correlated well with the clinical stage and prognosis. Mestdagh *et al.* concluded that MYCN/c-MYC signaling rather than *MYCN*-amplification alone underlies the differential expression of miRNAs in neuroblastoma. To identify mRNA targets down-stream of the MYCN/c-MYC-regulated miRNAs, they integrated mRNA and miRNA expression data sets from 40 neuroblastoma tumors and calculated correlations between each of the 50 miRNAs and around 15 000 mRNAs. In the group of mRNAs with inverse miRNA correlation, significant 3'UTR seed enrichment was only found for the 16 N-myc-activated miRNAs, indicating that these miRNAs have a widespread effect on differential gene expression in high-risk neuroblastoma. One third of the mRNAs were predicted targets of two or more MYCN/c-MYC-activated miRNAs, indicating a concerted action towards target gene suppression. Low expression of predicted mRNA targets in the tumors correlated with a particular poor patient prognosis. MYCN/c-MYC-activated miRNAs were predicted to repress several pathways known to be involved in neuroblastoma, including integrin signaling. In summary, the study by Mestdagh et al. comprehensively demonstrated widespread transcriptional repression of coding genes by MYCN/c-MYC through miRNA induction, serving as an additional mechanism of MYCN/c-MYC induced oncogenicity.

2.3.9 MiRNA expression and neuroblastoma differentiation

Several studies have used retinoic acid (RA)-treatment of MNA neuroblastoma cells as a model system to investigate the role of miRNAs during *MYCN* knockdown followed by

neuronal differentiation [156, 180-184]. During RA exposure of MNA neuroblastoma cells, N-myc is immediately down-regulated prior to the onset of morphological differentiation ([185], and chapter 2.1.3). Several individual miRNAs whose expression is changed during RA-induced differentiation have been functionally characterized. *Mir-152* was found to target *DNMT1* (*DNA methyltransferase 1*)[180]. Diminished DNMT1 leads to decreased promoter methylation, allowing transcriptional activation of target genes like *NOS1* (*nitric oxide synthetase*), a pro-differentiation signaling molecule. Strikingly, *mir-152* was found to be repressed by N-myc in an N-myc-repressible *in vitro* cell system, consistent with the model of RA-induced N-myc repression and consecutively up-regulation of *mir-152* during the differentiation process. *Mir-10a/b* was up-regulated during RA-treatment of both MNA [181] and non-MNA [186] neuroblastoma cells. *Mir-10a/b* was shown to target *NCOR2* (*nuclear receptor corepressor 2*), and both exogenous overexpression of *mir-10a/b* as well as siRNA-mediated knockdown of *NCOR2* in MNA neuroblastoma cells resulted in phenotypical changes compatible to RA-treatment, including indirect reduction of N-myc levels [181]. *Mir-9* and *mir-125*, up-regulated upon RA-treatment, target *t-TrkC*, a truncated form of *TrkC* (*tropomyosin-related kinase C*). Truncated-TrkC in turn abrogates the function of full-length TrkC whose expression in primary tumors is correlated with good prognosis. Thus, in the context of differentiation, *mir-9* seems to have onco-suppressive and pro-differentiation properties. On the other hand, as highlighted before, *mir-9* is directly activated by N-myc with pronounced oncogenic properties in proliferating neuroblastoma cells [165]. Laneve et al. demonstrated that during RA-differentiation and consecutively decreasing N-myc activity, the *mir-9* promoter is activated through phosphorylation of CREB and dismissal of repressing REST [187]. These studies illustrate that the same miRNA may exhibit opposing effects during distinct cellular processes, as a consequence of diverse promoter activation and executed by a different set of miRNA targets.

2.3.10 MYCN is targeted by miRNAs

Finally, *MYCN* itself is targeted by miRNAs. The functionally best characterized *MYCN*-targeting miRNA is *mir-34a* [188, 189], which is located at chromosome 1p36, a region frequently deleted in MNA neuroblastoma tumors [11]. MiRNA-regulation of *MYCN* has been the topic of **paper II** and is further discussed in chapter 6.3.

3 Aims

As outlined, *MYCN*-amplification (MNA) has a strong impact on survival in neuroblastoma. However, it is still not completely understood how N-myc contributes to the aggressive phenotype. MiRNAs are regulators of gene expression and, when deregulated, involved in cancer development. Therefore, the thesis addressed the following general questions:

1. **How does the proto-oncogene *MYCN* influence the expression of miRNAs in neuroblastoma?**

   For this purpose, we used an approach where we transiently down-regulated N-myc expression in MNA neuroblastoma cells by anti-*MYCN* shRNA, and analyzed subsequent changes in miRNA expression by miRNA microarray studies. The results are published in **paper I.**

   The establishment of anti-*MYCN* shRNA is described in a method paper (**appendix**).

2. **Are miRNAs, in turn, regulators of *MYCN* expression in neuroblastoma?**

   For this purpose, the *MYCN* 3'UTR was systematically screened for miRNA binding sites. Validated *MYCN*-regulating miRNAs were functionally characterized. The results are published in **paper II.**

3. **What are targets and cellular functions of N-myc-regulated miRNAs?**

   We focused on *mir-92,* which is regulated by N-myc (**paper I**) and belongs to the oncogenic mir-17-92 cluster, and experimentally investigated its predicted role to target the tumor suppressor *DKK3.* The results are published in **paper III.**

4 Material and methods

4.1 Standard methods

The following methods are regarded as standard methods in molecular and cellular biology, and therefore not discussed in detail in this thesis, but thoroughly described in each paper:

Cell culture techniques; cloning of expression plasmids; sequencing; transfection of cells with Lipofectamin; retroviral transduction of cells; qualitative and quantitative real-time RT-PCR; Western blotting; ELISA test; Alamar blue cell proliferation assay; clonogenic assay; flowcytometric cell cycle analysis.

4.2 Cell lines

The following cell lines were used in **paper I**, **II** and **III**: MNA neuroblastoma cell lines: Kelly (N206); SK-N-BE(2); SMS-KCN; SMS-KCNR; SMS-KANR; IMR-32, LAN-5. Non-MNA NB cell lines: SK-N-AS; SH-SY-5Y; SK-N-SH.

SH-EP-Tet21N cells are non-MNA neuroblastoma cells stably expressing high *MYCN* levels under the control of a tetracyclin repressor, meaning that the addition of tetracyclin, or its more stable homologue doxycycline, to the culture media switches-off *MYCN* expression [190].

In paper **II** and **III**, the human embryonic kidney cell lines HEK293 was used in luciferase reporter assays.

4.3 Patient tumor samples

For **paper II** and **III**, we received genomic DNA from neuroblastoma tissue samples obtained during surgery (biopsy) from neuroblastoma patients treated at the University Hospitals in Gothenburg and Stockholm, Sweden. Patient samples did not include patients' identification, but information about the *MYCN* status in the sample. Use of the material was approved by the ethical committees of the institutions (Karoliniska Institute, Gothenborg University).

4.4 Design of anti-MYCN shRNA molecules

The design, cloning, validation and cellular effect of anti-*MYCN* shRNA is described in detail in the appendix paper Henriksen JR, Buechner J, Lokke C, Flaegstad T, Einvik C: **Inhibition**

of gene function in mammalian cells using short-hairpin RNA (shRNA). *Methods Mol Biol* 2011, **703:**189-204. In **paper I**, we used the anti-*MYCN* shRNA aMN-887. In **paper III**, the retrovirally transduced shRNA aMN-1658 was used [52].

4.5 Computational miRNA target prediction

MiRNAs are only partially complementary to respective target mRNAs. The identification of miRNA target sequences in the genome is therefore challenging. For this purpose, several bioinformatics algorithms have been developed and build the basis for public available miRNA target prediction software programs (reviewed in [191]). Common for all algorithms is the inclusion of several known miRNA target site features to recognize miRNA target sites in the genome. These features include: seed site characteristics (e.g. 6-8mers, pairing in the 3'part of the miRNA, see chapter 2.2); site location (e.g. within a 3'UTR structure); conservation of the miRNA and the corresponding target site across species; site accessibility (e.g. secondary structure of the 3'UTR); the existence of several target sites within the same UTR; and plausible correlation between miRNA- and mRNA expression profiles (all reviewed in [191]). None of the currently available prediction programs incorporate all these features in their algorithms. Therefore, to increase specificity of the prediction, it is reasonable to intersect results from one prediction with results from other prediction programs.

In **paper II**, we combined three miRNA target prediction programs, TargetScan 5.1 [146] (www.targetscan.org), MiRanda [192] (www.microrna.org) and PicTar [193] (http://pictar.mdc-berlin.de) to identify *MYCN*-targeting miRNAs. Predictions from TargetScan, which uses site and miRNA conservation across different species as selection criteria, were intersected with the predictions from the MiRanda and PicTar programs. In **paper III**, TargetScan was combined with MicroCosm [132] (formerly mirBase target, http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5) and Diana MicroT v.3.0 [194] (http://diana.cslab.ece.ntua.gr/microT/).

4.6 MiRNA microarrays

In **paper I**, we performed 2 independent miRNA expression studies on SK-N-BE(2) cells transfected either with a plasmid containing the anti-*MYCN* shRNA aMN-887, or a control shRNA. As these transfections were of transient nature, cells were harvested three days after transfection; a time-point when *MYCN* expression is knocked down and neuronal differentiation becomes morphologically apparent (**appendix paper**). Total RNA was

isolated with the miRVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. The miRNA microarray assay started with 10 ng total RNA.

We used a commercial miRNA microarray service provider (lcsciences.com) to perform the microarray analyses, as this method is not yet established at the University of Tromsø. The technical details of the assay, including miRNA enrichment, fluorescent dye labeling and hybridization conditions, are described elsewhere [195]. LCSciences uses γParaflo Microfluidic Biochips. Compared to spotted microarrays, microfluidic chips have the advantage that the miRNA detection probes are synthesized by photochemistry directly in the microchambers that will be flooded with the custom RNA sample (see www.lcscience.com). This miRNA synthesis step immediately prior to each array experiment always guarantees the latest Sanger miRNA set to be investigated, and allows adding of custom small RNA probes.

The data analysis of the microarrays was part of the provider service, as this is a complex and highly expertise-demanding process, which includes subtraction of the background and a normalization step. After normalization, the p-values of the difference between the two fluorescent signals were calculated. Differentially expressed miRNAs were those with a p-value <0.01 in at least 50% of the array replicates (more details in **paper I)**.

Microarray results were confirmed by miRNA-specific RT-PCR on the same RNA preparation as used on the microarray, as part of the provider service.

4.7 miRNA-specific real-time RT-PCR

The expression of miRNAs and small RNA control molecules (SNORD38B) in **paper I** and **III** were measured using the Qiagen miScript SYBRGreen PCR Kit and specific primer sets (Qiagen). Results were analyzed using the ΔΔCT method with qBase software [196].

4.8 Luciferase reporter assay (LRA)

This assay was used in **paper II** and **III** to investigate if a miRNA binds to the *MYCN* and *DKK3* 3'UTR structures, respectively. For this purpose, the full-length 3'UTR structures were cloned into a firefly luciferase expressing plasmid (pMIR-REPORT) down-stream of the luciferase gene. Binding of miRNAs to the 3'UTRs will cause down-regulation of the luciferase activity. MiRNAs, the pMIR-3'UTR plasmids, and a plasmid expressing a Renilla luciferase (for luciferase normalization) were transfected into HEK293 cells. 48 hours after transfection, Renilla and Firefly luciferase activities were analyzed using the Dual Luciferase

Assay (Promega). Each miRNA transfection was done in triplets and independently repeated at least three times, resulting in at least nine (but up to > 30) LRAs for each individual miRNA. Luciferase activities were analyzed in duplicates. Normalization included two steps: first, the Firefly luciferase activity was normalized to the Renilla luciferase activity, and second, the normalized luciferase activity of transfected NC (pre-mir-346 or Negative Control mimic) was set as relative luciferase activity of 1. The PASW Statistics 18 software was used for data analyses and boxplot charts.

4.9 Site-directed mutagenesis

Site-directed mutagenesis was used to specifically mutate individual miRNA seed sequence of selected miRNAs (**paper II** and **III**, as described in detail). Two-base mismatch mutations, introduced within position 2-6 of the seed, have been shown to disrupt miRNA-binding capacity [197]. LRAs with either wild-type or mutated seed sequences were performed and compared. In case of a specific miRNA-mRNA interaction, destruction of the seed will cause a rescue in luciferase activity and validate the binding of the miRNA. In non-rescued cases, we extended the mutagenesis to include a complete seed mismatch (complete position 2-7).

4.10 Immunofluorescence confocal laser microscopy

Morphological changes and *in situ* N-myc expression were evaluated by immunostaining and confocal laser microscopy. Confocal laser microscopy, as compared to wide-filed standard immunofluorescence microscopy, has the advantage to serially produce thin optical sections through a fluorescent-stained specimen through incremental changes in the microscope fine focus mechanism. The serially acquired images can be computationally merged to generate a stacked image with a nearly three-dimensional perspective. The specimen can be stained with multiple combinations of different primary antibodies and secondary fluorescent antibodies, which can be distinguished on the same specimen by the use of filters in the microscope excluding unwanted fluorescence wavelengths. As SK-N-BE(2) neuroblastoma cells develop long neurite outgrowths during neuronal differentiation (**paper I**), we had to establish a protocol which prevents the detachment of cells and neurites during the repeated steps of staining and washing, in addition to prior transfection. We cultured cells on round poly-L-lysine coated glass slides, transfected them in six well dishes, and fixated three days after transfection with 4% paraformaldehyde. After permeabilization with ice-cold MeOH and blocking with BSA, cells were incubated with primary antibodies, and covalent bindings visualized by fluorescent secondary antibodies. Cell nuclei were stained with Draq5. We used

a Zeiss LSM500 confocal microscope, the software LSM Image Browser (Zeiss) and an Adobe Illustrator for image processing and preparation.

4.11 xCELLigence cell proliferation system

The xCELLigence System (Roche, Mannheim, Germany) monitors proliferation of cells continuously in real-time, without the need to incorporate any labels. The system is based on the measurement of electrical impedance across interdigitated micro-electrodes integrated on the bottom of special tissue culture plates (E-Plates, Roche). The impedance measurement provides quantitative information about the biological status of the cells. During proliferation, i.e. growth and division of cells, micro-electrodes are increasingly inter-connected, leading to changes of electrical impedance. These impedance changes can be calculated as a "cell index" that correlates to cell proliferation. An advantage of this method is the continuous, real-time documentation of cellular proliferation, which can be displayed as growth curves and make comparison of proliferation, or other growth parameters like doubling time, easily visible. As a prerequisite for this assay, cell density has to be optimized to insure that the cells do not reach confluence during the observation period.

In **paper II**, we seeded Kelly cells in 160 µl media (15 000 cells per well) in 16-well E-plates and transfected them in triplicates 4–6 h later with 60 µl of a transfection mix containing 0.2 µl Lipofectamine2000 and 0.6 µl miRNA mimic (20 µM). Proliferation was recorded automatically as cell index every 30 minutes for a minimum of 72 h.

5 Results

5.1 Paper I: Brief summary

Buechner J, Henriksen JR, Haug BH, Tomte E, Flaegstad T, Einvik C: **Inhibition of *mir-21*, which is up-regulated during *MYCN* knockdown-mediated differentiation, does not prevent differentiation of neuroblastoma cells.** *Differentiation* 2011, **81:**25-34.

In this study, we investigated the role of N-myc on miRNA expression in *MYCN*-amplified neuroblastoma cells. We performed a miRNA profiling study on SK-N-BE (2) cells, and determined differentially expressed miRNAs upon *MYCN* knockdown using anti-*MYCN* short-hairpin RNA (shRNA) technology.

MYCN knockdown induced strong neuronal differentiation of the SK-N-BE(2) cells, as demonstrated by morphology and the expression of neuronal markers. Two independent miRNA microarray analyses revealed 23 miRNAs consistently differentially expressed during *MYCN* knockdown-mediated neuronal differentiation. The expression changes were bidirectional, with 11 and 12 miRNAs being up- and down-regulated, respectively. Among the down-regulated miRNAs, we found several members of the oncogenic mir-17-92 family, including *mir-92a. Mir-21*, an established oncomir in a variety of other cancer types, was strongly up-regulated upon *MYCN* knockdown and subsequent differentiation. This observation was therefore further investigated by functional analyses of *mir*-21 in the *MYCN*-amplified cell lines SK-N-BE(2) and Kelly. Neither overexpression of *mir-21* in the high-*MYCN* neuroblastoma cells, nor repression of increased *mir-21* levels during *MYCN* knockdown-mediated differentiation had any significant effects on cell differentiation or proliferation.

In conclusions, we describe a subset of miRNAs that were altered during the *MYCN* knockdown-mediated differentiation of *MNA*-amplified neuroblastoma cells. In this context, N-myc acts as both an activator and suppressor of miRNA expression. *Mir-21* was up-regulated during cell differentiation, but inhibition of *mir-21* did not prevent this process. We were unable to establish a role for *mir-21* during differentiation and proliferation of the two neuroblastoma cell lines used in this study.

5.2 Paper II: Brief summary

Buechner J, Tømte E, Haug BH, Henriksen JR, Løkke C, Flægstad T, Einvik C: **Tumour-suppressor microRNAs *let-7* and *mir-101* target the proto-oncogene *MYCN* and inhibit cell proliferation in *MYCN*-amplified neuroblastoma.** *Br J Cancer* 2011, **105:**296-303.

MicroRNAs (miRNAs) regulate expression of many cancer-related genes through posttranscriptional repression of their mRNAs. In this study, we investigate the proto-oncogene *MYCN* as a target for miRNAs.

By genomic sequencing of the 3'-untranslated region (3'UTR) of *MYCN* in 7 *MYCN*-amplified neuroblastoma cell lines as well as 39 primary tumors (both *MYCN*-amplified and *MYCN* single-copy), we found only one single nucleotide polymorphism (SNP) in the 3'UTR of *MYCN*, demonstrating that mutations in the miRNA binding sequence are rare.

A luciferase reporter assay was used to investigate software-predicted miRNA target sites in the *MYCN* 3'UTR. The miRNAs were overexpressed in the HEK293 cell line by transfection of miRNA mimics or miRNA-expressing plasmids. Mutation of the potential target sites by site-directed mutagenesis was used to validate the *MYCN* 3'UTR as a direct target of several miRNAs.

To measure miRNA-mediated suppression of endogenous N-myc protein, as well as effects of *MYCN*-targeting miRNAs on proliferation and clonogenic growth, miRNAs were overexpressed in the MNA neuroblastoma cell line Kelly.

The study showed that *MYCN* is targeted by several miRNAs. In addition to the previously documented *mir-34a/c*, we experimentally validated *mir-449*, *mir-19a/b*, *mir-29a/b/c*, *mir-101* and *let-7e/mir-202* as direct *MYCN*-targeting miRNAs. These miRNAs were able to suppress endogenous N-myc protein in *MYCN*-amplified neuroblastoma cells. Especially *let-7e* and *mir-202* were strong negative regulators of *MYCN* expression. The *mir-101* and the let-7 family miRNAs *let-7e* and *mir-202* inhibited proliferation and clonogenic growth when overexpressed in Kelly cells.

5.3 Paper III: Brief summary

Haug BH, Henriksen JR, Buechner J, Geerts D, Tomte E, Kogner P, Martinsson T, Flaegstad T, Sveinbjornsson B, Einvik C: ***MYCN*-regulated miRNA-92 Inhibits Secretion of the Tumor Suppressor DICKKOPF-3 (DKK3) in Neuroblastoma.** *Carcinogenesis* 2011, **32:**1005-1012.

Dickkopf-3 (DKK3) is a secreted protein of the Dickkopf family of Wnt regulators. It functions as a tumor suppressor in a range of cancers, including neuroblastoma. *MYCN* was recently found to down-regulate *DKK3* mRNA. In this study, we investigated if the *DKK3* repression is mediated by *MYCN*-regulated miRNAs.

First, we demonstrated that *MYCN* knockdown in MNA neuroblastoma cell lines increases secretion of endogenous DKK3 protein to the culture media.

We then used miRNA target prediction software to identify *MYCN*-regulated miRNAs that could potentially target the 3'UTR sequence of *DKK3*. Luciferase reporter assays and seed mutagenesis were used to validate miRNA-*DKK3*-3'UTR interactions.

Several *MYCN*-regulated miRNAs were predicted to target *DKK3,* including *mir-92a* and *mir-92b* (**paper I**), and *let-7e*. Luciferase expression from a reporter vector containing the *DKK3*-3`UTR was decreased when this construct was co-transfected with *mir-92a*, *mir-92b* or *let-7e* in HEK293 cells. Site-directed mutation of the *mir-92* seed sequence in the 3'UTR completely rescued the observed decrease in reporter expression when co-transfected with *mir-92a* and *mir-92b*.

Antagomir and miRNA-mimic transfections in neuroblastoma cell lines confirmed that DKK3 secretion to the culture media is regulated by mir-92s.

Consistent with reports from other cancers, we found *DKK3* to be expressed in the endothelium of primary neuroblastoma samples and to be absent in tumors with MNA.

In conclusion, the study demonstrated that *MYCN*-regulated miRNAs are able to modulate the expression of the tumor suppressor *DKK3* in neuroblastoma.

6 Discussion

6.1 Knockdown of MYCN

MYCN is amplified in a subgroup of neuroblastomas with highly aggressive behavior [18]. Our group has previously established an efficient model system to selectively down-regulate *MYCN* expression in MNA neuroblastoma by specific anti-*MYCN* shRNA molecules (**appendix paper**). This approach allows us to investigate and compare cellular processes in both high- and low-*MYCN* neuroblastoma cells. In particular, this model system can be used to study neuronal differentiation in MNA neuroblastoma initiated by specific *MYCN* knockdown, as opposed to induced neuronal differentiation using protocols with RA, TPA or various combinations of growth factors (e.g. BDNF, NGF, IGF1, FGF) (see chapter 2.1.3 and review [45]). In **paper I**, we used plasmid-encoded anti-*MYCN* shRNA to induce transient *MYCN*-knockdown (as described in the **appendix paper**). In **paper III**, anti-*MYCN* shRNA was expressed in neuroblastoma cells using a tetracycline-inducible retroviral delivery system. This expression system allows us to conditionally repress *MYCN* expression in MNA neuroblastoma cells (as described in [52]).

6.2 MYCN knockdown alters miRNA expression in MNA cells

As thoroughly introduced in chapter 2.3, several studies have addressed the role of N-myc on the expression of miRNAs in neuroblastoma over the past few years. These studies were mainly performed by overexpressing *MYCN* in non-MNA neuroblastoma cell lines with or without the capacity to undergo neuronal differentiation [156-158, 161, 162, 165, 167, 173, 176, 180], or by comparing miRNA profiles in MNA versus non-MNA neuroblastoma tumors [22, 156-158, 160, 161, 164, 165, 167, 170, 175, 176, 198-201].

As outlined in chapter 2.3.9, the contribution of miRNAs to the neuronal differentiation processes in neuroblastoma has mainly been investigated in SH-SY-5Y cells induced to differentiate by the addition of TPA or RA alone, or RA in combination with BDNF [156, 169, 180-184, 186, 200, 202, 203].

In **paper I**, we analyzed the expression of 723 known human miRNAs during the differentiation of MNA SK-N-BE (2) cells upon *MYCN* knockdown. By comparing miRNA expression levels in high and low N-myc SK-N-BE (2) cells, we found 23 differentially expressed miRNAs. Twelve miRNAs (*mir-17*, *-18a*, *-20, -24, -25, -92a, -92b, -93, -103, -*

106a, -494 and *mir-495*) were down-regulated, and 11 miRNAs (*mir-21, -22, -126, -137, -181d, -218, -663, -671, let-7c, let-7d* and *let-7f*) were up-regulated.

Knockdown of *MYCN* expression in MNA neuroblastoma cell lines by small RNA molecules (siRNA or shRNA) initiates a distinct neuron-like differentiation process characterized by morphological (neurite outgrowth) and biochemical (up-regulation of neuronal markers) changes [52]. Two studies using anti-*MYCN* siRNA or shRNA to investigate miRNA expression during *MYCN* knockdown-induced differentiation of MNA neuroblastoma cells have been published ([156] and **paper I**). These studies report complementary rather than identical results most likely due to different knockdown techniques and cellular systems. While Chen and Stallings used the siRNA approach to confirm a correlation between N-myc and miRNAs differentially expressed in MNA versus non-MNA primary tumors, we used shRNA to investigate an unbiased pool of 736 miRNAs (**paper I**). In accordance with our data, Chen and Stallings found *mir-137*, *mir-181* and let-7 family members among the up-regulated miRNAs.

Both up- and down-regulation of miRNAs was observed upon *MYCN* knockdown (**paper I**). Among the down-regulated miRNAs, most were members of the oncogenic miRNA clusters that constitute the mir-17-92 family. It is well established that N-myc is a transcriptional activator by direct binding to the promoter regions of several miRNAs, including the mir-17-92 family clusters (chapter 2.3.1 - 2.3.4). In addition, miRNAs of the mir-17-92 family clusters have been shown to be down-regulated in non-MNA neuroblastoma cells in which differentiation was induced by various agents and growth factors [169]. In **paper I**, we showed for the first time that most miRNAs belonging to the mir-17-92 family are down-regulated upon the *MYCN* knockdown-mediated neuronal differentiation of MNA neuroblastoma cells. In **paper III**, two of the miRNAs positively correlated to *MYCN* expression (*mir-92a* and *mir-92b*) were shown to directly target the tumor suppressor *DKK3* (see chapter 6.4).

We also observed several miRNAs being up-regulated upon *MYCN* knockdown in SK-N-BE(2) cells, and most have previously been linked to a neuronal phenotype, or been shown to induce neuronal differentiation. Interestingly, *let*-7, a miRNA family reported to be involved in differentiation of neuroblastoma cells [180-182, 202], directly targets *MYCN* mRNAs, suggesting a feedback mechanism resulting in low N-myc levels during differentiation of *MYCN*-amplified cells (**paper II**, see below). The *let-7* family of miRNAs is highly

represented in miRNA populations in mouse, rat and primate brains [204, 205]. Moreover, the expression of *let-7*, *mir-218* and *mir-137* has been reported to increase during induced neuronal differentiation in mouse embryonic stem cells, mouse and human embryonic carcinoma cells and mouse neuronal stem cells (mNSC) [206-208]. Additionally, the exogenous expression of *mir-137* promoted neuronal-like differentiation in several mouse and human stem cells [207]. Very recently, *mir-663* and *mir-22* were reported to be up-regulated during ATRA-mediated differentiation of leukemic HL-60 cells [209]. In summary, these observations support the idea that miRNAs up-regulated during *MYCN* knockdown-mediated neuroblastoma differentiation are either directly involved in, or are a consequence of, the (neuronal) differentiation process.

Notably, *mir-21*, a miRNA with established oncogenic functions in other cancers (reviewed in [210]), was found prominently up-regulated during *MYCN* knockdown (**paper I**) and neuroblastoma cell differentiation [180, 181, 202]. We found *mir-21* expressed in all neuroblastoma cell lines investigated in this study and, interestingly, *mir-21* expression was correlated to the *MYCN* mRNA expression in these cell lines. We cannot exclude that the expression of *mir-21* is also influenced by variations in *mir-21* gene dosages, as *mir-21* is encoded on chromosome 17q which is frequently involved in unbalanced translocations in neuroblastoma cell lines [211]. However, an inverse correlation between *MYCN* and *mir-21* expression was also reported upon *MYCN*-induction in Tet21N cells [162] and primary neuroblastoma tumors [175]. *Mir-21* is a miRNA with putative anti-apoptotic and tumor promoting activities, expressed in a variety of solid tumors. Experimentally validated *mir-21* targets include several proteins with tumor suppressor functions (reviewed in [210]). However, overexpression of *mir-21* in SK-N-BE (2) and Kelly cells did not alter proliferation of these cell lines (**paper I**). In addition, *mir-21* target genes remained unchanged (**paper I**). Similar to our study, Folini *et al.* recently reported that changes in *mir-21* expression did not alter proliferation of prostate cancer cell lines [212]. Over-expression of *mir-21* alone did not induce neuronal differentiation in SK-N-BE (2) or Kelly cells (**paper I**). Using antagomir-21 to reduce *mir-21* increase had no effect on differentiation (**paper I**). These observations indicate that the observed increase in *mir-21* expression does not directly influence the neuronal differentiation process in MNA neuroblastoma cells induced to differentiate by *MYCN* knockdown. We suggest that the increase of *mir-21* is a consequence rather than a cause for the differentiation process. We were not able to establish a clear function or a target for *mir-21* in neuroblastoma cells (**paper I)**.

In conclusion, **paper I** fits in a series of several published studies elucidating the effect of N-myc on miRNA expression in neuroblastoma. The **Supplementary tables** give a current overview over miRNAs reported to be correlated with N-myc expression – both in primary tumors (**Suppl. table 1**) and cell lines (**Suppl. table 2**).

6.3 The expression of N-myc is regulated by miRNAs

The interaction between N-myc and miRNAs is mutual, as *MYCN* itself is targeted by miRNAs. This is the main finding in **paper II.** Here, we aimed to investigate how miRNAs contribute to *MYCN* regulation. We systematically investigated the *MYCN*-3'UTR sequence for potential miRNA binding sites. We used luciferase reporter assays to show that the 3'UTR sequence is directly targeted by several miRNAs (*mir-34a*, *-34c*, *-449*, *-19a*, *-19b*, *-29a*, *-29b*, *-29c, -101*, *-202* and *let-7e*). These miRNAs were further shown to decrease N-myc protein expression when overexpressed in the MNA neuroblastoma cell line Kelly. Finally, we showed that ectopic overexpression of *let-7e*, *mir-101* and *mir-202* efficiently inhibit proliferation and clonogenic cell growth in Kelly cells.

It has been reported that certain mutations and SNPs in the 3'UTR of cancer-related genes increase cancer susceptibility and may allow the cancer cell to escape miRNA regulation [213]. In **paper II**, we showed that mutations in the *MYCN* 3'UTR are rare, both in MNA and non-MNA neuroblastoma cells, as we detected only a single SNP (rs922) at position 250 (C250T) of the 3'UTR, which did not impair targeting of the *MYCN*-regulating miRNAs.

The functionally best characterized *MYCN*-targeting miRNA is *mir-34a*, which is located at chromosome 1p36, a region frequently deleted in MNA neuroblastoma tumors [214]. Overexpression of *mir-34a* in MNA neuroblastoma cell lines decreased N-myc levels, inhibited proliferation and induced apoptosis. Interestingly, *mir-34a* is transcriptionally activated by p53 [155], implicating that deletion of *mir-34a* has similar cellular consequences as p53 deficiency [189]. In addition to *MYCN*, *mir-34a* targets *BCL2* [188] and *E2F3* [184], making *mir-34a* to a multi-faceted tumor suppressor miRNA in neuroblastoma. *Mir-34b* and *-34c* are co-expressed from a locus at chromosome 11q23, another region commonly hemizygously deleted in neuroblastoma [108].

In **paper II**, we confirm that *mir-34a* directly targets the 3'UTR sequence of *MYCN*. Whereas Wei *et al.* concluded that two *mir-34* target sites were required to obtain maximum *MYCN* suppression by *mir-34a*, we found only one to be responsible for most of the suppressive

effect (2008). This result is supported by data from a study performed by Welch *et al*. [184]. We also verified that *mir-34c* and *mir-449*, but not *mir-34b*, target the *MYCN* 3'UTR similar to *mir-34a*.

We confirmed, as earlier indicated by Lewis *et al.* [146], *mir-101* as a *MYCN*-regulating miRNA and demonstrated its ability to suppress *MYCN* expression by binding to two predicted target sites. We further showed that *mir-101* inhibits proliferation of MNA Kelly cells. These data from neuroblastoma extend previous reports showing tumor suppressor properties of *mir-101* in different other cancer types [195, 215-219].

Xu *et al.* have previously shown that *mir-29* directly regulates B7-H3, a surface glycoprotein of the B7/CD28 family that is expressed on a wide variety of solid tumor cells, including neuroblastoma [220]. B7-H3 has immunoinhibitory effects protecting neuroblastoma cells from NK-mediated cytotoxicity [221]. In addition, B7-H3 is the target of the monoclonal antibody 8H9 [220] that showed promising results when used in compartmental radioimmunotherapy (cRIT) in a clinical trial for CNS-relapsed high-risk neuroblastoma ([118], and chapter 2.1.6). Compared with normal tissue, *mir-29* was found significantly lower expressed in neuroblastoma cells, contributing to a higher expression of B7-H3 on neuroblastoma cell surfaces [220]. It has been suggested that restoration of *mir-29* and subsequent translational inhibition of B7-H3 might therefore prove therapeutically beneficial, both by sensitizing neuroblastoma cells to NK/T-cell-mediated immunotoxicity and by protecting B7-H3 expressing normal tissue from 8H9-related toxicity [220]. Our data from **paper II** extend the therapeutical potential of *mir-29* as it was shown to directly target *MYCN*.

The human let-7 miRNA family consists of 10 different mature *let-7* sequences that are derived from 13 precursors [222]. Overexpression of *let-7* has been shown to inhibit proliferation of several cancer cell lines [223-228]. Several important cell cycle regulators, including cyclins, cyclin-dependent kinases (CDKs), Ras, HMGA2 and c-Myc have previously been confirmed to be targets of *let-7* [224-228]. The results from **paper II** have now added the *MYCN* oncogene to the list of cell cycle regulators targeted by *let-7*. The observed growth-inhibitory effect of *let-7e* on MNA neuroblastoma cells is most likely because of the combined suppression of several *let-7* targets involved in cell proliferation.

In summary, we were able to define a subset of miRNA that are able to regulate *MYCN* expression when overexpressed in MNA neuroblastoma cells. To what extent the N-myc protein is regulated by endogenous levels of these miRNAs, and if altered levels contribute to

neuroblastoma development, needs to be addressed in further studies. Recent data from miRNA profiling studies show that *let-7e*, *mir-29a* and *mir-29c* are significantly lower expressed in MNA primary tumors compared with non-MNA tumors [22], supporting the idea that they act as endogenous *MYCN* regulators.

6.4 N-myc-regulated miRNAs target DKK3

DKK3 is an established tumor suppressor gene that inhibits the proliferation of several cancers, including neuroblastoma. Koppen *et al.* demonstrated that in neuroblastic tumors arising from the sympathetic adrenal lineage, increased *DKK3* mRNA levels are strong markers of differentiation: high *DKK3* mRNA expression in differentiated ganglioneuromas and low *DKK3* mRNA expression in undifferentiated neuroblastomas, correlating with poor prognosis [96]. It has also been shown that *DKK3* mRNA levels are inversely correlated to *MYCN* mRNA expression in neuroblastic tumors and neuroblastoma cell lines [96, 97]. In **paper III**, we used two MNA neuroblastoma cell lines and induced *MYCN*-knockdown by retrovirally delivered anti-*MYCN* shRNA. For the first time, we were able to show that secretion of endogenous DKK3 protein into the cell culture media was increased upon *MYCN*-knockdown. Similar to the data presented by Koppen *et al.* [96] and Bell *et al.* [97], we also confirmed that *DKK3* mRNA levels are inversely correlated to *MYCN* repression in neuroblastoma cell lines. Despite the well documented inverse correlation between *MYCN* and *DKK3* expression, *DKK3* does not seem to be transcriptionally down-regulated by N-myc promoter binding, as ChIP analysis failed to reveal a direct interaction between N-myc and the *DKK3* promoter [96]. This suggests an indirect regulatory mechanism.

To investigate if the *DKK3* gene was inactivated by hypermethylation of the promoter region as reported from other cancers (see chapter 2.1.4.4), we analyzed the methylation status of the *DKK3* promoter in 10 neuroblastoma primary tumors and five neuroblastoma cell lines using methylation-specific PCR (MSP). The results revealed that neither the primary tumors nor the cell lines were hypermethylated at the investigated CpG island of the *DKK3* promoter.

Therefore, we aimed to investigate if *MYCN*-regulated miRNAs contribute to *DKK3* regulation. In **paper I**, we demonstrated that *mir-92a* and *-92b* are positively correlated to N-myc expression. The correlation between *mir-92a* and *MYCN* expression has also been confirmed in neuroblastic tumors [96]. Both *mir-92a* and *mir-92b*, in addition to *let-7e*, were predicted by *in silico* analyses to target the *DKK3*-3'UTR sequence. **In paper III**, we were able to show that all three miRNAs efficiently decreased expression of a luciferase reporter

containing the 3'UTR sequence from *DKK3*. The predicted target seed sequence for *mir-92a* and *mir-92b* in the *DKK3*-3'UTR sequence was validated by mutagenesis. However, mutation of the putative *let-7e* seed sequences, separately or in combination, could not rescue the *let-7e* luciferase repression. This observation could be explained either by as yet unidentified and unpredicted *let-7e* seed sequences in the *DKK3* 3'UTR, other targets of *let-7e* indirectly influencing *DKK3* expression, and/or off-target effects. By the use of miRNA mimics and antagomir treatment, we further demonstrated that both *DKK3* mRNA expression and protein secretion into the media were inversely correlated to *mir-92a*, *mir-92b* and *let-7e* expression in neuroblastoma.

Mir-92a is a member of the oncogenic mir-17-92 cluster that has been shown to be aberrantly expressed and to promote tumorigenicity in neuroblastoma (see chapter 2.3.2). With the exception of *mir-92a*, and to some extent *mir-19a* and *-19b*, none of the other *mir-17-92* members reduced expression of the *DKK3* 3'UTR luciferase reporter. We also reported a very robust inverse correlation between *mir-92a* and *DKK3* expression in a series of 95 neuroblastic tumors.

DKK3 has been shown to be involved in tumor vessel biology and to be highly expressed in tumor endothelium [229]. DKK3 was reported to stimulate vascular growth and increase vascular density in tumors. We observed that the expression of the DKK3 protein in neuroblastoma vasculature was significantly higher in non-MNA tumors and benign ganglioneuroma in comparison to MNA tumors. This is in line with the previous report by Koppen *et al.* [96] demonstrating higher levels of *DKK3* mRNA expression in both non-MNA neuroblastoma tumors and ganglioneuromas. However, the observation that a pro-angiogenic factor is down-regulated in high-stage tumors compared to lower stages is surprising. The mir-17-92 cluster has been related to angiogenesis [230]. Recently, it has been demonstrated that the *in vivo* inhibition of *mir-92a* enhanced blood vessel formation [231]. Our novel findings provide one possible explanation for this observation since DKK3 has been shown to stimulate angiogenesis [229]. The link between increased *mir-92* expression in MNA neuroblastoma cells and reduced DKK3 expression in the endothelial cells in the tumor is unclear.

It has been reported that miRNAs not only functions as intracellular regulators, but also as secreted effectors with paracrine and endocrine effects [232]. We therefore speculate that the tumor endothelial cells could be affected by *mir-92* released from MNA tumor cells in

aggressive neuroblastomas. It should be investigated whether secreted *MYCN*-regulated miRNAs might be responsible for the low DKK3 expression in the endothelium of most aggressive tumors.

Very recently, De Brouwer *et al.* published a study similar to paper **III** in this thesis, confirming *mir-92a* and *mir-19* as a *DKK3*-regulating miRNA [233].

7 Conclusions

Neuroblastomas with *MYCN*-amplification are characterized by aggressive biology and poor survival of the patients. To improve future treatment options, it is of fundamental interest to understand *MYCN's* role in tumorigenesis and determine factors regulating *MYCN* expression.

In this thesis, we focused on the interactions between *MYCN* and miRNAs, a group of endogenous small regulatory RNA molecules that can act as both tumor suppressors and oncogenes.

In **paper I**, we knocked-down *MYCN* in MNA cell lines by shRNA (**appendix paper**) and performed a miRNA expression profiling study to elucidate miRNAs that are correlated to *MYCN* expression. This approach is different from other studies used to investigate the role of N-myc on miRNAs, as *MYCN* knockdown in addition induces significant neuronal differentiation of the cells. We observed both up- and down-regulation of miRNAs. MiRNAs with positive correlation to *MYCN* included members of the oncogenic mir-17-92 cluster. One of the most prominently up-regulated miRNAs upon MYCN knockdown was *mir-21*. However, we were not able to establish a functional role for this miRNA during differentiation.

Mir-92a and *mir-92b* were both positively correlated to *MYCN* expression. In **paper III**, we demonstrated that both miRNAs target the tumor suppressor *DKK3* in neuroblastoma and repress secretion of the DKK3 protein.

Finally, we demonstrated in **paper II** that the interaction between *MYCN* and miRNAs is mutual, as the *MYCN* mRNA itself is targeted by several miRNAs (**paper II**). Some of these miRNAs showed anti-proliferative properties. Re-establishment of these miRNAs in *MYCN*-amplified neuroblastoma may prove to be of therapeutic value.

8 References

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9 Supplementary tables

Supplementary table 1:

Differentially expressed miRNAs between MNA and non-MNA primary tumors

Supplementary table 2:

Correlation between miRNA and N-myc expression in neuroblastoma cell lines

Suppl. Table 1: Differentially expressed miRNAs between MNA and non-MNA primary tumors. U=up-regulated in MNA tumors. D=down-regulated in MNA tumors.

Studies: 1=Chen 2007; 2=Schulte 2008; 3=Afanasyeva 2008; 4=Fontana 2008; 5=Mestdagh 2009; 6=Evangelisti 2009; 7=Bray 2009; 8=Mestdagh 2009; 9=Loven 2010; 10=Ma 2010; 11=Foley 2010; 12=Schulte, NAR 2010; 13=Schulte, IJC 2010; 14=Lin 2010; 15=Swarbrick 2010; 16=Afanasyeva 2011

Study	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
MIRNA																
let-7a-2*												U				
let-7b		U														
let-7d																
mir-1												U				
mir-7-2*												U				
mir-9							U	U		U						
mir-9*							U									
mir-15a														D		
mir-15b								U								
mir-15b*												U				
mir-17-5p		U	U	U	U		U		U	U		U				
mir-18a		U			U			U	U	U						
mir-18a*					U		U	U				U				
mir-19a					U		U	U	U	U						
mir-19b					U		U			U						
mir-20a		U	U		U		U	U		U			U			
mir-20b							U	U								
mir-21														D		
mir-22													D			
mir-23b														D		
mir-25		U					U					U				
mir-26a								D								
mir-26b								D						D		
mir-27a												U				
mir-27b	D											U		D		
mir-28								D								
mir-29a							D									
mir-29c							D									
mir-30a-5p								D					D			
mir-30a-3p							D						D			
mir-30b	D							D					D	D		
mir-30c	D							D					D	D		
mir-30d								D								
mir-30e	D							D						D		
mir-30e-3p							D						D			
mir-34a*												U				
mir-34b												U				
mir-92	U	U			U		U	U		U			U			
mir-93		U														
mir-95								D				D	D	D		
mir-98														D		
mir-99a		U										U				
mir-99b													D			
mir-103-2*												U				
mir-103								D								
mir-105												U				
mir-105*												U				
mir-107	D															
mir-106a		U					U									
mir-126														D		
mir-128a						D		D					D	D		
mir-128b														D		

Suppl. Table 1: Differentially expressed miRNAs between MNA and non-MNA primary tumors. U=up-regulated in MNA tumors. D=down-regulated in MNA tumors.

Studies: 1=Chen 2007; 2=Schulte 2008; 3=Afanasyeva 2008; 4=Fontana 2008; 5=Mestdagh 2009; 6=Evangelisti 2009; 7=Bray 2009; 8=Mestdagh 2009; 9=Loven 2010; 10=Ma 2010; 11=Foley 2010; 12=Schulte, NAR 2010; 13=Schulte, IJC 2010; 14=Lin 2010; 15=Swarbrick 2010; 16=Afanasyeva 2011

Study	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
mir-129-5p												D				
mir-129	D							D						D		
mir-130								U								
mir-135a														D		
mir-135a*												D				
mir-135b																
mir-137	D							D				D		D		
mir-140								D					D	D		
mir-142														D		
mir-143																
mir-146a	D						D									
mir-148a								D					D	D		
mir-148b								D					D			
mir-149	D						D	D				D	D	D		
mir-150	D															
mir-152							D						D			
mir-153								D					D			
mir-181a	U	U			U			U				U	U			
mir-181a*												U				
mir-181a-2*												U				
mir-181b	U	U			U											
Mir-181d							U						U			
mir-184								D			D		D			
mir-186	D												D			
mir-189	D															
mir-190	D						D	D				D	D	D		
mir-192*												U				
mir-195														D		
mir-193b												U				
mir-194												U				
mir-195*												U				
mir-196a												D				
mir-197								D				D	D	D		
mir-199a		U														
mir-199a-5p												U				
mir-199a_AS		U														
mir-199b-5p												U				
mir-200a	D															
mir-214								U				U				
mir-215								D								
mir-221		U														
mir-224*												U				
mir-296-5p												D				
mir-302a	D															
mir-323	D						D									
mir-323-5p												D				
mir-324-5p	D						D	D				D	D	D		
mir-324-3p								D								
mir-325												U				
mir-326	D							D								
mir-328								D					D			
mir-329							D									
mir-330	D						D	D					D			

Suppl. Table 1: Differentially expressed miRNAs between MNA and non-MNA primary tumors. U=up-regulated in MNA tumors. D=down-regulated in MNA tumors.

Studies: 1=Chen 2007; 2=Schulte 2008; 3=Afanasyeva 2008; 4=Fontana 2008; 5=Mestdagh 2009; 6=Evangelisti 2009; 7=Bray 2009; 8=Mestdagh 2009; 9=Loven 2010; 10=Ma 2010; 11=Foley 2010; 12=Schulte, NAR 2010; 13=Schulte, IJC 2010; 14=Lin 2010; 15=Swarbrick 2010; 16=Afanasyeva 2011

Study	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
mir-331-3p												D				D
mir-331	D							D					D	D		
mir-335	D						D									
mir-339-3p												D				
mir-340								D				D		D		
mir-340*												D				
mir-342																
mir-380-5p															D	
mir-378												U				
mir-379							D									
mir-379*												U				
mir-383												U				
mir-409-5p							D									
mir-411*												D				
mir-431*												U				
mir-432												D				
mir-450a												D				
mir-455							D									
mir-485-5p												D	D			
mir-488							D	D					D			
mir-488*												D				
mir-491							D	D					D			
mir-494							D									
mir-496							D									
mir-500								D								
mir-501													D			
mir-504												D				
mir-526b*								U								
mir-539							D					D	D			
mir-542-5p							D					D	D			
mir-543-3p												D				
mir-550												U				
mir-550*												U				
mir-551b							U									
mir-565																
mir-566												U				
mir-572							U	U								
mir-575												U				
mir-576																
mir-601								U								
mir-610								U								
mir-615								D								
mir-616													D			
mir-627												U				
mir-628								D					D			
mir-628-3p												D				
mir-628-5p												D				
mir-641												U				
mir-645								U								
mir-654							D						D			
mir-654-5p												D				
mir-660																
mir-665												U				

Suppl. Table 1: Differentially expressed miRNAs between MNA and non-MNA primary tumors. U=up-regulated in MNA tumors. D=down-regulated in MNA tumors.

Studies: 1=Chen 2007; 2=Schulte 2008; 3=Afanasyeva 2008; 4=Fontana 2008; 5=Mestdagh 2009; 6=Evangelisti 2009; 7=Bray 2009; 8=Mestdagh 2009; 9=Loven 2010; 10=Ma 2010; 11=Foley 2010; 12=Schulte, NAR 2010; 13=Schulte, IJC 2010; 14=Lin 2010; 15=Swarbrick 2010; 16=Afanasyeva 2011

Study	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
mir-744												D				
mir-766												D				
mir-935												U				
mir-1179												U				
mir-1248												U				
mir-1249												D				
mir-1259												U				
mir-1287												U				
mir-1290												U				
mir-1308												U				
mir-2110												D				

Suppl. Table 2: Correlation between miRNA and N-myc expression in neuroblastoma cell lines. pos=positive miRNA-N-myc correlation; neg=negative (invers) miRNA-N-myc correlation. MYCN=experimental MYCN change

Studies: 1=Chen 2007 (Kelly, siRNA); 2=Schulte 2008 (SHEP-MYCN-ER); 3a=Fontana 2008 (SKNAS vs SHSY5Y, LAN5, IMR32); 3b=Fontana 2008 (SHEP-Tet21N); 4=Mestdagh, Gen Biol, 2009; 5=Bray 2009 (SHEP-Tet21N); 6a=Mestdagh, Oncogene, 2009 (SHEP-Tet21N); 6b=Mestdagh 2009 (SHEP-MYCN-ER); 6c=Mestdagh 2009 (ChIP on 6 cell lines); 7=Hu 2010 (LAN vs CHLA cell lines); 8=Loven 2010 (SHEP-Tet21N); 9=Ma 2010 (SHEP-MYCN-ER); 10=Foley 2010 (SHEP-Tet21N); 11=Das 2010 (SHEP-Tet21N); 12=Buechner 2010 (Kelly, shRNA)

Study	1	2	3a	3b	4	5	6a	6b	6c	7	8	9	10	11	12
MYCN	↓	↑	h vs l	↓	h vs l	↑	↑	↑	nc	h vs l	↑	↑	↓	↑↓	↓
MIRNA															
let-7a	neg														
let-7b	neg	pos													
let-7c		pos													neg
let-7d		pos				pos									neg
let-7f		pos													neg
mir-7											pos				
mir-9							neg	pos	*			pos*			
mir-15b							neg	pos	*						
mir-17-5p		pos	pos	pos*	pos*	pos					pos*				pos
mir-18a			pos	pos*	pos*	pos	pos	pos	*		pos*				pos
mir-18a*							pos	pos	*						
mir-18b											pos*				
mir-19a			pos	pos*	pos*	pos	pos	pos	*		pos*				
mir-19b						pos									
mir-20a			pos	pos*	pos*	pos	pos	pos	*		pos*				pos
mir-20b							pos	pos	no		pos*				
mir-21											neg				neg
mir-22						neg					neg				neg
mir-23a											neg				
mir-23b						neg					neg				
mir-24															pos
mir-25						pos									pos
mir-26a											neg				
mir-26b															
mir-27a											neg				
mir-28						neg									
mir-29a											neg				
mir-30b	neg														
mir-30d		pos													
mir-31		pos									neg				
mir-31*											neg				
mir-92a							pos	pos			pos*				pos
mir-92		pos	pos	pos*	pos*	pos			*						
mir-93		pos				pos					pos*				pos
mir-98						neg									
mir-99a		pos													
mir-99b						neg									
mir-100		pos									neg				
mir-103															pos
mir-106a		pos									pos*				pos
mir-106b											pos*				
mir-107						pos									
mir-125a-5p											neg				
mir-125b											neg				
mir-126															neg
mir-129	neg														

*=binding to miRNA promoter confirmed by ChIP; (*)=binding to host gene promoter (ChIP); h vs l=high-MYCN state vs low-MYCN state

Suppl. Table 2: Correlation between miRNA and N-myc expression in neuroblastoma cell lines. pos=positive miRNA-N-myc correlation; neg=negative (invers) miRNA-N-myc correlation. MYCN=experimental MYCN change

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Study	1	2	3a	3b	4	5	6a	6b	6c	7	8	9	10	11	12
MYCN	↓	↑	h vs l	↓	h vs l	↑	↑	↑	nc	h vs l	↑	↑	↓	↑↓	↓
mir-130							neg	pos	*						
mir-135b						neg									
mir-137	(neg)														neg
mir-140						neg									
mir-141	neg														
mir-142						neg									
mir-143						neg									
mir-145		(pos)													
mir-146b-5p											pos				
mir-150	neg														
mir-152														neg	
mir-181a							pos		*						
mir-181d															neg
mir-183											pos				
mir-184	neg												neg		
mir-186	(neg)														
mir-187	neg														
mir-189						neg									
mir-190											neg				
mir-193a-3p											neg				
mir-199a/b-3p											neg				
mir-199a-5p											neg				
mir-200c	neg														
mir-214							neg	neg	*						
mir-216	neg														
mir-218															neg
mir-221		pos													
mir-222											neg				
mir-302a	pos														
mir-320		pos													
mir-323	pos														
mir-326	neg														
mir-330	pos														
mir-335						neg									
mir-342						pos									
mir-374b											neg				
mir-378						pos									
mir-421										pos*					
mir-422a											pos				
mir-494															pos
mir-495															pos
mir-500						pos									
mir-501						pos									
mir-503											neg				
mir-504											neg				
mir-526b*															
mir-565						pos									

*=binding to miRNA promoter confirmed by ChIP; (*)=binding to host gene promoter (ChIP); h vs l=high-MYCN state vs low-MYCN state

Suppl. Table 2: Correlation between miRNA and N-myc expression in neuroblastoma cell lines. pos=positive miRNA-N-myc correlation; neg=negative (invers) miRNA-N-myc correlation. MYCN=experimental MYCN change

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Study	**1**	**2**	**3a**	**3b**	**4**	**5**	**6a**	**6b**	**6c**	**7**	**8**	**9**	**10**	**11**	**12**
MYCN	↓	↑	h vs l	↓	h vs l	↑	↑	↑	nc	h vs l	↑	↑	↓	↑↓	↓
mir-572							pos								
mir-576						pos									
mir-601								neg	(*)						
mir-610							neg	neg	(*)						
mir-615						neg									
mir-645															
mir-660						pos									
mir-663															neg
mir-671															neg
mir-768-3p											neg				
mir-801											pos				

*=binding to miRNA promoter confirmed by ChIP; (*)=binding to host gene promoter (ChIP); h vs l=high-MYCN state vs low-MYCN state

Paper I

Paper II

Paper III

Appendix

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