Searching for WT1 target genes
in leukemic cell lines

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Helena Jernmark Nilsson



DOCTORAL DISSERTATION

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| **Abstract**Wilms’ tumor gene 1 (*WT1*) encodes a protein involved in the pathogenesis of the childhood kidney cancer Wilms’ tumor. The gene and its protein are also overexpressed in a big proportion of acute myeloid leukemias (AMLs). Experimental, as well as clinical, data indicate that WT1 plays an oncogenic role in AML, suggesting that WT1 could be subject for targeted therapy. As a transcription factor WT1 is, however, a difficult target. Relevant target genes downstream of WT1 in AML could therefore be more promising candidates for drug development. In this thesis, novel target genes of WT1 are identified and characterized.In **Paper I** we find that the suppressor gene *IRF8*, a previously known target gene of WT1, is upregulated in AML cells treated with cytarabine in cooperation with the co-factor ZNF224, through repression of WT1 activity. In **Paper II**, we report the novel WT1 target gene, *NAB2*. WT1 and NAB2 proteins bind to each other and NAB2 modulates the function of WT1. **Paper III** describes *QPRT*, which is another target gene, the expression of which is highly correlated to that of WT1 in AML. We found that QPRT, which has a key enzymatic function in the *de novo* NAD+ synthesis pathway, may have anti-apoptotic properties in leukemic cells, protecting against pharmacological drugs. **Paper IV** describes FSCN1, a protein known to be involved in cellular movement and the spreading of cancer cells. Both WT1 and FSCN1 are highly expressed in AML and our results raise questions whether cellular mechanisms important for the metastatic behavior of cancer cells, also have a role in leukemias such as AML. |
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| Cover photo by Helena Jernmark NilssonThe cover photo shows a real-time PCR amplification plot from the analysis of *NAB2* and *WT1* levels in K562 cells after knock down with shRNA directed against *NAB2*. Copyright Helena Jernmark NilssonPaper 1 © Elsevier Ltd. Paper 2 © Helena Jernmark Nilsson Paper 3 © Elsevier Ltd. Paper 4 © by the authors (Manuscript unpublished) Faculty of MedicineDepartment of Hematology and Transfusion MedicineISBN 978-91-7619-832-2ISSN 1652-8220Lund University, Faculty of Medicine Doctoral Dissertation Series 2019:103Printed in Sweden by Media-Tryck, Lund UniversityLund 2019  |

*“If the string breaks, try another piece of string.”*

*– Winnie-the-Pooh by A. A. Milne*

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# Abstract

Wilms’ tumor gene 1 (*WT1*) encodes a protein involved in the pathogenesis of the childhood kidney cancer Wilms’ tumor. The gene and its protein are also overexpressed in a big proportion of acute myeloid leukemias (AMLs). Experimental, as well as clinical, data indicate that WT1 plays an oncogenic role in AML, suggesting that WT1 could be subject for targeted therapy. As a transcription factor WT1 is, however, a difficult target. Relevant target genes downstream of WT1 in AML could therefore be more promising candidates for drug development. In this thesis, novel target genes of WT1 are identified and characterized.

In **Paper I** we find that the suppressor gene *IRF8*, a previously known target gene of WT1, is upregulated in AML cells treated with cytarabine in cooperation with the co-factor ZNF224, through repression of WT1 activity. In **Paper II**, we report the novel WT1 target gene, *NAB2*. WT1 and NAB2 proteins bind to each other and NAB2 modulates the function of WT1. **Paper III** describes *QPRT*, which is another target gene, the expression of which is highly correlated to that of WT1 in AML. We found that QPRT, which has a key enzymatic function in the *de novo* NAD+ synthesis pathway, may have anti-apoptotic properties in leukemic cells, protecting against pharmacological drugs. **Paper IV** describes FSCN1, a protein known to be involved in cellular movement and the spreading of cancer cells. Both WT1 and FSCN1 are highly expressed in AML and our results raise questions whether cellular mechanisms important for the metastatic behavior of cancer cells, also have a role in leukemias such as AML.

# Introduction

## The disease - acute myeloid leukemia

John Hughes Bennet (1812-1875) wrote the first publication describing leukemia in 1845 and only six weeks later Rudolf Virchow (1821-1902) published another article on the subject. In 1847, Virchow coined the term *Weisses Blut* (white blood) and talked about a disorder characterized by pus in the vessels and blood cells with diverse nuclear appearance (Piller 2001). The disease was also later described, among many other blood diseases, by William Osler (1849-1919) in his landmark textbook *The Principles and Practice of Medicine* in 1892 (Piller 2001; Stone 2003).

Already in 1849 Virchow divided leukemia into two main types defined by the starting site of the disease: splenic and lymphatic forms (Piller 2001). In 1868, Ernst Neumann made the fundamental discovery that the bone marrow is the source of leukocyte formation. The medical student Paul Ehrlich (1854-1915) made between 1877 and 1881 investigations with cell staining, which enabled his discovery of all the white blood cells and the classification of leukemia into a myeloid and a lymphoid group (Piller 2001; Androutsos 2004). Wilhelm Ebstein (1836-1912) introduced in 1889 the term “acute leukemia” and proposed the division into acute and chronic forms. In 1900 Otto Naegeli (1871-1938) supported Ehrlich’s view that there exist different cell lineages in the blood and stated that the presence of myeloblasts and lymphoblasts in the circulating blood defines acute leukemia (Piller 2001).

Hematopoietic cancers, including leukemias, form a group of cancer types which originates from hematopoietic stem cells and precursor cells (De Kouchkovsky and Abdul-Hay 2016; Arber et al. 2016; Swerdlow et al. 2016). Today, the term leukemia is used for neoplasms that present with involvement of the bone marrow. Most often malignant cells are also present in the peripheral blood (Steffen et al. 2005; De Kouchkovsky and Abdul-Hay 2016). The latest definition by the World Health Organization (WHO) from 2016 defines acute myeloid leukemia (AML) as a condition where at least 20% of the blood cells in the bone marrow or peripheral blood consists of blasts of myeloid origin. The percentage could be lower, if recurrent cytogenetic translocations are present (Arber et al. 2016). Although the treatment of AML has improved during the last decades, the clinical outcome is often poor (Dohner, Weisdorf, and Bloomfield 2015). An increased knowledge on the molecular pathophysiology of AML is therefore needed. A large number of genetic aberrations have been identified as driver events in the development of AML (De Kouchkovsky and Abdul-Hay 2016; Dohner, Weisdorf, and Bloomfield 2015). One of these AML-associated anomalies concerns the Wilms’ tumor gene 1 (*WT1*), commonly overexpressed in AML, but also showing AML-associated recurrent mutations.

## The gene - Wilms’ tumor gene 1 and its encoded protein

Wilms’ tumor gene 1(*WT1*) was first identified in 1990 as an inherited predisposition allele with a key function in the pathogenesis of Wilms’ tumor, a childhood kidney cancer (Hastie 2017; Call et al. 1990; Gessler et al. 1990) affecting 1 out of 10,000 infants (Hastie 2017). Wilms’ tumor is a disease in which *WT1* is defined as a typical tumor suppressor gene, with both alleles lost for tumors to develop (Hastie 2017). However, the majority of Wilms’ tumors express wild-type *WT1*, sometimes to high levels. Most cases of Wilms’ tumor are sporadic and almost entirely caused by large deletions or intragenic mutations of *WT1* (Yang et al. 2007). Familial cases of Wilms’ tumor account for only about 2% of all cases (Ruteshouser and Huff 2004).

The molecular attributes of WT1 are by now well-characterized. *WT1* encodes a zinc finger protein composed of ten exons, generating a 3 kb mRNA. The protein consists of 449 amino acids with a molecular weight of 52-54 kDa, due to alternative splicing. WT1 is structurally resembling the proteins of the early growth response (EGR) gene family. The N-terminal of WT1, which is negatively charged due to glutamine and proline-rich residues, harbors two of each other independent transcription domains one with a repressive function within residues 84-179 and one with an activating function between residues 180 and 294. Moreover, the N-terminal holds two self-association domains, residing within residues 1-45 and 157-253 (Yang et al. 2007; Toska and Roberts 2014; Ullmark, Montano, and Gullberg 2018; Hastie 2017; Mrowka and Schedl 2000).

## WT1 isoforms

The WT1 C-terminal includes four zinc fingers. Zinc finger proteins constitute an abundant group of proteins, interacting with DNA, RNA, poly-ADP-ribose, and proteins (Krishna, Majumdar, and Grishin 2003; Cassandri et al. 2017; Klug 2010; Eom, Cheong, and Lee 2016). Zinc finger proteins are involved in a multitude of cellular processes, such as transcription, degradation, signaling, actin targeting, DNA repair, and cell migration. At present, there are 30 zinc finger types approved by the HUGO Gene Nomenclature. The zinc fingers are divided into eight fold-groups based on their structure. The four zinc fingers of the WT1 protein are of the most widespread Cys2-His2 (C2H2)-type. Here, a zinc ion (Zn2+) coordinates two cysteines from two β-sheets and two histidines from an α-helix. Together with an inner structural hydrophobic core, the amino acids make a small, but stable, protein domain. The resulting secondary, three-dimensionally folded, structure, called ββα fold, is fundamental for interactions with DNA, RNA, lipids, proteins, and small molecules (Eom, Cheong, and Lee 2016; Klug 2010) and allows the zinc fingers to bind their targets with very high affinity by interacting with specific bases at the major groove of DNA. As in the case of WT1, zinc fingers are often linked in tandem, increasing the binding specificity of the protein, as well as making it possible to recognize targets of varying lengths (Krishna, Majumdar, and Grishin 2003; Cassandri et al. 2017; Klug 2010; Eom, Cheong, and Lee 2016).

The zinc fingers of WT1 bind preferably to GC-rich DNA target sequences, more specifically to a consensus site 5’ – GCGGGGGCG – 3’ of the major groove and each zinc finger interacts specifically with the DNA by recognizing a 3 base pair triplet sequence (Ullmark, Montano, and Gullberg 2018). The first zinc finger assists WT1 in anchoring to the DNA, but does not specifically bind to it. Recent observations lean towards that zinc finger 1 being able to distinguish between minor and major DNA grooves and detecting electrostatic potential variations of the DNA (Hastie 2017).

In mammals, at least 36 isoforms of WT1 (Hastie 2017) may result from a combination of alternative translational start sites, alternative RNA splicing, and RNA editing, but the functional relevance of many of the isoforms is unclear. The four main isoforms of the WT1 protein are generated either by alternative splicing of exon five, resulting in inclusion or exclusion of 17 amino acids (17AA) in the N-terminal, or the other major alternative splicing event resulting in inclusion or exclusion of three amino acids (±KTS) between zinc fingers 3 and 4 (Loeb and Sukumar 2002; Haber et al. 1991; Haber et al. 1990). The presence or absence of the three amino acids lysine, threonine, and serine (KTS) is probably of major physiological importance, since the splicing is conserved from zebrafish to human (Davies, Bratt, and Hastie 2000), while the 17AA splicing event is only seen in mammals (Toska and Roberts 2014). The functional consequences of the KTS insertion has been thoroughly examined (Ullmark, Montano, and Gullberg 2018). In the WT1(-KTS) isoform, zinc fingers 2 through 4 determine the sequence-specificity of DNA binding, whereas the function of zinc finger 1 remains elusive. The insertion of the tripeptide KTS between the third and fourth zinc fingers increases the flexibility of the protein, making zinc finger 4 unable to bind to its target DNA. On the other hand, the same isoform rather interacts with RNA, an interaction in which also zinc finger 1 is involved. The WT1(-KTS) isoforms, have a more rigid carboxyl-terminal giving the zinc finger-DNA interaction more stability. These different features of the isoforms, leave them with slightly different cellular roles, differing in the selective domain for DNA binding.
WT1(-KTS) isoforms rather bind DNA and are transcription factor-like, while WT1(+KTS) isoforms have a role in RNA binding (Ullmark, Montano, and Gullberg 2018; Nurmemmedov et al. 2009).

The fact that *WT1* is able to produce several isoforms, all with the potential to dimerize, results in a plentitude of protein products working in solo or as homo- or heterodimers, all with different as well as overlapping functions, through many different steps in the transcriptional and translational sequences.

## Subcellular localization of WT1

The majority of the WT1 protein is found in the nucleus, but all isoforms shuttle between the nucleus and cytoplasm (Niksic et al. 2004; Larsson et al. 1995). The subcellular localization of WT1 depends on “classical” nuclear localization signals (NLSs) held by zinc fingers 1 through 3 (Bruening et al. 1996), which interact with nuclear import receptors importins β and α1, and to a lesser extent α4, in the subcellular shuttle mechanism (Depping et al. 2012). Despite the main nuclear localization, all isoforms are broadly distributed between mRNP complexes, ribosomal subunits, and actively translating polysomes (Niksic et al. 2004; Ladomery et al. 1999), although WT1(+KTS) associates rather directly with splicing factors, such as U2AF65 (Davies et al. 1998).

The subcellular distribution of WT1 is also cell type specific, and in some cells up to 50% of the WT1 amount can be found in the cytoplasm (Depping et al. 2012; Niksic et al. 2004). In malignant cells the distribution appears to be skewed and a larger part of the total protein amount is cytoplasmic (Depping et al. 2012). This subcellular distribution raises the question if the distribution itself could be causal to the pathology of cancer.

## WT1 in development and homeostasis

Mice experiments have shown that Wt1 regulates the development and homeostasis of several mesodermal tissues, such as bone, fat, cartilage, or muscle (Chau and Hastie 2012; Wilm and Munoz-Chapuli 2016; Hastie 2017). During embryogenesis, *WT1* is mainly expressed in the urogenital system, but also in the liver, thymus, brain, spinal cord, and abdominal wall musculature. The WT1 levels are age-dependent, and in adults only low levels are found in kidneys, ovaries, endometrium, testes, spleen, and normal hematopoietic progenitor cells (Depping et al. 2012; Niksic et al. 2004).

Transitions between epithelial and mesenchymal cell states are critical for organ development during embryogenesis (Thiery et al. 2009; Lamouille, Xu, and Derynck 2014). During nephrogenesis, expression of *WT1* is associated with differentiation of the proliferating mesenchyme into epithelial components of the nephron, called mesenchymal-epithelial transition, MET (Martinez-Estrada et al. 2010). A direct role of WT1 in MET has not yet been proven, but WT1 is able to induce fibroblasts to mature into Sertoli cells via MET. The development of the heart and diaphragm is dependent on the reverse process called epithelial-mesenchymal transition, EMT, in which WT1 plays a crucial role. WT1 activates the transcription of *Snail* and impairs *Cdh1* (E-cadherin) leading to cell shape changes, loss of cell-cell adhesion, and altered cell polarity (Hastie 2017).

In normal cells, a stable ratio between WT1(+KTS) and WT1(-KTS) isoforms of about 2:1 is present (Haber et al. 1991; Renshaw, King-Underwood, and Pritchard-Jones 1997). Even if mice displaying a discrepancy between the ±KTS variants survive until birth, they do not develop normally, showing the importance of proper isoform balance. A lack of Wt1(+KTS) leads to a reduction of *SRY* and a male-to-female sex reversal with defective kidneys. Individuals lacking the
Wt1(-KTS) splice variant have severe developmental defect of the kidneys and gonads. Both phenotypes die short after birth (Hammes et al. 2001).

In humans, germline mutations of *WT1* are associated with malformations of the urogenital system (Bruening et al. 1992) and predisposition to early Wilms’ tumor development (Szychot, Apps, and Pritchard-Jones 2014). The *WT1* gene was identified as a classical tumor suppressor gene in Wilms’ tumor in 1990 and mapped to chromosome 11p13 (Call et al. 1990; Haber et al. 1990). However, the gene defects in *WT1* account for only a small portion, 10-15%, of Wilms’ tumor cases, the remaining cases showing expression of wild-type WT1 protein (Hastie 2017).

*WT1* mutations in germ cells cause WAGR syndrome, Denys-Drash syndrome, and Frasier syndrome. WAGR (Wilms tumor-Aniridia-Genitourinary malformation-Retardation) syndrome was the first *WT1*-associated disorder described. The position of the *WT1* gene was identified in children with the WAGR syndrome, by large deletions on chromosome 11p13. Deletion of the *WT1* gene in this syndrome causes genital anomalies and the loss of several neighboring genes, including the *PAX6* ocular development gene, which results in aniridia and increased risk for Wilms’ tumor (Fischbach et al. 2005).

The Denys-Drash syndrome (DDS) manifests with quickly advancing kidney-failure, male pseudohermaphroditism, and Wilms’ tumor (Mueller 1994). The nephropathy manifests in early life, often before two years of age. The first symptom is often uni- or bilateral Wilms’ tumor. The patients are genetically male, but external genitals are neither clearly male nor female. Nearly all DDS patients have heterogeneous germline mutations, most often characterized by *WT1* missense mutations within exon 8 or 9, encoding zinc fingers 2 and 3, respectively. The most frequently observed mutation changes the arginine of zinc finger 3 to tryptophan (R394W) (Niaudet and Gubler 2006). WT1 DDS mutants seem to have a dominant negative effect, being unable to bind the wild-type WT1, which lessens the transcriptional capacity (Little et al. 1993).

The disease picture of Frasier syndrome (FS) is similar to that of DDS, and the two diseases can be hard to distinguish although FS patients do not develop Wilms’ tumor (Koziell and Grundy 1999). The FS patient manifests with gonadoblastoma, dysgenetic gonads, and abnormal sexual differentiation (Frasier, Bashore, and Mosier 1964). The classical definition includes only 46,XY patients with female or ambiguous external genitalia, slow progressive course of renal failure, and neoplasm of the germ cells (Dai et al. 2011; Barbaux et al. 1997). The phenotype is caused by point mutations in the donor splice site in intron nine of *WT1*, leading to nucleotide substitution in the *WT1* protein and subsequent loss of WT1(+KTS) isoforms (Barbaux et al. 1997; Klamt et al. 1998). Thus, the ±KTS isoform ratio is inverted. Since the FS does not predispose to Wilms’ tumor, it has been suggested that the WT1(-KTS) isoform, which is found in abundance in FS patients, may function as a tumor suppressor defending the individuals against development of Wilms’ tumor (Barbaux et al. 1997). Moreover, the sex reversal in these patients could be caused by WT1(+KTS) deficiency, since the WT1(+KTS) isoforms and GATA-4 activate *SRY* (Hastie 2017; Hammes et al. 2001), which initiates male development in humans (Koopman et al. 1991).

## WT1 in hematopoiesis

Hematopoiesis is a procedure during which a small pool of hematopoietic stem cells (HSCs) generate multi-lineage blood cells under precisely regulated forms (Orkin and Zon 2008; Ng and Alexander 2017). The blood production continues throughout life and in adults hematopoiesis mainly occurs in the bone marrow (BM), but can also take place in extramedullary organs, such as the liver, thymus, and spleen. The HSC, the common ancestor of all human hematopoietic cell types, has two defining features, namely multipotency and self-renewal (Seita and Weissman 2010). HSCs are multipotent, meaning that every single HSC is capable of generating any and all of the essential hematopoietic cell types. The HSC with its unique features, was recognized and described by James Till, Ernest McCulloch, and Andy Becker in 1963 in the journal *Nature* (Becker, McCulloch, and Till 1963; Weissman 2014). The first cell-surface marker used to enrich human HSCs was CD34, a ligand for L-selectin (DiGiusto et al. 1994).

In human adults, a small fraction, only 1.2%, of the bone marrow CD34+ cells express WT1, while CD34- cells and more mature lineage-specific cells do not normally express WT1 above the limit of detection (Hosen et al. 2002), indicating a role for WT1 in early hematopoiesis. In support of this notion, overexpression of WT1 in CD34+ human umbilical cord cells affected their differentiation and also gave an increased amount of quiescent primitive progenitors (Ellisen et al. 2001; Svedberg, Richter, and Gullberg 2001).

Mice have been studied to increase our knowledge about the role of Wt1 in normal hematopoiesis *in vivo*. In mice, at day 12.5 post conception, the liver is the main site of blood cell formation and Wt1 is by then expressed in these cells (Fraizer et al. 1995). The expression of WT1 during hematopoiesis was also examined in a knock-in reporter GFP mouse model, showing that WT1 expression was absent in the long-term HSCs and that less than 1% of the multipotent progenitor cells expressed it (Hosen et al. 2007). Knockout of *Wt1* in mice is difficult to study, since the genotype is lethal and the mice die *in utero* (Kreidberg et al. 1993). The embryos of *Wt1*-deficient mice lack coronary vasculature, and form no kidneys, gonads, adrenal glands, or spleen (Kreidberg et al. 1993; Hammes et al. 2001). To overcome this difficulty, lethally irradiated mice were transplanted with hematopoietic cells from *Wt1-/-* embryos. These mice were able to reconstitute hematopoiesis after transplantation, suggesting that Wt1 is not critical for hematopoiesis. However, when chimeric mice were created, the *Wt1* negative HSCs could not compete with the *Wt1* positive cells (Alberta et al. 2003), indicating that *Wt1* provides the HSCs with some advantage over the *Wt1* negative cells. Another early study performed by Inoue et al., investigated how forced expression of *Wt1* in murine myeloid 32D cl3 cells affected their ability to proliferate. In response to granulocyte colony-forming-stimulating factor (G-CSF), the transcription factor Stat3 was activated, leading to inhibition of myeloid differentiation and instead their proliferation was promoted (Inoue et al. 1998).

Performed *in vitro* and *in vivo* experiments thus indicate that WT1 expression during normal hematopoiesis is phase-dependent, with expression falling as the blood cells mature. Further, WT1 does not appear to be essential for blood formation, since Wt1 negative cells can recreate hematopoiesis, although being outcompeted by wild-type cells. WT1 most probably influences proliferation, viability, and differentiation of hematopoietic cells during immature developmental stages (King-Underwood et al. 2005).

## WT1 levels and AML

Acute myeloid leukemia (AML) form a heterogeneous group of malignant diseases, characterized by an abnormal proliferation and differentiation of a clonal population of myeloid stem or progenitor cells (Watts and Nimer 2018; De Kouchkovsky and Abdul-Hay 2016; Dohner, Weisdorf, and Bloomfield 2015). As for other cancer forms, AML is caused by genetic aberrations which are most commonly acquired. Chromosomal translocations, insertions, deletions, mutations, and epigenetic modifications all contribute to the pathogenesis of the disease causing a transcriptional control in disarray and a disordered signal transduction of growth factor receptors (Chen, Odenike, and Rowley 2010; Grimwade, Ivey, and Huntly 2016; De Kouchkovsky and Abdul-Hay 2016; Steffen et al. 2005; Gruszka, Valli, and Alcalay 2017). A transcription factor network implies all transcription factors of a cell, the regulatory elements of their targets, and all the multiple interactions they represent, joined in a molecular circuit (Blais and Dynlacht 2005; Wilkinson, Nakauchi, and Gottgens 2017). In AML, translocations and mutations commonly result in the expression of aberrant transcription factors and dysregulated transcription factor networks (Steffen et al. 2005; Shima and Kitabayashi 2011; Wilkinson, Nakauchi, and Gottgens 2017), leading to defect cell fate decisions (Klemm, Shipony, and Greenleaf 2019; Lambert et al. 2018; Gottgens 2015). Thus, dysregulation of the transcription factor networks is a common event in leukemia and transcription factors are included among overexpressed oncogenes.

When first identified in Wilms’ tumor, the transcription factor *WT1* was described as a prototypical tumor suppressor gene. However, the majority of Wilms’ tumors express wild-type WT1, sometimes to high levels, consistent with the function of an oncogene (Yang et al. 2007). Wild-type WT1 is also overexpressed in a variety of other human cancer forms, supporting an oncogenic function of WT1 (Yang et al. 2007; Hastie 2017).

When compared to healthy controls, most AML patients overexpress *WT1* in bone marrow and/or peripheral blood (Lyu et al. 2014; Inoue et al. 1994; Miyagi et al. 1993; Miwa, Beran, and Saunders 1992). High WT1 levels are associated with a poor prognosis (Bergmann et al. 1997; Garg et al. 2003; Karakas et al. 2002; Trka et al. 2002; Inoue et al. 1994). However, some studies with *de novo* AML patients do not show any prognostic value in the presence or absence of *WT1* overexpression (Gaiger et al. 1998; Schmid et al. 1997; Yanada et al. 2004). One study of AML in children even showed a positive correlation between high *WT1* levels and survival (Rodrigues et al. 2007). However, although conflicting data exist, most clinical studies support an oncogenic function for the gene.

WT1 levels are not only high in AML patients, but also in most leukemic cell lines (Miyagi et al. 1993; Miwa, Beran, and Saunders 1992).When WT1 levels were reduced by treatment with WT1 antisense oligomers, leukemic cells stopped growing and became apoptotic, consistent with an oncogenic function of WT1 (Algar et al. 1996). Others have inhibited WT1 expression with shRNA in the leukemic K562 cells, resulting in cell cycle arrest and leukemogenesis block (Li et al. 2014). Other early experiments with cell lines overexpressing WT1 resulted in differentiation arrest, and/or enhanced proliferation, further supporting an oncogenic role of WT1 in leukemogenesis (Inoue et al. 1998; Svedberg et al. 1998). Besides effects on differentiation and proliferation, forced expression of WT1 can also entail cellular resistance to cytostatic compounds (Svensson et al. 2007; Shen et al. 2007; Simpson et al. 2006; Montano et al. 2013; Ito et al. 2006). In the K562 cell line, Ito et al. showed that specifically cells positive for the 17AA insertion acquired an anti-apoptotic phenotype when treated with drugs (Ito et al. 2006). The mechanisms by which WT1 exerts chemoresistance are not clear, but may be caused by altered regulation of the tumor suppressor IRF8 and the zinc finger protein ZNF224 (Montano et al. 2013; Montano et al. 2016; Vidovic et al. 2010), repression of the pro-apoptotic gene *BAK* (Morrison, English, and Licht 2005), or induction of the growth factor receptor EGFR (Menke et al. 1997). On the other hand, in mice the 17AA insertion does not seem to give an advantage; animals lacking the 17AA insertion develop normally and the adult mice were fertile, which lessens the importance of the amino acids (Natoli 2002), in spite of their function as a transcriptional activator and its pro-survival properties in K562 cells.

Forced expression of *Wt1* in mice also supports an oncogenic function of Wt1. Mice overexpressing Wt1 in the bone marrow have enlarged amounts of immature hematopoietic cells, suggesting that Wt1 maintains self-renewal of the cells. Moreover, when a “second hit” was engineered with a virus carrying the leukemogenic *AML1-ETO* fusion gene, Wt1 cooperated with the fusion protein causing a rapid induction of AML (Nishida et al. 2006). Also, it was recently demonstrated that depletion of Wt1 can cooperate with the oncogenic Flt3-ITD mutation in induction of AML, demonstrating an oncogenic role also of reduced expression of Wt1. Remarkably, Wt1-haploinsufficient, but not completely Wt1-deficient, hematopoietic cells progressed to leukemic transformation, indicating the importance of the gene dosage of Wt1 (Pronier et al. 2018).

Finally, to describe *WT1* as either a tumor suppressor gene or an oncogene is obviously complicated. *WT1* behaves as a chameleon gene influencing differentiation on account of cell type, differentiation status, gene dosage, presence of other gene alterations, and microenvironment (Huff 2011).

## Transcriptional control

Transcriptional regulation, enabling gene expression, is the key process that makes cellular life possible. In eukaryotes, the process of DNA being copied into an RNA molecule, is dependent upon RNA polymerase II, a variety of general transcription factors (GTFs), and a large number of diverse complexes that act as co-activators, co-repressors, chromatin modifiers, and remodelers (Sainsbury, Bernecky, and Cramer 2015; He et al. 2013; Gupta et al. 2016). Transcriptional regulation occurs at two interconnected levels, one involving transcription factors and the transcription apparatus, and the other involving chromatin and its regulators (Lee and Young 2013). The transcription cycle is a discontinuous process, but can be divided into three phases: initiation, elongation, and termination, with the elongation phase including RNA polymerase II pausing (Engel, Neyer, and Cramer 2018; Mayer, Landry, and Churchman 2017). The core promoter is the minimal part of the DNA sequence necessary for transcription initiation, and spans about ±50 base pairs relative to the transcription start site (Goodrich and Tjian 2010; Duttke et al. 2015).

The RNA polymerase II makes a complex (the pre-initiation complex, PIC) with transcription factors, chromatin remodeling factors, and co-factors (Lambert et al. 2018). The pre-initiation complex binds to the DNA and initiates transcription by inducing DNA melting and strand separation, resulting in an “open” complex (Sainsbury, Bernecky, and Cramer 2015; Gupta et al. 2016).

The co-factors are either co-activating (e.g. Mediator complex, histone methyltransferases, histone acetyltransferases, and chromatin-modifying complexes) or co-repressing (e.g. histone demethylases, histone deacetylases, and polycomb complexes) (Lambert et al. 2018; Wilkinson, Nakauchi, and Gottgens 2017). The transcription factors are able to make a complex with co-activators, as well as with co-repressors simultaneously. Therefore, a transcription factor can function as an activator in one context, and as a repressor in another (Lambert et al. 2018).

Chromatin is a protein structure consisting of eight histones wrapped and held together by about 147 base pairs of DNA, together called the nucleosome (Kornberg 1974; Klemm, Shipony, and Greenleaf 2019; He et al. 2010). The chromatin appears either in an indolent form (heterochromatin) or in an active form (euchromatin), as originally based on its cytological properties (Passarge 1979). Within the facultative and constitutive heterochromatin, the nucleosomes are densely arranged, while the active chromatin is rather poorly endowed with the octamers. The regulatory capacity of chromatin is very dynamic, and its accessibility moves between closed, permissive, and open, owing to nucleosome remodelers (Klemm, Shipony, and Greenleaf 2019; Becker and Workman 2013). There is a vast amount of chromatin modification proteins; 443 different forms have been described, which help change chromatin state and make the DNA accessible, or inaccessible, for the transcription complex (Lambert et al. 2018).

## WT1 as a transcription factor

A transcription factor binds to a specific sequence of the DNA and regulates transcription (Lambert et al. 2018). WT1 is a typical zinc finger transcription factor with four DNA-binding C2H2 zinc fingers in the C-terminal, and target genes important for cellular growth and metabolism, including extracellular matrix components, growth factors, and other transcription factors have been identified. WT1 contains both activating and repressing domains and functions either as a repressor or an activator, depending on isoform, WT1 level, transcription start site, and cell type (Yang et al. 2007; Toska and Roberts 2014). As an example, *Wnt4* can be either activated or repressed during development of the kidney in a tissue-dependent manner, involving change of chromatin state (Essafi et al. 2011).

WT1 interacts with several protein partners, many of which are also transcription factors that regulate WT1 in feed-back mechanisms (Toska and Roberts 2014). The partners of WT1 can be divided into five categories depending on their function. The first two groups comprise proteins involved in transcriptional regulation, including DNA-binding transcription factors and transcriptional co-regulators, while the three other groups concern proteins with effects on post-translational regulation, proteolysis, and epigenetic regulation. Known protein partners of WT1 include BASP1, p53, Par-4, CBP, and WT1 itself (Toska and Roberts 2014).

BASP1 (Brain acid-soluble protein 1) regulates WT1 activity by binding to a suppression domain (residues 71-101), of the WT1 N-terminal. The proteins associate in the cell nucleus, and BASP1 functions as a co-repressor by inhibition of WT1 activity (Carpenter et al. 2004). The tumor suppressor p53 protein binds to the first and second zinc fingers of WT1 in the C-terminal. Interactions of WT1 with p53 modifies the pro-apoptotic properties of both proteins and the cells escape p53-mediated cell death (Maheswaran et al. 1993). Par-4 (prostate apoptosis response-4) represses WT1 transcriptional activity by interactions with the zinc fingers. In associations between Par-4 and the +17AA isoforms of WT1, Par-4 functions as a co-activator regulating cell growth (Richard et al. 2001). The transcriptional co-activator CREB-binding protein (CBP) interacts with WT1 in a direct mode, and WT1 binds by the first and second zinc fingers to CBP (Wang 2001).WT1 can also self-associate (Moffett et al. 1995) through two N-terminal domains (Holmes et al. 1997), and through the first zinc finger in the C-terminal (Wang et al. 2018). However, the purpose of homodimerization is still elusive.

## Candidate WT1 target genes in AML

WT1 is a multifunctional transcription factor that, depending on circumstances, activates or represses transcription of its target genes, including itself (Toska and Roberts 2014). Primarily the WT1(-KTS) isoforms work as transcription factors on known or proposed target genes involved in cellular processes such as development, tissue homeostasis, and disease (Toska and Roberts 2014; Hastie 2017). Toska and Roberts divide the target genes of WT1 into groups encoding proteins involved in eight different cellular mechanisms, defined as “growth and development”, “differentiation”, “Wnt signaling”, “MAPK signaling”, “apoptosis”, “epigenetic regulation”, and last group designed as “other functions” (Toska and Roberts 2014). Several target genes of these groups have been implicated in hematopoiesis, and may therefore potentially be involved in the development of WT1-driven leukemia. Some examples of such candidate target genes are described below.

### Growth and development

The gene *CXXC5* is transcriptionally controlled by WT1 during kidney development (Hartwig et al. 2010; Kim et al. 2010) and *encodes* retinoid-inducible nuclear factor (RINF) (Bruserud et al. 2015; Kuhnl et al. 2015), a zinc finger protein of the CXXC-type, important for normal myelopoiesis (Bruserud et al. 2015). CXXC5 affects both intra- and extracellular signaling and high CXXC5 levels inhibit the Wnt signaling pathway (Kuhnl et al. 2015). *CXXC5* has a possible role in leukemogenesis and is a candidate for a tumor suppressor gene, preventing leukemia progression through inhibiting the Wnt signaling pathway. The Wnt signaling pathway promotes leukemic transformation, proliferation and survival of leukemic cells *in vitro* (Kuhnl et al. 2015). In many AMLs, the gene *CXXC5* is lost, and epigenetic silencing of its promoter is frequently seen. Moreover, loss of the *CXXC5* locus leads to a changed phosphorylation grade of intracellular proteins, leading to dysfunctional cell growth and development (Kuhnl et al. 2015; Stoddart et al. 2016; Bruserud et al. 2015). Elevated levels of the gene transcript are associated with raised levels of other genes implicated in leukemogenesis (*WT1*, *GATA2, RUNX1, LYL1, DNMT3, SPI1,* and *MYB*) (Kuhnl et al. 2015; Bruserud et al. 2015).

Erythropoietin (Epo), encoded by the *EPO* gene, and the Epo-receptor, encoded by the *EPOR* gene, are regulated by Wt1 in mice in a tissue-specific manner (Dame et al. 2006; Kirschner et al. 2008). Epo signaling is required for normal erythropoiesis. Patients with the preleukemic disorder myelodysplastic syndrome (MDS) are often treated with Epo to increase erythropoiesis. However, Epo also has the ability to drive MDS into AML (Bunworasate et al. 2001) and increased Epo signaling in preleukemic cells could therefore increase the risk of AML development.

The membrane-bound receptor TrkB, a member of the neurotrophic tyrosine receptor kinase (NTRK) family, is encoded by the *NTRK2* gene which is a target gene of WT1. *NTRK2* is dependent on Wt1 for normal development of the heart in mice (Wagner et al. 2005). TrkB regulates cellular growth via the MAPK pathway. The MAPK pathway is deregulated in many AMLs due to constitutive phosphorylation (Milella et al. 2001). High WT1 levels could keep TrkB active leading to overactive cellular growth in AML cells.

The insulin growth factor receptor (*IGF1R*) is transcriptionally controlled by WT1 (Werner et al. 1994; Werner et al. 1995). The signaling pathway of the receptor is dysfunctional in many cancers. In AML, IGF-1 promotes cell growth and survival via PI3K/Akt signaling (Chapuis et al. 2010).

The *CCNE* gene encoding cyclin E is also a proposed WT1 target gene (Loeb et al. 2002). As a cell-cycle regulatory protein, cyclin E makes a complex with CDK2, which pushes the cell cycle from G1 to S-phase (Cheng and Tse 2018). Many AMLs encounter high levels of cyclin E (Iida et al. 1997). Thus, cyclin E is important for cell proliferation, but its overexpression may also lead to chromosome instability and tumorigenesis (Spruck, Won, and Reed 1999).

### Differentiation

The *SNAI2* gene was first identified as encoding a protein controlling cell migration in chicken embryos (Nieto et al. 1994). It has later been established that the *SNAI2* gene is a direct target gene of WT1 (Kim et al. 2007). The zinc finger protein SNAI2 is well known to promote EMT (Savagner, Yamada, and Thiery 1997), a process important for development and homeostasis. SNAI2 plays also an essential role in tumorigenesis and tumor invasion by acting in an anti-apoptotic manner (Vega et al. 2004; Fenouille et al. 2012) and inducing AML in mice (Li et al. 2017). Moreover, SLUG protects hematopoietic cells against radiation- and p53-triggered apoptosis (Inoue et al. 2002; Wu et al. 2005).

WT1 is a negative regulator of its target gene c-*MYB*, which encodes the oncogene and transcription factor c-MYB. The protein regulates hematopoiesis, and a reduction of c-MYB results in impaired hematopoietic cell proliferation (McCann et al. 1995). c-MYB controls genes important for lineage determination, cell proliferation, and cell differentiation (Uttarkar et al. 2016). *c-MYB* has a central role in development and progression of AML (Walf-Vorderwulbecke et al. 2018). A mutation in the WT1-binding site of the *c-MYB* promoter, makes WT1 binding impossible resulting in an overactive c-MYB and subsequent leukemogenesis progression (McCann et al. 1995).

*HOXA10*, a homeobox gene found on chromosome 7, encodes a transcription factor involved in gene expression, morphogenesis, and differentiation (Magnusson et al. 2007). *HOXA10* was recognized as a direct target gene of WT1 in gynecological tumors, as WT1 binds to the promoter and inhibits *HOXA10* expression (Andikyan and Taylor 2009). The protein Hox-A10, is found at highest levels in myeloid progenitor cells, more specifically Hox-A10 has an important role in hematopoietic lineage commitment (Magnusson et al. 2007). In AMLs, the gene is often overexpressed, leading to uncontrolled proliferation of progenitor cells (Shah et al. 2011).When the WT1 binding site has been mutated, WT1 was unable to bind to and repress *HOXA10* (Andikyan and Taylor 2009). Since AML cells have high WT1 levels, it is possible that the *HOXA10* promoter is mutated in AMLs.

### Wnt signaling

Wnt signaling is essential for embryonic development and adult homeostasis (MacDonald, Tamai, and He 2009). The pathway seems to have an important role in the development and homeostasis of blood and immune cells as well. Leukemic cells use the pathway to self-renew and proliferate, which is why active Wnt signaling is essential for the progression of AML (Staal et al. 2016). WT1 modulates Wnt signaling in development and tumorigenesis (Kim et al. 2009) and its deregulation could therefore be of importance in tumorigenesis.

The cell cycle regulating gene *CCND2*, is a direct target of WT1 (Kim et al. 2009). *CCND2* encodes cyclin D2 and mutations in the gene are recurrent in AML patients (Eisfeld et al. 2017). In *Wt1*-depleted mice, the Wnt signaling pathway is diminished and the target levels, including *CCND2*, decreased (von Gise et al. 2011). Cyclin D2 is a necessary subunit of active CDK4 and 6. The active complex stimulates cell cycle progression (Wang et al. 2007). Deregulated cyclin D2 can induce leukemogenesis by abnormal cell proliferation.

The β-catenin interacting Lymphoid enhancer factor 1 (*LEF1*) (Fu et al. 2014) is a target gene of WT1 (Kim et al. 2009). LEF1 has a crucial role in normal hematopoiesis and its absence leads to defective myelopoiesis (Skokowa et al. 2006). In some subtypes of AML, high LEF1 levels seem to be a favorable prognostic factor (Metzeler et al. 2012; Fu et al. 2014). A balanced level of LEF1 seems to be crucial for normal hematopoiesis (Petropoulos et al. 2008), proposing a role in disturbing the equilibrium for WT1, leading to AML.

The *JUN* gene, encoding the transcription factor JUN, is a direct target gene of WT1 (Kim et al. 2009). *JUN* is often overexpressed in AML and seems to be essential for AML cell survival and progression (Zhou et al. 2017). WT1 could possibly contribute to leukemogenesis in *JUN* expressing cells by maintaining high levels of this target gene.

WT1 modulates the transcription of the Nemo-like kinase (*NLK*) gene by direct association with its promoter (Kim et al. 2009). NLK has an important role in the initiation and progression of many human cancers, and seems to act as an oncogene in some situations and as a tumor suppressor gene in others (Huang et al. 2015), in line with the chameleon role of WT1 in cancer, including leukemia. Moreover, the NLK protein product regulates the Wnt/β-catenin pathway negatively by phosphorylating its aforementioned target protein LEF1, leading to transcriptional suppression of Wnt target genes (Zhang et al. 2015).

### MAPK signaling

The mitogen-activated protein kinase (MAPK) pathway is important for cellular growth, survival, and death in both physiological and pathological processes (Low and Zhang 2016). An extracellular signal activates the MAPK phosphorylation cascade, leading to changes in protein function and gene expression, rendering in a proper biological response. There are three main MAPK families in humans, i.e. ERKs, JNKs, and p38/SAPKs, concerning slightly different processes (Morrison 2012).

Several *DUSPs* (Dual-specificity phosphatases) are target genes of WT1, namely *DUSP5* (Kim et al. 2009), *DUSP6* (Morrison et al. 2008), and *DUSP16* (Kim et al. 2009).The DUSP proteins regulate MAPKs in a negative manner, by dephosphorylating threonine and/or tyrosine residues (Low and Zhang 2016). DUSP5 regulates ERK1/2 of the MAPK pathway (Keyse 2008) and the abnormal ratio between DUSP5 and DUSP5 pseudogene 1 (DUSP5P1) is associated with leukemia, by inhibition of apoptosis (Staege et al. 2014). DUSP6 recruits WT1 to its promoter (Kim et al. 2009) and inactivates ERK1/2 through a complicated negative-positive feedback loop (Morrison et al. 2008). DUSP16 reduces myeloid cell proliferation, by negatively regulating JNK kinases and low cellular DUSP16 levels are associated with AML (Zaidi et al. 2009). Moreover, a recurrent deletion of chromosome 12p, the location of the *DUSP16* gene, is often seen in leukemia (Hoornaert et al. 2003; Wiemels et al. 2008).

With very high WT1 levels in AML cells, the MAPK pathway may become overactivated resulting in abnormal cell proliferation, survival and differentiation.

### Apoptosis

The B-cell lymphoma 2 (BCL2) protein family is an important regulator of apoptosis, and includes both pro- and anti-apoptotic members. BCL2 proteins are often deregulated in cancer (Vogler 2012). The founding member BCL2, encoded by the *BCL2* gene, is a target gene of WT1 and upregulated in WT1 expressing cells, making them resistant to drug-induced apoptosis (Morrison, English, and Licht 2005). Removing *BCL2* from AML cells might eliminate the cancer (Konopleva and Letai 2018).

Other proposed WT1 targets are the *BAK* and *BCL2A1* genes (Morrison, English, and Licht 2005). The BCL2 Antagonist/Killer (BAK), a member of the BCL2 protein family, has pro-apoptotic functions (Reichenbach et al. 2017). BCL2A1 is another member of the BCL2 family, with pro-survival functions and is mainly expressed in the hematopoietic system. *BCL2A* is upregulated in leukemia and the protein also provides resistance to chemotherapy (Vogler 2012).

Moreover, WT1 influences apoptosis by regulation of its target gene *MYC*, by either repressing or activating it, depending on the cellular context (Li et al. 1999; Han et al. 2004). The proto-oncogene *MYC*, encoding a “master regulator” of cellular growth and metabolism, is often overexpressed in cancer cells, upregulating the production of the building blocks for rapid cellular proliferation. MYC induces gene expression leading to cellular proliferation, and is able to maintain the “transformed phenotype” of tumor cells. MYC is regulated by a vast amount of proteins and further controls many pathways by itself, therefore its dysregulation affects the pathological phenotype in numerous ways (Miller et al. 2012).

### Epigenetic regulation

The *SPRK1* target gene of WT1 was revealed during analyses of mutated WT1 in the context of angiogenesis. Mutated WT1 represses *SRPK1* expression, leading to reduced levels of the anti-angiogenic splice isoform of VEGF. The lack of VEGF isoform balance results in enhanced angiogenesis in WT1-mutated cells. Furthermore, wild-type WT1 is able to reverse the altered VEGF splicing and tumor progression (Amin et al. 2011). Inhibition of the splicing kinase gene *SRPK1* leads to altered isoform levels of several proteins involved in leukemogenesis. Moreover, downregulation of SRPK1 leads to cell cycle arrest, leukemic cell differentiation, and prolonged survival of mice with AML (Tzelepis et al. 2018).

Abnormal DNA methylation is a characteristic of many tumors, including hypermethylation of tumor suppressor genes and hypomethylation of proto-oncogenes (Kulis and Esteller 2010). DNMT3 is a methyltransferase important for embryonic development, imprinting, and X-chromosome inactivation, encoded by the *DNMT3* gene, a proposed target gene of WT1 which regulates *DNMT3A* expression by direct transactivation (Szemes et al. 2013). Somatic mutations in *DNMT3A* are frequent in *de novo* AMLs and associated with poor prognosis, indicating the importance of methylation for proper control of hematopoiesis (Ley et al. 2010). The most frequent missense mutation in *DNMT3A* affects amino acid R882, reducing DNA methylation dramatically *in vitro* (Spencer et al. 2017; Ley et al. 2010; Russler-Germain et al. 2014). Recently, the role of WT1 in methylation of DNA and its further modifications by hydroxymethylation has gained high attention.

## WT1, methylation and hydroxymethylation of DNA

DNA methylation, the addition of a methyl group to a cytosine base, with the purpose to change the properties of DNA activity, without changing its nucleotide sequence, is essential to genome stability, transcription, and development (Reik, Dean, and Walter 2001). Methylation occurs mainly on the 5’ carbon of cytosine (5mC) within a CpG dinucleotide and is characterized as a repressive mark (Lyko 2018). The CpG dinucleotides cluster in the CpG islands found near the transcription start site (Deaton and Bird 2011). Methylation of DNA is performed by a number of different members of the DNA methyltransferase (DNMT) family, that includes five members (Lyko 2018). In mammals three DNMTs are active, namely DNMT1, DNMT3A, and DNM3B (Law and Jacobsen 2010). Generally, the DNMT3 enzymes methylate the DNA *de novo* in the embryo, after demethylation (mark removing) performed by TET enzymes (Lyko 2018; Law and Jacobsen 2010), while DNMT1 enzymes maintain DNA methylation. Most likely, the division cannot be this well-defined, since DNMT3 enzymes have been shown also to contribute to maintenance and dynamic remodeling of DNA methylation (Lyko 2018).

Methylation of cytosines in gene regulatory elements, such as promoters, has for long been associated with transcriptional silencing (Lyko 2018; Law and Jacobsen 2010; Klose and Bird 2006; Deaton and Bird 2011). Methylation at enhancers is believed to confine transcription factor binding and regulate developmental genes (Lyko 2018), although new findings have opened for the possibility that DNMTs also have functions in transcriptional activation (Yin et al. 2017).

The methylation of cytosines can be further modified into hydroxymethylation (5hmC). Conversion of 5mC into 5hmC was previously regarded as a step towards demethylation, but the presence of 5hmC is increasingly recognized to actively mediate transcription (Richa and Sinha 2014). Oxidation of 5mC into 5hmC is catalyzed by the TET (Ten-Eleven Translocation) family of methylcytosine dioxygenases (Ito et al. 2011), maintaining hydroxymethylation in CpG islands of gene regulatory elements, thus also maintaining active transcription (Jeschke, Collignon, and Fuks 2016). TET2 enzymes are recruited to DNA by specific transcription factors, directing the catalytic capacity of TET2 as a regulatory element. Recently, much interest has been raised for the role of TET2 as a WT1-interacting partner in transcriptional control. WT1 binds directly to TET2 and recruits the dioxygenase to the transcription start site and CpG islands of WT1 target genes, to control the epigenetic status (Wang et al. 2015; Pan et al. 2015; Rampal and Figueroa 2016). Moreover, WT1 and TET2 cooperate to suppress leukemic cell proliferation and colony formation in AML (Wang et al. 2015). An important role of TET2 in hematopoiesis is indicated by recurrent loss-of-function mutations of TET2 in AML (Bullinger, Dohner, and Dohner 2017).

*Isocitrate dehydrogenase (IDH) 1* and *2* encode two proteins with key functions in cellular metabolism, epigenetic regulation, redox states, and DNA repair (Molenaar et al. 2018). *IDH1/2* are often mutated in cytogenetically normal (CN) AML (Montalban-Bravo and DiNardo 2018). Most mutations acquire novel gain-of-function, leading to simultaneous fall and rise in α-ketoglutarate (α-KG) and 2-hydroxyglutarate (2-HG) levels, respectively. 2-HG is a competitive inhibitor of histone demethylases and TET proteins (Xu et al. 2011). When 2-HG accumulates in AML due to IDH mutations, TET2 therefore becomes inhibited resulting in modifications of DNA and histone methylation, and AML with genetic mutations in *TET2* or in *IDH1/2* is characterized by a common DNA hypermethylation phenotype (Rampal and Figueroa 2016).

In consistence with functions in the same molecular pathway, *TET2* and *IDH* mutations are mutually exclusive in AML (Figueroa et al. 2010). Interestingly, it was recently found that also recurrent mutations of *WT1,* identified in AML, are most often not occurring together with either *TET2* or *IDH* mutations (Rampal and Figueroa 2016), indicating the importance of also WT1 in the regulation of these methylation and hydroxymethylation processes.

## Mutations of WT1 in AML

Acquired somatic *WT1* gene mutations, present at diagnosis in 10-15% of adult CN-AMLs, are independent predictors of poor clinical outcome (Owen, Fitzgibbon, and Paschka 2010; Krauth et al. 2014). Mutations cluster in exon 7 and 9, the former predominantly causing frame shift mutations, encoding proteins devoid of the potential to bind DNA. Exon 9 mutations rather result in recurrent amino acid substitutions in zinc finger 3, most commonly exchanging basic Arg394 or His397 for uncharged Trp and Tyr, respectively (Krauth et al. 2014). Arg394 and His397 are both predicted to participate in the DNA interactions (Stoll et al. 2007), suggesting reduced or lost DNA-binding ability due to the mutations. Thus, the heterozygous mutations of *WT1* found in AML are in most cases predicted to result in loss-of-function, indicating that haploinsufficiency of WT1 is an important mechanism for the way *WT1* mutations contribute to leukemia development. The functional interactions with TET2 proteins mentioned above, and their consequences for methylation and hydroxymethylation, provide a framework in which mutant WT1 may cause epigenetic changes supporting the development into leukemia and has driven the progress in finding IDH inhibitors for use in the clinic (Medeiros et al. 2017). Interestingly, the notion of reduced WT1 levels as a pro-leukemogenic factor is consistent with recent experimental data, which state that haploinsufficiency of WT1 cooperates with the *FLT3-ITD* mutation to induce AML in a mouse model (Pronier et al. 2018). However, it should be recalled that most cases of AML are characterized by overexpression of wild-type WT1, seemingly in contradiction to the notion of loss-of-function.

# The present investigation

## Aim of the thesis

As described above, WT1 is commonly overexpressed in AML. Clinical, as well as experimental, data support an oncogenic function of WT1. To understand how WT1 perturbs cellular physiology into a leukemic phenotype, a better knowledge of mechanisms downstream of the transcription factor WT1 is called for. Given that WT1, as a transcription factor, is a poor target for molecular therapy, downstream components may be possible novel therapeutic targets. Although some candidate target genes in this context have been proposed, our knowledge in this area is incomplete.

The aim of this thesis is therefore to search for novel target genes of WT1 in AML and to characterize their functional role, using experimental cellular models.

# Results and general discussion

In Paper I, we investigate mechanisms by which WT1 exerts transcriptional control of the *IRF8* gene. *IRF8* was previously characterized as a target gene of WT1 (Vidovic et al. 2010; Montano et al. 2013) and has been reported as a tumor suppressor in leukemic cells (Holtschke et al. 1996; Burchert et al. 2004; Tamura et al. 2003). WT1 represses transcription of *IRF8*, consistent with an oncogenic role with antagonistic effect on IRF8 (Vidovic et al. 2010).

ZNF224, a Krüppel-like zinc finger protein, has been identified as WT1-interacting partner, participating in the transcriptional control of a number of target genes (Florio et al. 2010; Montano et al. 2013). ZNF224 favors expression of
pro-apoptotic genes, thus potentiating the cytotoxic response to the chemotherapeutic agent cytarabine, a common drug in the treatment of AML (Montano et al. 2013). In Paper I, we investigate how WT1 and ZNF224 regulate transcription of *IRF8* and its relation to treatment with cytarabine. Our results showed that when we treated K562 cells with cytarabine, *ZNF224* mRNA levels were elevated, as well as *IRF8* levels. When we instead silenced the *ZNF224* with shRNA, *IRF8* levels also fell. Cells with suppressed ZNF224 prior to treatment with cytarabine, reacted in a similar way with reduced increase of IRF8 in response to cytarabine.

The *IRF8* promoter is suppressed by WT1 (Vidovic et al. 2010). To investigate the possibility that ZNF224, a co-factor of WT1 in the regulation of other genes, could relieve the suppression on transcription of *IRF8* by WT1, we performed luciferase experiments in HEK293 cells. HEK293 cells do express very low amounts of endogenous WT1 and by transfecting the cells with the *IRF8* promoter and WT1 we found that the promoter was suppressed. When transfection was performed with increasing *ZNF224* levels together with *WT1*, ZNF224 indeed relieved the WT1-mediated *IRF8* promoter suppression, consistent with the notion of ZNF224 as a co-regulator of WT1 on the *IRF8* promoter. The performed immunoprecipitation revealed an interaction between WT1 and ZNF224 in the nucleus, but not in the cytosol. A chromatin immunoprecipitation (ChIP) assay verified the interaction on the promoter of *IRF8*.

In conclusion, our results add more data to previous findings that ZNF224 and WT1 can be interacting partners in the transcriptional regulation of WT1 target genes. WT1-mediated suppression of the *IRF8* was previously shown (Vidovic et al. 2010). Here we show that cytarabine induces expression of *IRF8* and that ZNF224 contributes to that upregulation of *IRF8*, correlating to the response to cytarabine. Our data support previous notions that ZNF224 favors expression of pro-apoptotic genes in response to cytarabine (Montano et al. 2013). Together with a WT1-mediated repression of ZNF224 (Montano et al. 2015) our data emphasize the notion of WT1-ZNF224-IRF8 mechanisms in leukemic cells.

The main aim of this thesis was to identify new target genes of WT1 in leukemic cells. In Paper II, III, and IV, we report the novel target genes *NAB2*, *QPRT*, and *FSCN1*, respectively. *NAB2* was hypothesized to be a WT1 target given the fact it is transcriptionally controlled by EGR1, a transcription factor of the C2H2 zinc finger type with similarities to WT1, including 51% sequence similarity in the zinc finger domains (Rauscher et al. 1990). *QPRT* was recognized as a potential WT1 target gene because of the strong positive correlation between the expression of *WT1* and *QPRT* in AML samples. *FSCN1*, finally, was brought to our attention both by finding that its expression correlated to that of *WT1* in AML, and the recent reports of a role of WT1 in EMT and cellular migration (Artibani et al. 2017; Park et al. 2019; Kaverina et al. 2017; von Gise et al. 2011; Essafi et al. 2011), cellular phenomena in which FSCN1 may be involved (Li et al. 2018). Below follows a summary of our findings that led us to conclude that *NAB2*, *QPRT*, and *FSCN1* are target genes of WT1, followed by a discussion of the potential role of these genes in leukemic cells.

## Correlation between the expression of target genes and WT1 in leukemic samples

The cDNA microarray technology, has enabled analyses of transcriptomes and the quantification of a vast amount of gene transcripts from a cell or tissue samples (Govindarajan et al. 2012). We analyzed microarray data collected from the microarray database NCBI Gene Expression Omnibus (GEO) available to the public. We evaluated the correlation between *WT1* and putative target genes using Pearson’s correlation coefficient test, that evaluates the linear association of two variables. The microarray data came from 5,302 leukemia patients. Additionally, we analyzed for partial correlation between WT1 and putative target genes in 3,844 AML patient samples using the Ultranet tool. The resulting genes were ranked using Ultranet (Jarvstrat et al. 2013). In a total of 20,311 genes, *QPRT* was ranked as number 2, *NAB2* as number 5, and *FSCN1* as number 22. The strong correlation between expression of *WT1* and *QPRT, NAB2*, and *FSCN1* inthis large number of primary leukemic samples indicated them as putative target genes and called for more detailed analyses.

## Overexpression of *WT1* in cells

Experiments in this thesis have relied on leukemic cell lines and in some cases primary progenitor cells as experimental model to examine and illustrate the molecular mechanisms and phenotypic effects of our interest. Cell lines have many advantages as experimental models. They are inexpensive, easy to use and access, and offer an unlimited resource of material.

The K562 cell line is derived from a patient with chronic myeloid leukemia (CML) in blast crisis and therefore resembling AML. K562 cells express the *bcr-abl* hybrid gene that drives the expression of WT1 (Svensson et al. 2007). Importantly for our purpose, K562 cells express *WT1* at high levels and are also dependent on the protein for proliferation and survival (Svensson et al. 2007; Montano et al. 2015).

The myeloid cell line U937 does not express *WT1* and therefore functions as a system for analyzing target gene responses after forced *WT1* expression, which confers a resistance to differentiation induction, indicating an oncogenic action of WT1 in these cells (Svedberg et al. 1998). The cells are derived from a patient with histiocytic lymphoma with cell differentiation stopped at the monocytic level.

One adherent cell line has been used, the embryonic kidney cell line 293T/17 (HEK293T/17), a highly transfectable clone of the 293T cell line. The kidney cells express very low amounts of *WT1*, and are easy to work with. However, it is not a hematological cell line, thus in some aspects less relevant for our purposes.

We have enriched CD34+ cells from umbilical cord blood of healthy donors. Using the cell surface marker CD34, hematopoietic stem and progenitor cells can be identified. Only a small minority of the CD34+ cord blood cells express *WT1* at levels comparable to those in leukemic cells (Hosen et al. 2007), so CD34+ cells can be regarded as WT1-negative to use for forced *WT1* expression.

For forced expression, we have primarily used the WT1+/- (+17AA, -KTS) isoform, since it is considered the most efficient transcription factor, holding a transcriptional activation domain and without the KTS insertion, which aggravates DNA binding (Toska and Roberts 2014).

By transducing the virtually *WT1*-negative CD34+ and U937 cells with WT1+/-, *WT1* mRNA and the corresponding protein levels raised. Subsequently, we measured the mRNA and protein levels of *NAB2*, *QPRT*, and *FSCN1* and conclude that WT1+/- regulates *NAB2*, *QPRT*, and *FSCN1* expression under the circumstances investigated. Moreover, our finding that these effects of WT1 are dependent on its DNA-binding zinc fingers, since a truncated form of WT1 lacking the zinc fingers, WT1(delZ) did not show any effect, indicate that WT1 works as a transcription factor for the target genes. It should however be emphasized that the experiments do no demonstrate that WT1 directly transactivates the target genes, since indirect mechanisms may be present.

## Suppression of WT1 in cells

After screening five different shRNA constructs we chose those two showing most prominent suppression of WT1 protein expression. When WT1 levels were knocked down in K562 cells using these shRNA, the result was a significant decline of *QPRT* and *NAB2* mRNA levels, as well as protein levels. These results are consistent with those above, and supports the notion of WT1 as a transcriptional regulator of the target genes.

## Promoter analyses

The identification of the promoter is an important step in understanding transcriptional regulation of genes. The promoter is the genomic sequence proximal to the transcription start site. There is a vast amount of promotor and transcription factor binding site prediction programs to obtain (Liu and States 2002), and we have used the Matinspector tool from Genomatix (Quandt et al. 1995) to predict WT1 binding sites in the target genes.

NAB2 (NGFI-A-binding protein 2) is a co-repressor of the transcription factor
EGR1 which binds to very similar DNA-binding motifs as those WT1 binds to (Srinivasan et al. 2006). EGR1 binds directly to the *NAB2* promoter which is located from -679 to -74 base pairs, relative to the transcription start site (Kumbrink, Gerlinger, and Johnson 2005).

The *FSCN1* core promoter, binding the transcription factors cAMP response element-binding protein (CREB) and aryl hydrocarbon receptor (AHR) (Hashimoto, Loftis, and Adams 2009), is found 100 base pairs upstream from the transcription start site (Bros et al. 2003) and Lu and colleagues identified a Sp1 binding site at -70 to -60 base pairs (Lu et al. 2010). The gene harbors evolutionarily highly conserved motifs between human and primate (Hashimoto, Loftis, and Adams 2009).

In the case of the *QPRT* promoter, no previously published information was available. To predict the promoter area, we performed a bioinformatic analysis as described in Paper III. Briefly, we used publicly available Chip-Seq data to define regions in the *QPRT* gene enriched in transcription factor binding sites, and with histone modifications consistent with transcriptional regulatory elements. The work led us to define the -440 to +1760 base pair area as the putative functional promoter of *QPRT*.

The Matinspector tool (Quandt et al. 1995) made it further possible to predict putative WT1 binding sites within and in the proximity of each promoter area defined. This way, four, six and two potential WT1-binding sites were identified in the promoters of *QPRT*, *NAB2*, and *FSCN1*, respectively.

In the case of *NAB2* and *FSCN1*, promoter-luciferase experiments were performed to investigate the functional effect of WT1, showing a positive response on the *NAB2* promoter, while no (or negative) response was seen on the *FSCN1*-promoter. These data support the notion of *NAB2* as a direct target gene of WT1, while suggesting that the effects on the *FSCN1* gene are mediated by regulatory regions outside the investigated area, or by indirect effects.

The direct binding of WT1 to the *NAB2* and *QPRT* promoters, was demonstrated by chromatin immunoprecipitation-PCR experiments, in further support of WT1 as a direct regulator of these two target genes. Moreover, ChIP-analyses demonstrated that WT1 recruits NAB2 protein to the *IRF8* promoter, showing that NAB2 works as a co-factor of WT1 on this target gene.

Taken together, our analyses of the target gene promoters strongly support that WT1 has direct effect on *QPRT* and *NAB2*. The prediction of specific binding sites supports this notion although the binding sites were not exactly defined. In the case of *FSCN1*, we did not find support for direct effects of WT1 in the investigated area of the *FSCN1* gene.

## Does *QPRT* play a role in apoptosis in leukemia?

*QPRT* (Quinolinate phosphoribosyltransferase) had the highest correlation to *WT1* in our screen. *QPRT* encodes an enzyme with the same name, with important functions in the *de novo* nicotinamide adenine dinucleotide (NAD+) synthesis pathway (Liu et al. 2016). In blood cells, NAD+ synthesis descents predominantly from one of the two precursors: nicotinic acid or nicotine amid (NaM) by the Preiss-Handler pathway (Preiss and Handler 1958; Foster et al. 1985). NAD+ has functions in many human pathways, such as glycolysis, citric acid cycle, mitochondrial respiratory chain, and DNA-repair (Ying 2008; Schreiber et al. 2006; Krishnakumar and Kraus 2010; Imai and Guarente 2014; Chalkiadaki and Guarente 2015).

QPRT in leukemia is not an area previously studied. QPRT is active in normal platelets and erythrocytes (Foster et al. 1985), as well as in monocytes but not lymphocytes (Jones et al. 2015). An experimental drug, FK866, reduces NAD+ levels by inhibiting the NAD+ synthesis pathway through NAMPT blockage. FK866 was used on glioma tumors, but did not give the desired effects (Thakur et al. 2013). In leukemic cell lines (Thakur et al. 2013) and in leukemia patient samples (Gehrke et al. 2014; Cea et al. 2009) the result was the reverse, indicating that QPRT does not seem to be able to keep up the NAD+ production in leukemic cell lines treated with FK866.

Most cancers are highly proliferating tissues with a huge need of cellular energy and synthesis of biomass (Keibler et al. 2016) and therefore show an incessant need of NAD+ biosynthesis. Cancer cells need a lot of energy which they get through NAD+, that boost glycolysis (Yaku et al. 2018). Moreover, DNA-damage in cancer cells with genetic instability (Lengauer and Issa 1998) generates a high need of NAD+ dependent PARP1-mediated DNA repair. Further, oxidative stress induced by irradiation or alkylating agents during cancer therapy increases the need of sirtuin-mediated defense and thus the demand of NAD+ synthesis (German and Haigis 2015).

We found a correlation between high levels of *WT1* and *QPRT* in leukemic cells and as described above, our further analyses showed that WT1 positively regulates *QPRT* transcription, suggesting that QPRT may mediate oncogenic effects in leukemia. Interestingly, our data show an increased resistance to imatinib in K562 cells after forced expression of *QPRT*. Those results are in line with the anti-apoptotic properties already ascribed QPRT (Liu et al. 2016). Oxidative stress may stimulate resistance to imatinib (Synowiec et al. 2015) and NAD+ levels rise in cells exposed to oxidative stress (German and Haigis 2015). However, in our experiments, neither in control cells, nor in imatinib-treated cells, forced *QPRT* expression did affect NAD+ levels, indicating that the observed effect of QPRT on viability is independent of NAD+. By sequestering active caspase 3, QPRT has been shown to suppress cell death (Ito et al. 2006). It is therefore appealing to hypothesize that QPRT downregulates, alone or in collaboration with WT1, functional caspase 3.

## The functional role of *NAB2* on WT1

NGFI-A-binding protein 2 (NAB2), also known as melanoma-associated delayed early response (MADER) protein, is found in the nucleus. *NAB2* is a delayed early response gene and is stimulated by growth factors and cytokines (Svaren et al. 1996; Lucerna et al. 2003). *NAB1*, a related gene of *NAB2*, shares a high level of homology, but is not induced by the same stimuli. A gene homologous to the human *NAB1* and *NAB2* genes has been found in *C. elegans*, suggesting that NAB molecules are conserved throughout evolution (Svaren et al. 1996).

The *NAB2* gene caught our interest initially due to its co-regulation of Early growth response-1 (*EGR1*), an immediate early response gene, encoding a zinc finger transcription factor influencing cellular differentiation, proliferation, and cell death (Sevetson, Svaren, and Milbrandt 2000; Min et al. 2008). Moreover, EGR1 has a role in the regulation of hematological stem cell homeostasis (Min et al. 2008). *NAB2* is a target gene of EGR1 (Kumbrink, Gerlinger, and Johnson 2005) and NAB2 modulates transcription induced by EGR1 (Rauscher et al. 1990; Svaren et al. 1996; Lucerna et al. 2003; Russo, Sevetson, and Milbrandt 1995). The proteins interact directly with each other, establishing a negative feedback loop (Kumbrink, Gerlinger, and Johnson 2005; Kumbrink, Kirsch, and Johnson 2010; Sevetson, Svaren, and Milbrandt 2000). The binding between NAB2 and EGR1 results mainly in repressed gene expression (Svaren et al. 1996), even though the opposite has been demonstrated (Sevetson, Svaren, and Milbrandt 2000; Collins et al. 2006). NAB2 mediates repression via two conserved domains, NCD1 (Svaren et al. 1996) and NCD2 (Swirnoff et al. 1998), as well as via the Nucleosome Remodeling and Deacetylase (NuRD) complex (Srinivasan et al. 2006). NAB2 interacts with EGR1 through the chromodomain helicase DNA-binding protein 4 (CHD4)-interacting domain (CID), recruiting the NuRD complex (Srinivasan et al. 2006). The NuRD complex is a chromatin remodeling complex, involved in the control of gene expression (Basta and Rauchman 2015).

Given that *NAB2* is a target gene of EGR1 and that NAB2 binds to EGR1 and modulates its transcriptional activity, and our finding that *NAB2* is a target gene also of WT1, we wanted to elucidate if NAB2 functionally interacts also with WT1. From co-precipitation experiments, we could conclude that NAB2 and WT1 bind to each other in the nucleus, and our luciferase-reporter experiments indicated that NAB2 interfered with the transcriptional repression performed by WT1 on the *IRF8* promoter. Moreover, our ChIP-PCR analyses further supported the conclusion that WT1 recruits NAB2 to the *IRF8* promoter, and consistently, forced expression of *NAB2* in WT1 expressing K562 cells led to increased *IRF8* levels.

Thus, our data suggest that NAB2 may influence the leukemic phenotype by counteracting WT1-mediated suppression of the tumor suppressor *IRF8*. Moreover, forced *NAB2* expression diminished mRNA levels of *VDR* (vitamin D receptor), *CCND1* (cyclin D1), and *QPRT*, all target genes of WT1 (Maurer et al. 2001; Ullmark et al. 2017; Xu et al. 2013). VDR mediates the effects of vitamin D, influencing proliferation and differentiation of HSCs (Pezeshki et al. 2018). Cyclin D1 is important for cell cycle progression, and high levels are seen in cancer (Hydbring, Malumbres, and Sicinski 2016; Asghar et al. 2015) and some leukemias (Aref, Mossad, et al. 2006; Aref, Mabed, et al. 2006; Fernandes et al. 2018). Possible anti-apoptotic role of QPRT is discussed above.

Taken together, our data suggest that NAB2 has the potential to interfere with the function of WT1 in leukemic cells with increased expression of the tumor suppressor IRF8, and decreased expression of *VDR*, *CCND1*, and *QPRT*, encoding proteins with potentially pro-leukemogenic effects. Although robust evidence is lacking, our results therefore indicate that NAB2 may have anti-leukemia effects.

## Is WT1-driven expression of *FSCN1* a part of EMT-related mechanisms in leukemia?

Zinc finger proteins are involved in a vast amount of cellular processes (Cassandri et al. 2017). The zinc finger transcription factor WT1 plays important roles in development, homeostasis, and disease (Hastie 2017). The epithelial-mesenchymal transition (EMT) is an essential process throughout embryogenesis (Thiery et al. 2009) and e.g. during formation of the heart, WT1 modulates gene transcription (Hastie 2017), leading to cell shape changes, loss of cell-cell adhesion, and altered cell polarity (Lamouille, Xu, and Derynck 2014). When the kidneys are formed, WT1 is involved in the reverse process, mesenchymal-epithelial transition (MET) (Scharnhorst, van der Eb, and Jochemsen 2001). Thus several observations indicate that WT is involved in EMT, although, the specific function of WT1 in these processes is not fully clarified (Hastie 2017).

WT1 controls *SNAI2* (Kim et al. 2007), which encodes a protein controlling cell migration (Nieto et al. 1994) and induces AML in mice (Li et al. 2017). Cell migration is an essential process during embryogenesis, leading to development of different organs (Scarpa and Mayor 2016). Migration is also an important hallmark of cancer invasiveness and metastasis, reflected by abnormal expression of filopodia, finger-like protrusions built-up by tight parallel bundles of actin. In normal cells, filopodia have important functions in cell migration, neurite outgrowth, and wound healing (Kuhnl et al. 2015).

FSCN1, an actin-bundling protein, is a member of the fascin three-member family consisting of fascin-1, fascin-2, and fascin-3. The fascin molecules are 55 kDa globular proteins comprising four tandem FSCN domains (Mao, Duan, and Jiang 2016). FSCN1 was one of the first actin-bundling proteins to be biochemically characterized (DeRosier and Edds 1980). FSCN1 expression is transcriptionally controlled, but also post-transcriptionally as phosphorylation of a serine residue (S39) leads to decrease in actin-bundling *in vitro* (Vignjevic et al. 2006)*.*

A dramatic increase in *FSCN1* expression has been noted in some cancers, lymphocytic disorders, and hyperplasias. The amount of *FSCN1* rises in parity with cancer stage and high levels of *FSCN1* are associated with poor prognosis (Mao, Duan, and Jiang 2016). Among the filopodia-regulating proteins, FSCN1 has the strongest implications in cancer progression and metastasis to date (Arjonen, Kaukonen, and Ivaska 2011). Efficient bundling of actin filaments within filopodia is essential for filopodia formation (Vignjevic et al. 2003; Vignjevic et al. 2006; Cohan et al. 2001). Formation of filopodia are dependent upon *FSCN1* expression and several studies have indicated that FSCN1 is able to increase the migratory ability of cells (Yamashiro et al. 1998; Jawhari et al. 2003). Thus, by participating in filopodia formation, FSCN1 may promote cell migration and invasiveness.

FSCN1 is involved in cancer cell migration, invasion, and metastasis (Kuhnl et al. 2015). On the transcriptional level FSCN1 directly or indirectly activates the *NF-kB* promoter in breast cancer cells. The NF-kB pathway controls metastasis by regulating expression of urokinase-type plasminogen activator (uPA), that in turn activates matrix metalloproteases (MMP)-2 and MMP-9, leading to invasion (Al-Alwan et al. 2011). These functions are tightly associated with EMT (Mao, Duan, and Jiang 2016; Zhao et al. 2016).

*TWIST*, regulated by WT1, is an actor in the Wnt pathway (Corbin et al. 2009) and a strong inducer of EMT (Wang et al. 2016). In murine experiments, Twist facilitated leukemic cell migration (Yang et al. 2004) and seems to have a role in human leukemogenesis as well (Chen et al. 2015). In *Drosophila*, FSCN1 is expressed in motile hemocytes (blood cells of invertebrates) (Machesky and Li 2010).

With such strong associations between FSCN1 and disease progression and outcome in cancer, one can hypothesize that FSCN1 plays a role also in leukemia. In cancer, increased expression of FSCN1 is correlated to chemoresistance (Ghebeh et al. 2014; Barnawi et al. 2016; Li et al. 2018) and our data indicate that *FSCN1* is strongly expressed in AML. So far, however, our investigations do not support that FSCN1 provides chemoresistance also in leukemia, although treatment with FASCIN-G2, a pharmacological inhibitor of FSCN1, resulted in some resistance to imatinib and cytarabine. Therefore, we cannot for the moment draw firm conclusions regarding the pathogenic role, if any, of FSCN1 in leukemia.

# Future perspectives - target genes and their relevance for AML

In this thesis one previously identified, and three novel target genes of WT1 are presented. The target gene of a transcription factor is defined as a gene of which the expression is controlled by that transcription factor. The term target gene usually implies direct binding of the transcription factor to the gene, but indirect regulation may be included (Taverner, Smith, and Wardle 2004). Given the apparent role of WT1 in AML, if possible, intervention in WT1-pathways has therapeutic potential. As a transcription factor, however, WT1 is not an easy target for direct molecular therapeutic intervention. Potentially, identification of critical target genes of WT1 may open for new therapeutic interventions. Therefore, it is important to define the relevant target gene repertoire in leukemic cells.

Several target genes of WT1 have to date been isolated, and this thesis has expanded this knowledge, calling for a demonstration of which ones that are indeed relevant for AML. Genes emphasized for their involvement in leukemogenesis, are genes associated with treatment response and progress of the disease. To prove this involvement is, however, not trivial. Various factors, endogenous and exogenous, contribute to AML. Dividing genes into groups and defining them as causative or not of AML, is hard because of the huge network of genes cooperating and affecting each other in different ways, dependent on cellular and environmental circumstances. Below, some strategies to demonstrate AML-relevance are discussed.

## Functional phenotype in cell culture

Transformed and immortalized leukemic cell lines have been extensively used as experimental models for AML. The use of leukemic cell lines as cellular models for leukemia has many advantages. The cell lines are cost effective, easy to use, offer an unlimited supply of identical cell material, results can easily be repeated worldwide, their use lessens the need for animal experiments, and ethical concerns are bypassed. Cell lines as experimental models are, however, handicapped by some obvious weaknesses. In transformed and immortalized cell lines important cellular mechanisms may differ from primary leukemic cells. Also, the lack of interactions between the cells and the local environment is absent in *in vitro* experiments (Kaur and Dufour 2012; Drexler, Quentmeier, and MacLeod 2004). Some conditions should be fulfilled in a cell line used for investigation of the relevance of WT1 target genes. Firstly, expression of WT1 should be important for viability and proliferation of that cell line, or overexpression of WT1 should confer some quantifiable oncogenic effect. Secondly, the expression of the target gene should be regulated by WT1. One example of a cell line meeting this criterion is the K562 line. In this thesis, a few analyses to reveal the functional relevance of the target genes have been performed, but additional experiments could certainly be of value.

## Animal models for leukemia

Genes are often conserved through evolution (Siepel et al. 2005) and that is why we can draw conclusions from animal experiments and apply them on the human phenotype. Experiments in animals give a broader picture of gene function, since the gene is not isolated in a cellular model but can be studied in a physiological context (Barré-Sinoussi and Montagutelli 2015).

The most commonly used non-human animal models for the study of hematopoiesis and leukemia is the mouse, which has proven to be a good model organism, even if we cannot transfer the results straight off to man (Almosailleakh and Schwaller 2019; Parekh and Crooks 2013).

There are today a large number of animal models for the study of AML available (Skayneh et al. 2019). AML can be remodeled in genetically engineered mouse models, in which foreign genetic material is transferred into murine germ cells (Almosailleakh and Schwaller 2019). The technique of engineering genetically manipulated mouse models enables us to test hypotheses about tumorigenesis. It becomes possible to *in vivo* study oncogenesis due to loss, mutation, underexpression, or overexpression of a particular gene (Walrath et al. 2010).

### Conventional transgenic AML models

These “classical” models depend on the direct insertion of foreign DNA fragments into the pro-nucleus of fertilized oocytes. A foster-mice then carries the oocytes and the transgene is randomly integrated. There are several classical transgenic AML models investigating e.g. the fusion genes *PML-RARA* or *RUNX1-ETO* controlled by promoters such as *CD11b*, *hCG*, or *hMRP8* to analyze the leukocytic phenotype by targeting myeloid cells. Unfortunately, however, the classical models do not necessarily recapitulate the desired leukemic phenotypes very well (Almosailleakh and Schwaller 2019). In the case of WT1, transgenic mice overexpressing WT1 in hematopoietic cells were generated by the insertion of a 4.5 kb *WT1* transgene into oocytes under the control of the mouse *tec* promoter, active in hematopoietic progenitors. These mice, overexpressing WT1 alone, did show increased myelopoiesis, including splenomegaly indicating leukemogenic effect of WT1, but did not, however, develop signs of acute leukemia. To study the role of WT1 in AML further, progenitor cells from these WT1 transgenic mice were retrovirally transfected with the *AML1-ETO (RUNX1-ETO)* fusion gene, after which the cells were transplanted into lethally irradiated recipients (Nishida et al. 2006). *AML1-ETO* is a translocation frequently seen in AML which by itself does not generate acute leukemia in transgenic models (Nimer and Moore 2004). The combination of *WT1* and *AML1-ETO* did however indeed cause a rapid induction of AML development. Furthermore, a dose-response was observed since mice from strains expressing high levels of WT1 developed AML faster than mice from low-level strains (Nishida et al. 2006). This model, and additional yet to be developed mouse models, could potentially be used to investigate the relevance of target genes by investigating the consequences from suppression or deletion of the target gene under study.

### Transgenic AML models by homologous recombination in ES cells

Homologous recombination can be used to circumvent the random integration in classical transgenic models. The technique offers insertion of the gene of interest at a specific locus. As an example, the successful integration of the driver fusion gene *MLL-AF9* resulted in rapid development of AML (Almosailleakh and Schwaller 2019). More often, the technique of homologous recombination has been used with the purpose of functional deletion of particular genes, often called “knockout mice”. WT1 has been deleted in the germ line of conventional transgenic mice. Many of those WT1-mouse models have been generated for the purpose of studying the kidney (Ozdemir and Hohenstein 2014). A major problem with mouse WT1-knockout models when it comes to study hematopoiesis is that the mice die *in utero* around day 14 due to defect kidney function (Kreidberg et al. 1993). To get around this problem, conditional expression models have been developed.

### Conditional transgenic AML mouse models

Creating a conditional expression mouse model allows the scientist to decide when and where the gene of interest should be expressed. With the conditional models, it is possible to analyze the role of particular proteins in the development of leukemia when overexpressed or deleted at a desired time point, and also in a chosen tissue (Almosailleakh and Schwaller 2019).

Chau et al. generated a tamoxifen-inducible exon 1 *Wt1* knockout model, enabling them to study the role of Wt1 in the physiology of all tissues of adult mice. The model pictured the need of Wt1 for kidney homeostasis, but also for the integrity of pancreas, spleen, fat, and bone tissue. Interestingly, Wt1 was also critical for normal erythropoiesis since the knockout of Wt1 resulted in extensive cell death of erythroid progenitors (Chau et al. 2011).

Recently, Pronier et al. made a conditional knockout mouse model, specific for the study of hematopoietic cells. The authors made use of a conditional *Wt1*-knockout mouse model being either heterozygous (*Wt1fl/+*) or homozygous (*Wt1fl/fl*) for the *Wt1* allele. After transplantation of bone marrow cells from these mice to new recipients, leukemogenesis was studied. This way, the group analyzed the WT1 dose-dependency and could conclude that mice carrying one Wt1 allele showed signs of enhanced stem cell renewal, while *Wt1*-deficient individuals did not. Upon serial transplantation, animals being engrafted with *Wt1fl/+* cells either developed acute T cell leukemia or died of bone marrow failure, while *Wt1fl/fl* individuals were unaffected. The slow progression to leukemia indicated the need of additional genetic events. Consistently, a subset of aged primary Wt1 haploinsufficient mice developed myeloid malignancies, and further experiments showed that Wt1 depletion in combination with homozygous AML-associated *FLT3-ITD* mutations caused rapid progression to AML (Pronier et al. 2018). This finding, that the level of a gene can be crucial for disease development, adds another dimension to the problem. Knocking out a putative target gene with no apparent effect on the phenotype, one could misjudge the result and assume that a haploinsufficiency of the gene studied would neither affect the disease studied. The experiment states that gene levels are crucial for the disease picture.

### Transfer of patient-derived cells into mice

To observe human leukemic cells *ex vivo* is challenging, since the natural environment of leukemic cells cannot be replaced in a test tube in most cases leading to rapid cell death. In a small number of cases, however, it has been possible to establish immortalized cell lines, as discussed above. To be able to examine primary AML cells from patients, researchers have transplanted these cells into immunodeficient mice, such as nude (nu), severe combined immunodeficient (SCID), non-obese diabetic (NOD), NON-SCID, and NON-SCID-IL2rγnull (NSG) strains, making humanized mice models. These models make it possible to examine malignant hematopoiesis and the pathology of leukemia *in vivo* (Theocharides et al. 2016; Almosailleakh and Schwaller 2019). The humanized mouse models offer natural-like circumstances for the disease to develop and researchers to examine its characteristics, heterogeneity, and to test therapeutic responses. Not even this model is however able to reconstitute the original environment, but offers good context for studies of leukemia (Almosailleakh and Schwaller 2019).

Since 1976, when the first transgenic mouse was engineered, the murine models have developed tremendously (Ericsson, Crim, and Franklin 2013). Good mouse models are probably the key for clarifying the signaling pathway downstream of WT1 and applying a role to WT1 target genes in leukemogenesis. Studies of the role of WT1 target genes could be studied in the established WT1/AML1-ETO or WT1/FLT3-ITD models described above. The most obvious strategy would be to delete or suppress the target gene of interest in the mice and to study the impact on AML-development. Possible problems with embryonic lethality may be circumvented by conditional deletion. Additional mouse models could certainly also be developed.

# Populärvetenskaplig sammanfattning

Blodet består av tre huvudkomponenter: de röda blodkropparna (syretransport), de vita blodkropparna (immunförsvaret) och blodplättarna (blodet levrar sig). Men alla blodceller kommer från en och samma blodstamcell, som senare utvecklas till specialiserade blodceller som är bäst på just sitt område. Denna nybildning av mogna och specialiserade blodceller från omogna stamceller pågår ständigt livet ut. Leukemi, eller blodcancer, är en cancerform, som drabbar blodet. Leukemicellerna stannar då i sin mognadsprocess och kommer inte att kunna fungera normalt. Vilken typ av blodcancer som blir resultatet beror på i vilket utvecklingsstadium processen stannar upp. Blodcancer kan vidare delas in i en akut eller kronisk form. Blodcancer kan drabba alla åldrar och även om många botas är behandlingsresultaten i många fall tyvärr dåliga. Vår forskning handlar om en av de vanligaste akuta formerna av blodcancer hos vuxna, akut myeloisk leukemi (AML). Den heter så därför att en grupp vita blodkroppar, de myeloiska cellerna, drabbas. AML-cellerna stannar i ett tidigt skede av mognadsprocessen mot vita blodkroppar och är därför väldigt omogna och kallas blaster. Det blir brist på normala vita blodkroppar och blasterna fungerar inte som utvecklade celler och därför drabbas den sjuka ofta av infektioner, då immunförsvaret försämras.

Kroppens ”byggstenar”, cellerna, innehåller all information om vår utveckling i form av arvsmassan (DNA). DNA består av tusentals gener och fungerar som en slags instruktion för var, när och hur processer skall sättas igång eller stoppas, vilket ger olika slags specialiserade celler, som kan bygga upp en fungerande människa. Ibland kan det dock uppstå felaktigheter i arvsmassan. Koden för en särskild gen kan vara fel, vilket gör att det protein som genen kodar för inte kopieras alls eller kopieras, men fungerar felaktigt. En gen kan också kopieras allt för många gånger och finns då i överflöd.

I AML har man upptäckt att det ofta finns ett överskott av proteinet WT1 i de sjuka blodcellerna, men även att det i vissa fall är förändrat. I min avhandling har jag undersökt hur WT1 fungerar i blodcancercellerna. WT1 är ett protein, som reglerar många andra gener i cellerna vilket kan vara viktigt vid uppkomst av blodcancer. Jag har därför särskilt fokuserat på vilka gener WT1 påverkar och på vilket sätt. Vi har därmed letat efter så kallade målgener för WT1 med någon koppling till blodcancer. Vi hittade tre gener, *NAB2*, *QPRT* och *FSCN1*, som på olika sätt samverkar med WT1 i blodcancer. Genom att öka mängden av målgenerna, har vi funnit visst stöd för att de kan göra cancercellerna mer aggressiva och motståndskraftiga mot läkemedel (cytostatika). Vi har också funnit att de kan förändra egenskaperna hos WT1. Vi har även undersökt ett annat protein (ZNF224), som ökar i blodet vid cytostatika-behandling. ZNF224 binder då till WT1 och aktiverar på så sätt den cancerhämmande genen *IRF8*. Cancerpatienter behandlas oftast med någon slags cytostatika, vilket kan vara en effektiv behandling. Tyvärr är eller blir vissa cancerformer motståndskraftiga mot cytostatika, vilket gör det svårt att hitta ett läkemedel som fungerar.

Genom att öka vår förståelse av hur WT1 fungerar i AML i samarbete med andra gener och under olika förhållanden hoppas vi på att i förlängningen kunna bidra till utvecklingen av nya och effektivare läkemedel.

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