SET and SET-CAN in human leukemia

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Chapter 4

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Submitted

Chapter 5

Scope of this thesis

Cancer is driven by the sequential acquisition of genetic alterations involving constitutive activation of protooncogenes, and inactivation of tumor suppressor genes. These changes release cells from their normal growth constraints and give rise to clonal expansion of mutated cells that eventually invade tissues normally consisting of other cell types. Based on the tissue and cell type from which they arise, cancers are broadly classified into two main groups involving solid tumors, which are composed of epithelial or mesenchymal cells that normally are immobile, and hematopoietic cell-originated leukemias or lymphomas whose precursors are normally mobile.

Recurrent, balanced chromosome translocations, a characteristic of many leukemias as well as solid tumors, often result in the generation of chimeric genes that encode novel fusion proteins contributing to the malignant phenotype. Understanding of the normal function of the proteins encoded by the fusion partners provides insights into the mechanisms with which these chimeric proteins transform normal cells into tumor cells. The translocation t(6;9)(p23;q34), mostly associated with the M2 or M4 subtypes of acute myeloid leukemia (AML), fuses the *DEK* and *CAN* genes on chromosome 6 and 9, respectively, and results in the expression of the DEK-CAN fusion protein. Another chromosome rearrangement found in a patient with acute undifferentiated leukemia (AUL), fuses *CAN* to another partner, *SET,* which is located on the same chromosome as *CAN.* The studies in this thesis are aimed to understand the function of SET in hematopoietic cells and to analyze how these functions are altered by SET-CAN fusion protein.

First, the effects of SET and SET-CAN expression on the growth, survival and differentiation of a human promonocytic cell line were determined (Chapter 2). Second, through identification of the genes that are affected by SET overexpression, the mechanism underlying the SET induced phenotype is analyzed further (Chapter 3). Finally the role of SET-CAN on hematopoiesis and leukemogenic transformation was examined by creating SET-CAN transgenic mice (Chapter 4)

Chapter 1

INTRODUCTION

Hematopoiesis

Stem cells, which can give rise to multiple cell types while maintaining their self renewal capacity, may be classified into two groups consisting of pluripotent and multipotent stem cells. Pluripotent stem cells (e.g. embryonic stem and embryonic germ cells) can differentiate into all three germ layers including endoderm, ectoderm and mesoderm whereas multipotent stem cells, which may be isolated from different types of fetal and adult tissues, are lineage specific and include hematopoietic stem cells (HSCs), mesenchymal stem cells, neuronal stem cells, hepatic stem cells, etc.[1]

The hematopoietic system of vertebrates is derived from the mesodermal germ layer. Blood cell development (hematopoiesis) begins in the extra-embryonic yolk sac (primitive hematopoiesis) at embryonic day 7.5 (E7.5) and in the aorta/gonad/mesonephros (AGM) region of the mouse embryo (definitive hematopoiesis) at E8.5[2,3]. Yolk sac or AGM derived HSCs, the common ancestors of all types of blood cells, migrate to the fetal liver at E10-E11 and by E12 fetal liver becomes the major site of hematopoiesis in the embryo. Hematopoiesis switches to the bone marrow through migration of HSCs from fetal liver to the bone marrow at E16-E17, which soon after birth becomes the predominant site for hematopoiesis continuing into adult life[4,5].

Based on their self renewal activity, HSCs, can be divided into two subsets consisting of long-term HSCs (LT-HSC), highly self renewing cells that are competent to reconstitute the hematopoietic system of an animal for its entire life span, and a short-term subset (ST-HCS) that reconstitute the animal for a limited period of time (Figure 1)[6].

Hematopoiesis is initiated by asymmetric division of HSCs resulting in one daughter cell that retains the HSC identity, while the other daughter cell proliferates and differentiates to yield committed progenitors. Committed progenitors are restricted to a certain cellular fate as they differentiate toward mature elements of the blood including lymphoid, myeloid, erythroid and megakaryocytic cells (Box1 and Figure 1)[1,6]. Cell surface markers and

metabolic properties can be used to identify the lineage or stage of maturation of hematopoietic cells. For example all HSC activity is contained within the c-Kit^{+}, Sca-1^{+} lineage marker$^{low/-}$(Lin$^{low/-}$) component of the bone marrow and most of these cells represent ST-HSCs, multilineage progenitors or lineage-committed progenitors (Figure 1)[7-10]. In addition to the cell surface marker phenotype, HSCs can also be identified as a side population (SP) after staining with the DNA binding dye Hoechst-33342 and selection of unstained cells using the fluorescence activated cell sorter (FACS)[11]. The SP profile is determined by Hoechst-33342 dye efflux, which is a consequence of P-glycoprotein/ABC transporter (Bcrp1/ABCG2) activity on the surface of HSCs[11,12]. The SP fraction accounts for 0.07% to 0.1% of murine bone marrow and represents a minimal 1000-fold enrichment of LT-HSCs[11].

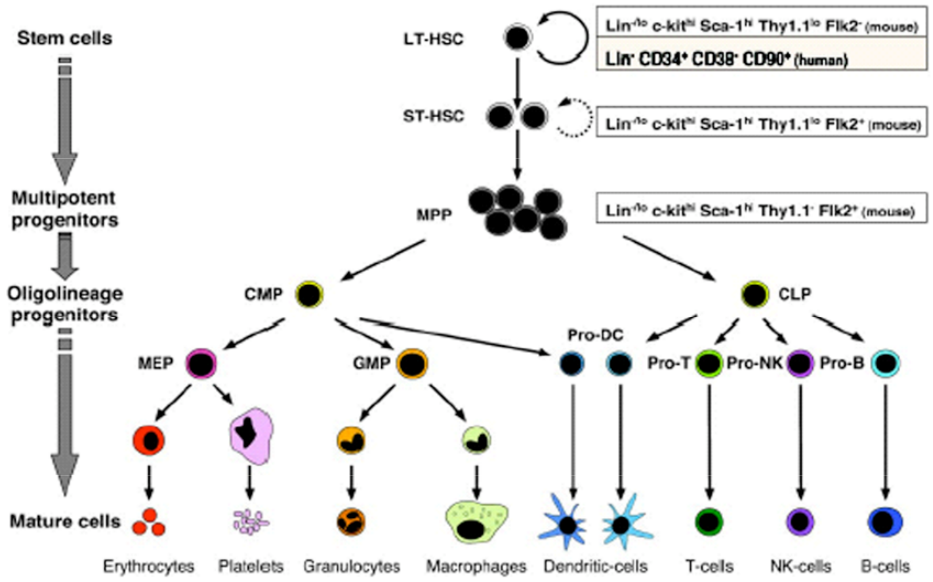


Figure 1. Differentiation pattern of hematopoietic cells (Adapted from ref.[6]).

The decision between self-renewal or lineage commitment of HSCs is mainly regulated by the integrated effects of extracellular signals and the differential expression of transcription factors (reviewed in ref. [13] and [14]).

Although the transcription machinery that orchestrates HSC self-renewal and differentiation is highly complex, accumulating data indicate that the transcription factors HoxB4[15,16], Ikaros[17], activated nuclear form of Notch1[18,19] and TGF/BMP-4 family members[20,21] might be involved in the maintenance or promotion of the HSC renewal, while co-expression of PU.1, GATA-1 and C/EBPα directs commitment towards the common myeloid precursor (CMP)[22]. PU.1 expression is also essential for HSCs commitment towards the common lymphoid precursor (CLP) via the regulation of expression of the IL-7 receptor, a signaling molecule essential for the development of lymphoid lineages. Commitment decisions of HSCs towards the myeloid or lymphoid lineages, two major groups of hematopoietic cells relevant for leukemia, is effected by the expression level of PU.1, with higher doses favoring commitment towards myeloid differentiation[23].

In addition to transcription factors, extrinsic cytokines produced within the stromal compartment of bone marrow also affect the lineage decisions of HSCs. For example, concomitant overexpression of the receptors for GM-CSF and M-CSF is able to reprogram CLPs towards monocytes or granulocytes[25] and IL-7 receptor expression is required for proper development of lymphoid cells[23]. Thus, key transcription factors and cytokines play an important role during normal hematopoiesis and aberrant expression or function of these factors contributes significantly to leukemogenesis (reviewd in ref. [14]).

Leukemic Hematopoiesis

Hematopoiesis is normally regulated by homeostatic mechanisms that control the production of blood cells according to the requirements of the organism. Leukemia results from uncontrolled proliferation of hematopoietic cells and an altered capacity to differentiate into functional cells as a result of accumulating genetic changes that affect both these processes[6,26].

Since unlimited self-renewal capacity, the main characteristic of leukemia and all other cancer types, is also a defining feature of stem cells, it has been proposed that leukemic process might be initiated and sustained by a few leukemic stem cells (LSC), which possess indefinite proliferation potential and drive formation and progression of leukemia. This hypothesis is confirmed by the finding that human acute myeloid leukemia (AML) stem cells of different French-American-British (FAB) subtypes are able to undergo self-renewal, which sets them apart from the bulk of leukemic cells that do not retain this capacity[27,28]. Subsequent studies provided more evidence for the origin of LSC and results from different experimental models showed that both HSCs[29] and developmentally more restricted progenitors[30] are capable of transformation into LSCs as a result of accumulating mutations. The initial mutations might occur in survival, apoptosis and differentiation pathways in HSCs, which already have self-renewal ability whereas in progenitor cells lacking this ability the initial mutations might likely serve to restore their self-renewal activity thereby allowing enough time to the cells to accumulate subsequent mutations that lead to a fully transformed LCS (reviewed in ref.[31]).

Genetic alterations in leukemia

Leukemias are classified according to cell identity and maturity. Acute leukemias occur in people of all ages and are characterized by mainly immature cells whereas chronic leukemias generally occur in adults and consist of more differentiated cells. Based on the cell type, the leukemias are subdivided into acute myeloid (AML), acute lymphoid (ALL), chronic myeloid (CML), or chronic lymphoid (CLL) disease[32]. The discovery of the Philadelphia chromosome[33] and the successive characterization of the t(9;22) that gives rise to this small chromosome marker[34] in CML opened a new era of cataloging leukemic karyotypes. Subsequent studies identified more than 350 recurrent balanced chromosomal abnormalities in hematologic malignancies (reviewed in ref.[35]).

Box 1 | Hematopoietic cell types*

- **Myeloid cells:** all non-lymphoid cells of the blood. In its most common usage 'myeloid cells' refers to granulocytes (neutrophils) and monocytes (macrophages).
  Polymorphonuclear leukocytes: white blood cells of the myeloid lineage (such as neutrophils) that have a lobed and variably shaped nucleus and a granular cytoplasm. These cells are 'professional' phagocytes that engulf and kill microorganisms.
  Eosinophils: polymorphonuclear leukocytes that are associated with helminth infection and hypersensitivity.
  Granulocytes (neutrophils): white blood cells that are dedicated to the ingestion and destruction of microorganisms (for example, bacteria).
  Monocytes: mononuclear phagocytes that circulate in the blood and migrate to tissues where they differentiate into macrophages.
  Macrophages: phagocytic cells that are derived from monocytes. These cells respond to foreign materials (for example, bacteria, protozoa or tumour cells) to release substances that stimulate other cells of the immune system. They are also involved in antigen presentation.
- **Lymphoid cells:**
  B cells: lymphoid cells that produce antibodies.
  T cells: lymphoid cells that act in cell-mediated immunity and stimulate B cells. They are divided into three subsets: T-helper, T-suppressor and cytotoxic T cells, depending on their function. T-helper cells are CD4+ T cells, meaning that they recognize antigens presented on major histocompatibility complex (MHC) class II molecules. They are subdivided into two classes: TH1 and TH2.
  TH1 cells: mediate delayed type hypersensitivity responses and provide protection against intracellular pathogens and viruses. These cells produce the cytokines interferon-gamma, interleukin-2 and tumour necrosis factor-beta.
  TH2 cells: provide help to B cells and help to eradicate helminths and extracellular parasites. These cells produce the cytokines interleukin-4, 5, 9 and 13.
- **Erythroid cells:** red blood cells (erythrocytes) that contain haemoglobin for oxygen delivery to tissues.
- **Megakaryocytes:** giant, polyploid cells that give rise to thousands of platelets each. These cells are called thromboblasts in chickens.

*Modified from ref. [24]

The main subtypes of ALL and AML are characterized by a large variety of genetic alterations including deletions, point mutations and gross chromosomal aberrations, such as hyperdiploidy or balanced translocations. These genetic changes help in the sub-classification of leukemias and provide prognostic information. In recent years, genome-wide analysis using microarray-based technologies showed that the different subtypes of leukemia associated with recurrent chromosomal translocations display distinct gene expression patterns, strongly correlated with specific morphologic, immunophenotypic, and prognostic parameters [36-39].

Cloning of the break points involved in chromosomal abnormalities led to identification of many known and unknown genes, and had an enormous impact on the differential diagnosis of leukemia, on the general understanding of the molecular mechanisms underlying leukemogenesis and on the design of novel therapeutic strategies. Chromosomal translocations in leukemia can result in either deregulation of an oncogene via association with constitutively active regulatory elements, e.g. translocation of MYC into the immunoglobulin heavy chain (IGH) or T-cell receptor (TCR) loci[40], or more commonly, by creation of an in-frame fusion gene that encodes the N-terminus of one protein fused to C-terminus of another protein. This usually results in the creation of an activated kinase or a novel transcription factor[41,42]. *MLL, TEL* and *AML1* are the most predominant targets of chromosomal translocations in leukemia, each of which can fuse with more than 15 alternative partner genes[43,44] and in the case of TEL and AML1 fusion also occurs with each other[45]. Leukemia related chromosomal translocations may be classified into four groups consisting of those involving the IGH and TCR loci, those involving tyrosine kinases, those involving transcription factors and those involving nucleoporins[43]. Due to the focus of my thesis research, I will concentrate on a description of the nucleoporins.

Nucleocytoplasmic transport and nucleoporins

Genetic material of the eukaryotic cells is present in the nucleus, and separated from cytoplasm by a nuclear envelope. Since transcription and translation occur separately in the nucleus and cytoplasm, nucleo-cytoplasmic exchange of RNAs, proteins, as well as other molecules to the site of action is an essential process for cellular maintenance. Nucleo-cytoplasmic transport occurs via nuclear pore complexes (NPC), the water-soluble channels formed between two nuclear membranes.

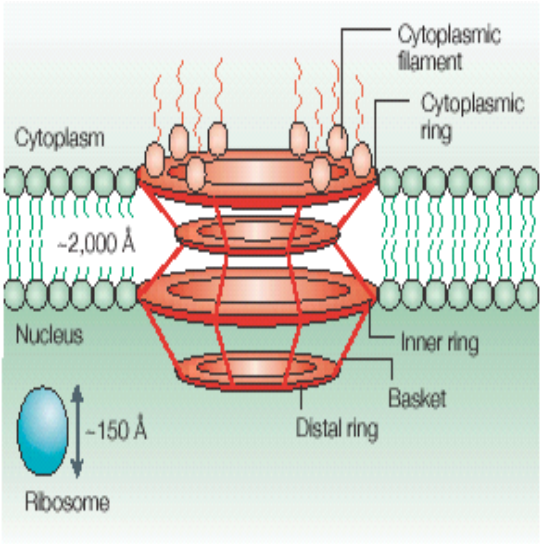


Figure 2. Diagram of nuclear pore complex (~200 nm in length) composed of nucleoporins (Adapted from ref. [48]).

Each cell contains ~3000 nuclear pores and the number of the functional pores varies depending on the growth state of the cell, which is usually proportional to the transcriptional activity[46,47]. The mammalian NPC is composed of about 30-50 different proteins called nucleoporins (NUPs)[49], which are arrayed in a basket-like structure of about 200nm in length and 10nm in diameter with a capability to dilate about two fold (Figure 2)

(reviewed in ref. [48])[50]. Although the nuclear pores allow the free diffusion of the molecules smaller than 10nm in diameter, or with a molecular mass up to 60kD, usually the transfer of both small and large molecules is mediated via active transport[51]. Nucleo-cytoplasmic distribution of the proteins is determined by the signals in their amino acid sequences, such as nuclear localization signals (NLS) or nuclear export signals (NES), which are recognized by continually shuttling transport proteins that deliver the cargo to the site of action [50].

For the nuclear import of proteins, adaptor protein importin a binds to the NLS-containing proteins and subsequently interacts with import receptor importin b which transfers the complex to the nucleus through the NPC. Once in the nucleus, importin β binds to RanGTP, which causes the release of the NLS-containing cargo. For the nuclear export, export receptors, such as CRM1, binds to NES-containing proteins and RanGTP in the nucleus and carries the cargo to the cytosol through the NPC. Subsequent hydrolysis of RanGTP to RanGDP in the cytosol results in the release of NES-containing cargo and the free export receptor recycles to the nucleus. Although asymmetrical localization of Ran guanine nucleotide exchange factor (RanGEF) in the nucleus and the Ran GTPase-activating protein (RanGAP) in the cytosol are the main factors that maintains the directionality of the transport via establishing a high RanGTP concentration in the nucleus and high RanGDP level in the cytoplasm, interaction of the transport receptors with nucleoporins also has an important impact on the bidirectional transfer of the molecules (reviewed in ref. [52] [48]).

Involvement of FG-repeat containing nucleoporins in chromosomal translocations: NUP98 and NUP214/CAN

Many nucleoporins (NUPs) are conserved in evolution from yeast to mammals and a number of NUPs contain FG amino-acid repeat motifs that serve as docking sites for transport receptors[53,54]. Two NUP family genes,

NUP98 and NUP214/CAN, are targets of chromosomal translocations in patients with leukemia implying that impaired nucleo-cytoplasmic transport might play a role in leukemic transformation}(Table 1)(reviewed in ref.[55])[56,57].

Table 1. Nucleoporin group of translocations

Genes	Rearrangement	Disease
NUP98 fusions		
HOXA9	t(7;11)(p15;p15.5)	AML, MDS, t-MDS/AML, CML
HOXA13	t(7;11)(p15;p15)	AML, MDS
HOXA11	t(7;11)(p15;p15)	CML
HOXC11	t(11;12)(p15;q13)	AML
HOXC13	t(11;12)(p15;q13)	AML
HOXD11	t(2;11)(q31;p15)	AML
HOXD13	t(2;11)(q31;p15.5)	AML, t-DMS/AML
PMX1	t(1;11)(q23;p15.5)	t-MDS/AML
DDX10	inv11(p15.5;q22)	AML, t-MDS/AML
TOP1	t(11;20)(p15.5;q11)	AML, t-MDS/AML
LEDGF	t(9;11)(p22;p15.5)	AML
RAP1GDS1	t(4;11)(q21;p15.5)	T-ALL
NSD1	t(5;11)(q35;p15.5)	AML
NSD3	t(8;11)(p11.2;p15)	AML
ADD3	t(10;11)(q25;p15)	T-ALL
NUP214/CAN fusions		
DEK	t(6;9)(p23;q34)	AML
SET	t(9;9)(q34;q34)	AUL
ABL1	Episomal amplicon	T-ALL

AUL, acute undifferentiated leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; T-ALL, T-cell acute lymphoblastic leukemia; t-MDS/AML, therapy-related acute myelogenous leukemia (Adapted from ref. [43,48]).

NUP98 is located on chromosome 11p15.5 and is transcribes a 7.0 kb mRNA encoding a nucleoporin 98 (NUP98)/nucleoporin 96 (NUP96) precursor protein. This precursor protein is subsequently cleaved to produce mature NUP98 and NUP96[58]. NUP98 resides on the nucleoplasmic side of NPC[59] and consists of two FG repeat domains separated by a GLEBS-like motif, a RNA-binding domain and a NLS located at the C-terminus of the protein (Figure 3). The GLEBS motif serves as a binding domain for RAE1, a mRNA specific carrier containing a NES[60]. }. Although NUP98 specific

antibodies block rRNA, snRNA and mRNA transport without effecting other functions[61], embryonic stem cells lacking NUP98 remain alive suggesting that none of the major transport pathways was completely inhibited[62].

NUP98, one of the most frequently involved genes in leukemic chromosomal translocations, is associated with both myeloid and lymphoid malignancies and can fuse to in total 15 different fusion partners (Table 1). Genes involved in NUP98 fusions can be divided into two groups: homeobox genes, a family of transcription factors (involving the members of *HOXA, HOXC and HOXD* genes) required for embryonic development, and non-homeobox genes (Table 1). In all fusions, the N-terminal FG repeat regions of NUP98 is retained and is fused to the C-terminus of the partner protein, which in the case of homeobox proteins contain the DNA-binding homeodomain (Figure 3) (Reviewed in ref.[63]).

Two NUP98-HOXA9 protein isoforms, encoded from the same fusion gene, transform NIH3T3 cells. Transformation requires FG-repeat rich motifs of NUP98 to recruit transcriptional coactivators CBP/p300 and acts as strong transactivators. Therefore, it has been anticipated that NUP98-HOXA9 proteins might be oncogenic transcription factors that deregulate HOX-responsive genes rather than oncogenic transport factors[64]. Although only a few NUP98 fusion genes have been used to create in vivo mouse models of leukemia[65,66], early findings imply that a block in differentiation might be a mechanism common to all known NUP98-HOX fusion genes involved in leukemic transformation (reviewed in ref. [63]).

Recently it has been reported that all non-homeobox genes involved in NUP98 fusions contain a potential coiled-coil region, which is retained in the fusion protein[67]. It is known that oligomerization via coiled-coil regions present in the fusion partners of RARa and AML1 activate the oncogenic potential of these fusion proteins, and oligomerization alone is sufficient for transformation[68]. Although further investigation is needed to determine the significance of the coiled-coil regions in the pathogenesis of the non-

homeobox fusion proteins, none of the NUP-98 homeobox fusions contain such regions suggesting that these two distinct groups of fusion proteins might act differently in leukemogenic transformation[67].

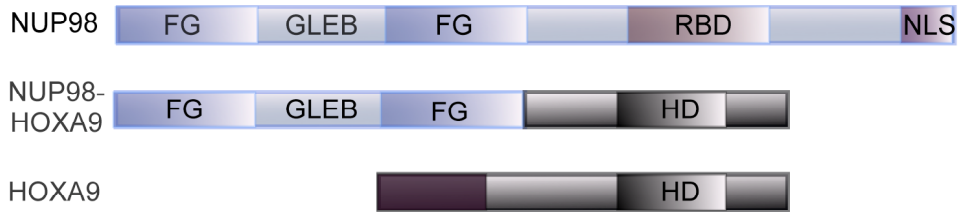
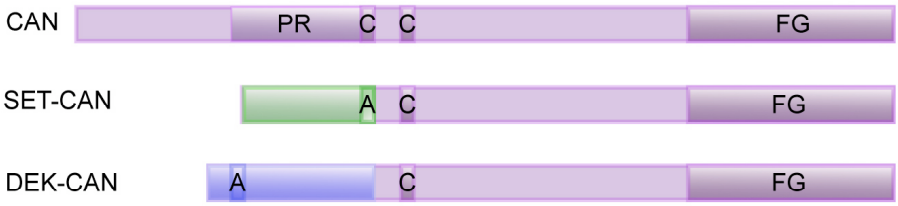


Figure 3. Functional domains of NUP98 and the fusion partner HOXA9. FG, Phe-Gly repeats; GLEB, RAE1 binding motif; RBD, RNA binding domain; NLS, nuclear localization signal; HD, homeodomain (Modified from ref. [55]).

CAN is another FG-repeat containing nucleoporin gene targeted by chromosome translocations in leukemia. It is located on human chromosome 9q34 and was initially identified as a partner in the t(6;9) translocation in which it is fused to *DEK* on chromosome 6p21. The DEK-CAN chimeric protein is expressed in a subgroup of patients with AML and myelodysplastic syndrome (MDS)[69,70]. In an isolated case of acute undifferentiated leukemia (AUL), *CAN* is fused to *SET* gene. SET is present in the same chromosomal region as *CAN*[71] at a distance of 2.51 Mb closer the centromere (Ensemble genome browser). In both translocations, the two-thirds of the C-terminal part of CAN, including its FG repeat domain, is linked to almost the entire DEK or SET protein[72](Figure 4A). DEK and SET do not show any sequence similarities except that they both contain highly acidic regions and coiled-coil domains[67,73]. Recently it was also shown that the N-terminus of *CAN*, containing the predicted coiled coil domain, also fuses to the C-terminus of

ABL1 through the formation of extra chromosomal (episomal) amplicons (Figure 4B)[74].

A.



B.

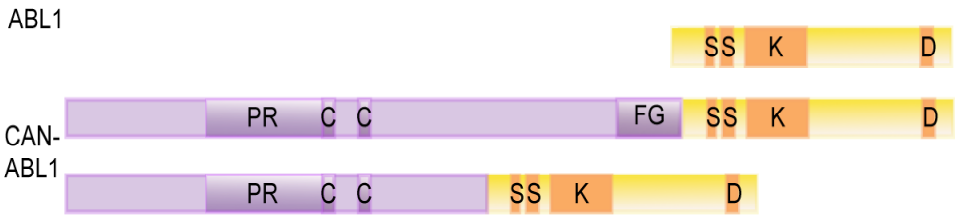


Figure 4. Schematic representation of CAN fusion proteins. A) SETCAN and DEK-CAN B) CAN/ABL1. PR, prolin rich; C, coiled coil; FG, FG repeat; A, Acidic tail; S, SH3 and SH2 domains, respectively; K, kinase domain; D, DNA binding domain.

CAN, a ubiquitously expressed phosphoprotein, mainly localizes to the cytoplasmic face of the NPC[75-77]. The central region, containing a coiled-coil domain, and the C-terminal FG-repeat region of CAN interacts with NUP88 and hCRM1, respectively, and mediates the attachment of NUP358/RanBP2 to the NPC (Figure 5). This is essential for the localization of hCRM1 to the nuclear envelope, and acts as a terminal binding site in NES-dependent hCRM1-mediated nuclear export[72,77].

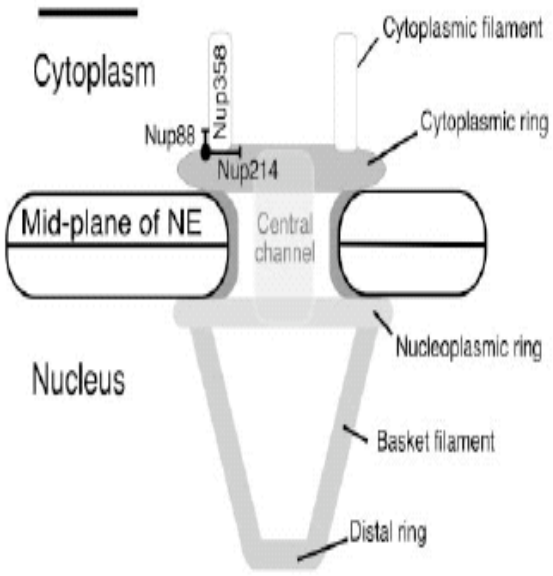


Figure 5. Schematic representation of the localization of CAN/NUP214 at nuclear pore complex. NE, nuclear envelope. Bar=50nm (Adapted from ref.[77]).

Similar to CAN, SET-CAN and DEK-CAN associate with hCRM1 through the FG-repeat region of CAN that is retained in the fusion proteins. However, the fusion proteins fail to interact with NUP88, probably due to loss of part of the central region of CAN[72]. Similar to DEK and SET, both DEK-CAN and SET-CAN fusion proteins localize in the nucleus, resulting in relocalization of CAN from nuclear envelope to the nucleus[76]. Overexpression of CAN, as well as mutants containing the hCRM1 interacting domain, cause growth arrest and apoptosis in the human U937 promonocytic cell line[57]. *Can*-knockout mouse embryos do not develop beyond E4.5 due to depletion of maternal *Can*. The absence of *Can* results in cell cycle arrest at G_2 followed by a block in nucleo-cytoplasmic mRNA/protein transport[78]. In addition to its essential role in RNA and protein transport, CAN is also involved in import of

adenovirus 2 (Ad2) DNA through direct interaction with the viral nuclocapsid[79].

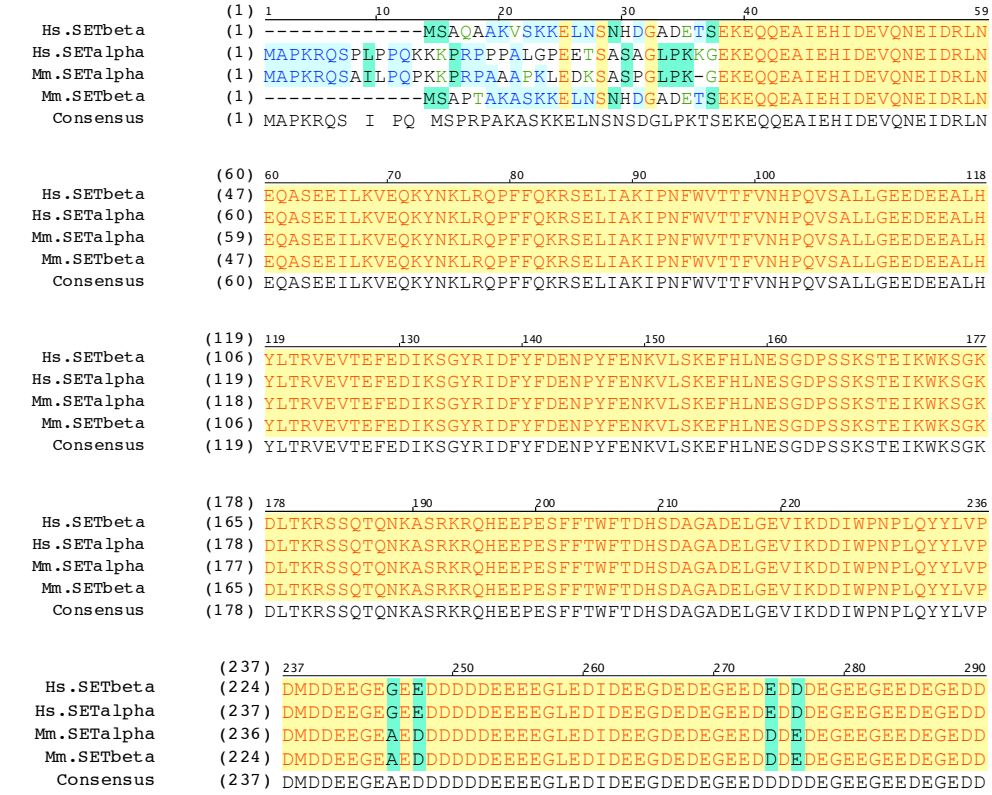
SET, a partner involved in CAN-fusions

Human *SET,* initially characterized as a component of the t(9;9) that gives rise to the *SET-CAN* chimeric gene in a patient with AUL[71], encodes a 39-kDa, predominantly nuclear, phosphoprotein[80]. Human and mouse *SET* are 94% identical in nucleotide sequence and show 98% similarity in amino acid sequences (Figure6A and B). The Ser9 and Ser24 residues at the N-teminus of SET are phosphorylated by an as yet unknown kinase and the biological relevance of this phosphorylation remains to be determined[81].

SET shows homology to the nucleosome assembly proteins (NAPs), which play a role in chromatin remodeling[71,82]. After its initial isolation, SET also has been identified as the template-activating factor I (TAF-I) b, which stimulates the replication and transcription of the adenovirus genome in a cell-free system together with TAF-Ia[83]. TAF-Iaand TAF-Ib mRNAs are transcribed from the *SET* gene but have different first exons. They encode proteins that exist mostly as homo- or hetero-dimers. Dimerization is mediated through their predicted coiled-coil region at the N-terminus (Figure 7)[73,83]. The chimeric gene in the patient with t(9;9) is transcribed as both a and b SET-CAN (Kandilci A and Grosveld G, unpublished data).

SET physically interacts with several protein complexes, suggesting that it has diverse functions including a role in chromosome remodeling[84], apoptosis[85,86], cell cycle regulation[87-89], transcriptional regulation [90] and mRNA stabilization[91]. SET binds to histones in vitro[82] and in vivo, as a subunit of the INHAT complex,[84] suggesting that it has a role in chromosome remodeling. The INHAT complex consists of SET, TAF-Ia and pp32, a putative HLA-associated protein I or I_1^{PP2A}. Binding of the INHAT complex to histones prevents their acetylation, thus preventing transcriptional activation[84]. Moreover, overexpression of SET can inhibit demethylation of ectopically

A.



B. Similarity table (%)

	Hs.SET β	Hs.SET α	Mm.SET α	Mm.SET β
Hs.SET β	100	88	88	98
Hs.SET α		100	94	87
Mm.SET α			100	89
Mm.SET β				100

Figure 6. Comparison of amino acid sequence of human and mouse SET using Vector NTI software (InforMax, Inc., Bethesda, MD). **(A)** Comparison between the alpha and beta isoforms of human (Hs) and mouse (Mm) SET (Hs.SETbeta: NP_003002.1, Hs.SETalpha: BAA08139.1, Mm. SETbeta: BAB31936.1, Mm.SETalpha: NP_076360.1). [color setup: conservative, similar, identical]. **(B)** Similarity table showing the percentage of amino acid similarities between human and mouse SET proteins.

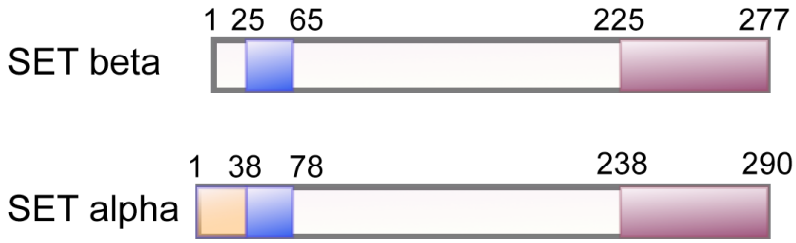


Figure 7. Illustration of SET-beta and SET-alpha proteins. First exons of SETb (1-25) and SETa(1-38), N-terminal coiled-coil motifs (in blue and) and C-terminal acidic domains (dark pink) are shown.

methylated DNA, thus resulting in gene silencing[92]. SET is involved in apoptosis through cleavage and removal of the acidic domain by the serine protease granzyme A at its Lys^{176} residue[93]. SET binds and blocks DNase activity of the tumor metastasis suppressor NM23-H1, an activity also negatively regulated by granzyme A cleavage[86]. NM23-H1 released of SET inhibition induces apoptosis by causing single-strand DNA nicking[86].

In addition, SET is also cleaved by caspases at the tetra amino acid sequence SNHD releasing the first 18 amino acids. SET is mostly targeted by caspase 7, either during radiation, etoposide treatment, or TNF induced apoptosis and it is a potential marker for cell death[85]. Overexpression of SET has the opposite effect on Granzyme A and Fas-mediated apoptosis, the latter is mediated via the inhibitory effect of SET on PP2A activity[87,94] and the ensuing activation of the MAPK/ERK1, ERK2 pathway[95].

It has been proposed that SET has opposite functions during different phases of the cell cycle. First, SET binds the cyclin-CDK inhibitor p21 and reverses its inhibitory effect on cyclin E–CDK2 complexes, suggesting a positive regulatory role for SET in G_1/S transition[88]. Second, SET interacts with cyclin B–CDK1 complexes [96] and overexpression of SET inhibits cyclin B–CDK1 activity, thereby blocking G_2/M transition[89]. In addition, the cell division autoantigen-1 (CDA1), another member of the NAP/SET family,

which shows 40% identity and 68% similarity of its acidic and basic domains to those of SET, also inhibits proliferation upon overexpression and decreases bromodeoxyuridine incorporation[97]. Similar to the effect on cell-cycle progression, the role of SET in transcriptional regulation also appears to be contradictory. On the one hand, SET inhibits acetylation of core histones via the INHAT complex and therefore promotes transcriptional repression via chromatin hypoacetylation[84,98].On the other hand, SET forms a complex with histones and the p300/CBP transcriptional coactivators thereby augmenting p300/CBP dependent transcription[99]. Besides its chromatin related activities, SET also inhibits the transcriptional activity of the transcription factor Sp1. SET interacts with the Sp1 DNA binding domain and blocks its binding to DNA[100]. Interestingly, the domain of Sp1 that binds SET also interacts with the acetyltransferase p300 and several other chromatin-related factors. The interaction with p300 stimulates the DNA binding activity of Sp1[100]. In addition to direct or indirect effects on transcription, SET has been reported to participate in post-transcriptional regulation of mRNA through interaction with HuR, a RNA binding protein that selectively targets and stabilizes mRNAs containing AU-rich elements (AREs) at their 3' untranslated regions (UTRs). AREs are present in the 3'UTRs of proto-oncogenes, cytokines and lymphokines[91]. SET, together with pp32 and the acidic protein rich in leucin (APRIL), binds to regions of HuR known to be essential for its nucleo-cytoplasmic shuttling of mRNAs and for mRNA stabilization. Therefore, SET might be important for HuR regulated stability and shuttling of ARE-containing mRNAs[91].

Despite the apparent role of SET as a multitasking protein, it is presently unclear how all these findings fit together in a cellular network of survival, proliferation and differentiation processes. It is likely that the cell type and the cellular context might be important for the specific function of SET. However, there is a possibility that the highly charged acidic tail of the protein

might create artifacts during protein purification procedures, suggesting interactions with complexes, which do not occur in vivo.

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Chapter 2

Effects of SET and SET-CAN on the differentiation of the human promonocytic cell line U937

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Effects of SET and SET-CAN on the differentiation of the human promonocytic cell line U937

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Human *SET* encodes a nuclear phosphoprotein with a highly acidic carboxyl-terminus, forming a *SET-CAN* fusion gene in a patient with acute undifferentiated leukemia. SET is highly conserved between species and is ubiquitously expressed, suggesting a widespread biological role. Even though SET is involved in chromatin remodeling and transcriptional activation, its precise role in hematopoietic cells and the contribution of SET-CAN to leukemogenesis remains unknown. We determined the effect of tetracycline-regulatable expression of SET, a deletion mutant of SET, and SET-CAN on the human promonocytic cell line U937T. The expression of SET and SET-CAN inhibited proliferation of these cells. SET accomplishes this through the induction of the differentiation program, an effect that depends on the presence of its acidic domain. SET-CAN most likely inhibits growth by interfering with hCRM1, but it also partially blocks differentiation. Our results are the first demonstration of a potential role of SET in hematopoietic differentiation.

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Introduction

Human *SET* was initially identified as a partner of the *SET-CAN* fusion gene, which resulted from a translocation or inversion involving chromosome 9q34 in a patient with acute undifferentiated leukemia (AUL).[1] SET-CAN expression is under the control of the SET promoter and it contains almost the entire SET open-reading frame lacking the last six amino acids, fused to the carboxyl-terminal two-third of CAN.[1] CAN is an essential nucleoporin involved in nucleocytoplasmic transport of protein and mRNA.[2] SET and CAN localize mainly to the nucleus and nuclear envelope, respectively, and both are expressed ubiquitously in mouse and human.[2–4] Mouse, rat[5] and human *SET*[6] are 94% identical in nucleotide sequence and encode a phosphoprotein containing an acidic C-terminal tail.[3] SET, shows homology to nucleosome assembly proteins (NAPs), which have a role in chromatin remodeling.[7] It has been reported that SET (TAF-Iβ) binds to histones *in vitro*[7] or *in vivo* as a subunit of the INHAT complex.[8] This complex consists of SET, TAF-Iα, a SET isoform containing a different 24 amino acids long N-terminus and pp32, a putative HLA-associated protein I or I_1^{PP2A}.[8] The binding of the INHAT complex to histones prevents their acetylation, which causes transcriptional repression.[8] Moreover, the overexpression of SET can inhibit demethylation of DNA, which results in gene silencing.[9] Granzyme A, a serine protease, cleaves SET after Lys^{176}, removing its acidic domain, which disrupts its NAP activity.[10] In addition, SET binds and blocks DNase activity of the tumor metastasis suppressor NM23-H1, an activity also negatively regulated by granzyme A cleavage.[10] NM23-H1 released of SET inhibition induces apoptosis by causing single-strand DNA nicking in cells.[10] Although there are several additional functions attributed to SET such as the inhibition of protein phosphatase 2A[11,12] and transcriptional activation,[13] the role of SET in hematopoiesis and the effect of SET-CAN on that function remains elusive. To determine the effect of SET and SET-CAN expression, we used the human promonocytic cell line U937T as a model system and studied the role of these proteins and that of a deletion mutant of SET lacking the acidic tail on the growth and differentiation of this cell line.

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Materials and methods

Constructs

Using PCR-based cloning techniques, we removed the C-terminal 51 amino acids of SET (SET Delta Acid, SdelAc), and added a Flag epitope to the N-termini of the open-reading frames of SET (Flag-SET, FS) and SDelAc (FLAG-SdelAc, FSDelAc). We cloned all constructs into the *Eco*R1 site of the TetVP16-responsive expression vector pUHD10S[2] and their integrity was confirmed by DNA sequencing.

Establishment of inducible cell lines

Stable U937T cell clones,[14] regulatably expressing FS, FSDelAc or SET-CAN (SC) were generated and maintained as described previously.[14]

Immunocytochemistry and Western blot analysis

After 72 h of FS, FSDelAc or SC expression, cells were fixed, permeabilized and stained with primary anti-Flag (1:500; Sigma, St Louis, MO, USA) or affinity-purified polyclonal anti-CAN antibody (1:500; directed against the carboxy-terminus of CAN), and a secondary Alexa 488-conjugated antibody (Molecular Probes, OR, USA). CD11b was detected with anti-CD11b antibody (1:10; Pharmingen, San Diego, CA, USA), followed by incubation with Cy3-conjugated FAB fragment (Jackson ImmunoResearch, PA, USA). FS, FSDelAc and SC protein levels in induced or uninduced cells were determined by separating cell lysates on 8% SDS-PAGE gels, followed by Western blot analysis using primary anti-Flag (1:5000) or rabbit polyclonal anti-SET[3] antibodies (1:4000) and a secondary HRP-conjugated antibody. Equal loading level of the proteins was confirmed by anti-actin antibody (1:1000, Santa Cruz Biotechnology, CA, USA).

Growth curve, apoptosis and cell cycle analysis

To remove tetracycline, cells were washed with RPMI 1640, and plated at a density of 4×10^5 cells/well in six-well plates and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in the presence (uninduced) or absence (induced) of tetracycline (three wells/time point). Cells were counted at consecutive days and after propidium iodide (PI) and Annexin V staining, we determined the percentage of dead and apoptotic cells by fluorescence activated cell sorter (FACS). DNA content and cell cycle distribution was analyzed by FACS as described before.[14]

Differentiation assay and FACS analysis

After 16 h of culture of induced and uninduced FS, FSDelAc or SC cells (2.5×10^5 cell/ml), differentiation was stimulated by the addition of vitamin D_3 (10^{-7} M in ethanol) or an equivalent amount of ethanol as negative control, and cells were cultured for an additional 48 h (total 64 h of culture). The expression of the CD11b marker was analyzed by FACS after staining with anti-MO1 monoclonal antibody (Beckman-Coulter, Miami, FL). SC cells were also analyzed 24 h after the addition of vitamin D_3 (total 40 h of culture in the presence or absence of tetracycline). The coexpression of CD11b and FS was detected by immunofluorescence, and cell morphology was determined using Giemsa-stained cytospin preparations.

Results

To investigate the effect of SET on the growth and differentiation of the human promonocytic U937T cell line (Boer *et al*[14]), we created tetracycline-inducible clonal lines expressing Flag epitope-tagged SET (FS), or a Flag-tagged deletion mutant of SET lacking the acidic domain (FSDelAc). We determined whether the effects of SET in U937T cells were different from those induced by its oncogenic counterpart SET-CAN, which would ascertain intrinsically different functions of these two proteins. Therefore, we also created tetracycline-inducible clonal U937T lines expressing SET-CAN (SC). The expression of all three proteins without tetracycline was confirmed by immunocytochemistry and Western blot analysis (Figure 1a and b). We determined the effect of FS, FSDelAc and SC expression on proliferation and viability of the cells by culturing for 5 days with (uninduced) or without (induced) tetracycline. We counted the cells daily and determined their cell cycle profile, viability and rate of apoptosis by FACS after staining with PI and Annexin V, respectively. Compared to uninduced U937T controls, cells expressing FS and SC, but not FSDelAc showed a 1.6- and 5.9-fold lower proliferation rate after 5 days of induction, respectively (Figure 1c). The inhibition of proliferation by FS was not caused by an increased rate of death and apoptosis, which remained similarly low to that of uninduced or U937T parental cells (Figure 1d). In contrast, growth suppression in SC-expressing cells was accompanied by an increasing percentage of cell death and apoptosis amounting to 90% at day 5 of induction (Figure 1d). These observations were further supported by flow-cytometric cell cycle analysis of U937T, FS, FSDelAc and SC cells at 5 consecutive days following induction. In agreement with the growth rate, this analysis shows that FSDelAc cells proliferate the same as control U937T cells, whereas FS cells progressively exit the cell cycle and accumulate in G0/G1 phase. By contrast, the surviving SC cells arrest during S phase of the cell cycle (Figure 1e). U937T parental cells slow down slightly because of the culture becoming confluent.

U937T cells can be induced to differentiate, resulting in cell cycle exit and the expression of the CD11b surface marker.[15] We reasoned that the progressive cell cycle exit of FS-overexpressing cells could be the result of the induction of differentiation. Indeed, by using FACS, we determined that FS induction resulted in a 25-fold upregulation of the CD11b differentiation marker, while the induction of FSDelAc or SC did not have this effect (Figure 2a). The latter was not caused by an intrinsic inability of the cells to differentiate, because addition for 48 h of the potent stimulator of monocytic differentiation, vitamin D_3,[15] to uninduced FS, FSDelAc and SC cultures caused 98% of the cells to express CD11b (Figure 2a and c). As judged by the stable expression of CD11b, the induction of FS or FSDelAc did not affect vitamin D_3-induced differentiation (Figure 2a c), whereas the expression of SC reduced the number of cells expressing CD11b in a dose-dependent manner to 37% after 64 h of SC induction (Figure 2a and d). As cell death at 64 h after induction measures 60%, we repeated the experiment at 40 h after the induction of SC, 10 h prior to the SC-induced rise in cell death. Also at this time point, SC expression inhibited vitamin D3-induced CD11b expression to 35% (Figure 2e). This suggests that SC expression inhibits vitamin D_3-induced differentiation. We also assessed the effect of vitamin D_3-induced differentiation on the levels of endogenous SET, which increased somewhat during the first 4 h of differentiation (Figure 2f) in both ethanol control and vitamin D3-treated cells and then remained stable. We conclude that vitamin D_3-induced the differentiation of U937T cells is not driven by a large increase in the level of endogenous SET.

Discussion

We have shown that forced expression of SET and SET-CAN inhibits proliferation and induces cell cycle arrest in U937T cells. From our data, we infer that SET induces cell cycle arrest of U937T cells through the induction of differentiation. This activity is dependent on the presence of the acidic tail of SET, which mediates histone and NM23-H1 interaction.[8,10] In contrast, SET-CAN inhibits differentiation, indicating that fusion of SET to CAN creates a protein with a distinct function. Boer *et al*[14] have shown that forced expression of DEK-CAN (which possesses the same portion of CAN as SET-CAN) induces cell cycle arrest, due to interference with the leucine-rich nuclear export sequence receptor hCRM1. In fact, the overexpression of CAN or any CAN mutant containing the C-terminal hCRM1 interaction domain exhibits this effect. The cell cycle arrest and enhanced cell death by SET-CAN is also likely to be caused by this interference. However, DEK-CAN did not inhibit the differentiation of U937T cells,[14] suggesting that the suppression of differentiation by SET-CAN might be due to an altered SET-related rather than a CAN-related function of the fusion protein. It is important to note that the inhibition of differentiation by SET-CAN is observed well before the SET-CAN-mediated effects of CRM1 inhibition become critical and cause cell death. This may reflect a biologically relevant function of SET-CAN, which was isolated from a patient with AUL. However, unlike the primitive progenitors in which SET-CAN was found, U937T cells are committed myeloid cells. Currently, we are developing a transgenic mouse model expressing SET-CAN to verify whether SET-CAN also inhibits the differentiation of primitive progenitors. As apoptosis in U937T cells caused by SET-CAN and DEK-

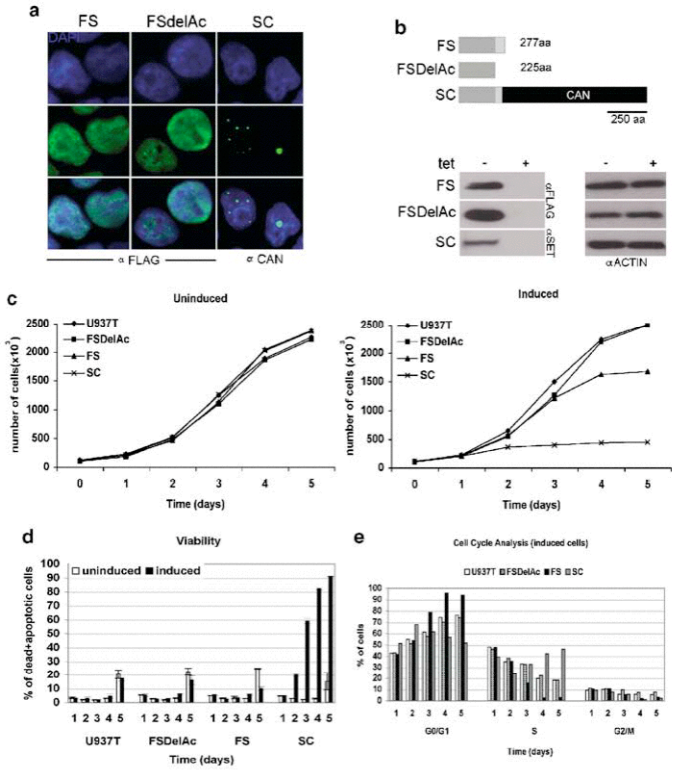

Figure 1 Effect of SET and SET-CAN on proliferation and cell viability. (a) Stably transfected U937T cells were washed with PBS and cultured for 72 h in the absence of tetracycline to induce Flag-SET (FS), Flag-SET Delta Acid (FSDelAc) and SET-CAN (SC) expression. Immunofluorescent detection of FS, FSDelAc and SC expression was performed either with the anti-Flag or anti-CAN antibody as described in the text. FS and FSDelAc showed dispersed nuclear staining, whereas SC showed punctuated staining (green, middle panel). Blue shows nuclear staining with DAPI. (b) Induced (tet−) and uninduced (tet+) cells were cultured for 72 h and proteins on Western blot were detected either with anti-Flag, anti-SET or anti-Actin antibody (see text). (c) FS, FSDelAc and SC cells were seeded at 1×10^5 cells/ml (4×10^5 cells/well in a six-well plate) with or without tetracycline and cultured for 5 days. Each day cells were counted and the mean value of triplicate cultures is shown in the growth curve. (d) To determine the viability of parental U937T cells, induced or uninduced FS and SC cells, approximately 3×10^5 cells were stained with Annexin V bound to fluorescein isothiocyanate and 1 μg/ml PI, and analyzed by FACS. (e) The same cells that were used for the growth curve and viability analysis were also further analyzed by FACS to determine the cell cycle profile for each day.

CAN seems paradoxical for an oncogene it is possible that, similar to MLL-fusion proteins,[16] DEK-CAN and SET-CAN are less well tolerated in U937T cells than in primitive progenitors. In addition, we have indications that SET-CAN in the bone marrow of a SET-CAN AUL patient[1] is expressed at a much lower level than in our SET-CAN U937T cell lines.

As a component of the INHAT complex, SET is involved in inhibiting histone transacetylation resulting in transcription silencing and chromatin compaction.[8,9] If the effect of SET in U937T cells is mediated via this complex, it is conceivable that SET inhibits the expression of genes stimulating growth, thereby promoting differentiation. Alternatively, SET might inhibit the expression of genes repressing differentiation. In both cases, the upregulation of CD11b would be an indirect effect merely reflecting the fact that the cells differentiate. It is also possible that the upregulation of CD11b is a more direct consequence of SET overexpression, for instance, if SET would inhibit the expression of a repressor of CD11b. We know that significant upregulation of endogenous SET is not involved in vitamin D_3-induced differentiation of U937T cells, but we believe that overexpression triggers changes in gene expression that in part mimic the effect of vitamin D_3 induction. At present, we have no answer to these questions but we are currently testing the different possibilities.

If indeed SET-CAN inhibits the differentiation of primitive progenitors, its expression could lead to the enlargement of this compartment increasing the likelihood of secondary mutations to occur resulting in leukemia.[17] We hypothesize that as a result of a chromosome translocation, haploinsufficiency of SET in the patient could enhance the differentiation inhibitory effect of

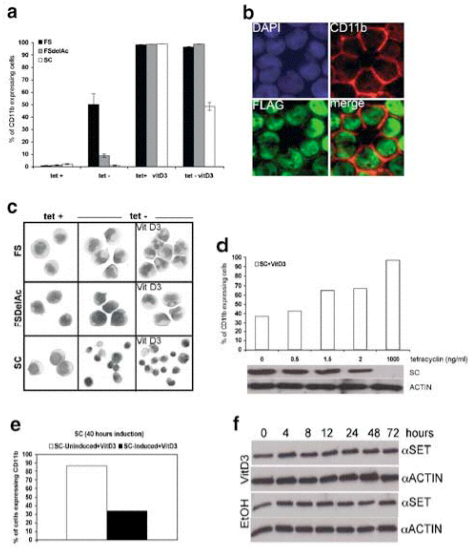

Figure 2 SET stimulates whereas SET-CAN suppresses differentiation. (a) For differentiation assays, FS, FSDelAc and SC cell lines were cultured as described in the text. Cells were stained with anti-MO1 (CD11b) monoclonal antibody and analyzed on a Becton Dickinson FACS Calibur flow. Mean value of triplicate experiments are shown in the graphic. (b) Coexpression of FS and CD11b was detected by immunofluorescent staining as described in the text. Blue represents nuclear staining with DAPI, green represents anti Flag staining for FS and red shows the CD11b expression. (c) Giemsa staining of U937T cells expressing FS shows a phenotype that is similar to FS or FSDelAc cells induced to differentiate with vitamin D3. The overexpression of FS and FSDelAc has no effect on vitamin D_3-stimulated differentiation, whereas SC expression causes the appearance of cells with an apoptotic morphology. (d) SC cells were cultured in the absence or presence of 0.5, 1.5, 2 and 1000 ng/ml tetracycline for 16 h and differentiation was stimulated by adding vitamin D_3 to the culture for an additional 48 h. CD11b expression of the viable cells (PI- and Annexin V-negative cells) was determined by FACS. Western blot analysis was performed as described in the text to show the expression of SC. Loading level of the proteins was determined by an anti-actin antibody. SC expression blocked vitamin D_3-stimulated differentiation in a dose-dependent manner. (e) To detect the inhibition of differentiation by SC prior to exhibiting its toxic effect, cells were cultured with or without tetracycline for 16 h, followed by 24 h of culture in the presence of vitamin D_3. Cells were analyzed by FACS to determine the expression of CD11b. At this time of induction (total 40 h) 80% of the cells stained negative for PI and Annexin V. (f) U937T cells treated with vitamin D_3 or vehicle (ethanol) were harvested at different time points of treatment and endogenous SET expression was analyzed by Western blot.

SET-CAN and therefore jointly contribute to leukemogenesis. We are currently developing a mouse model to test this hypothesis.

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Chapter 3

SET-induced calcium signaling and MAPK/ERK pathway activation mediate dendritic cell-like differentiation of U937 cells

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SET-induced calcium signaling and MAPK/ERK pathway activation mediate dendritic cell-like differentiation of U937 cells

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Human *SET*, a target of chromosomal translocation in human leukemia encodes a highly conserved, ubiquitously expressed, nuclear phosphoprotein. SET mediates many functions including chromatin remodeling, transcription, apoptosis and cell cycle control. We report that overexpression of SET directs differentiation of the human promonocytic cell line U937 along the dendritic cell (DC) pathway, as cells display typical morphologic changes associated with DC fate and express the DC surface markers CD11b and CD86. Differentiation occurs via a calcium-dependent mechanism involving the CaMKII and MAPK/ERK pathways. Similar responses are elicited by interferon-γ (IFN-γ) treatment with the distinction that IFN-γ signaling activates the DNA-binding activity of STAT1 whereas SET overexpression does not. In addition, unlike IFN-γ signaling, SET generated stress-induced p38/MAPK activity. Interestingly, IFN-γ treatment transiently upregulated endogenous SET in a dose-dependent manner. These results suggest that SET is part of both IFN-γ-mediated and stress-mediated cellular responses and that SET induces cell differentiation via calcium and MAPK/ERK pathways.

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Introduction

The human *SET* gene, a member of the *NAP/SET* family,[1] is located on chromosome 9q34. SET was initially identified as one partner in the *SET-CAN* fusion gene, which results from the t(9;9)(q34;q34).[2] *SET* encodes a 39-kDa ubiquitously expressed, predominantly nuclear phosphoprotein. One-third of its C-terminal acidic amino acids form an acidic tail.[2–4] SET (also known as template-activating factor I beta (TAF-Iβ)) physically interacts with several protein complexes, which suggests that it has diverse functions including Granzyme A induced apoptosis,[5,6] chromosome remodeling,[7] transcriptional regulation,[8] mRNA stabilization[9] and cell cycle regulation.[10–13]

SET also forms a complex with the MLL leukemic fusion protein and type-2A protein phosphatase (PP2A).[14] Among other functions, PP2A regulates cell cycle progression,[15,16] and one target of PP2A is the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) pathway.[17] SET inhibits PP2A activity,[10,12] and overexpression of SET activates MAPK and prevents Fas-mediated apoptosis.[17]

It has been proposed that SET has opposite functions during cell cycle progression:[13,18] First, SET binds p21. This interaction reverses the inhibitory effect of p21 on cyclin E CDK2 complexes and suggests a positive regulatory role for SET on G_1/S transition by allowing cyclin E CDK2 activity in the presence of p21.[11] Second, SET interacts with cyclin B CDK1.[19] Overexpression of SET inhibits cyclin B-CDK1 activity, which in turn, blocks the G_2/M transition; this finding suggests a negative regulatory role for SET in G_2/M transition.[13] Overexpression of cell division autoantigen-1 (CDA1), another member of the NAP/SET family, inhibits proliferation and decreases bromodeoxyuridine uptake in HeLa cells.[20] Acidic and basic domains of CDA1 show 40% identity and 68% similarity to SET.[20]

We have recently shown that overexpression of *SET* in the human promonocytic cell line U937 causes G_0/G_1 arrest and stimulates differentiation, an effect dependent on the acidic domain of SET.[21] Therefore, the level of expression of SET may affect cellular processes, as determined by cell type and context. Herein, we further analyzed the mechanisms of SET-induced differentiation of U937 cells and showed that it occurs along a dendritic pathway as a result of calcium signaling and MAPK/ERK activation.

Materials and methods

Cell culture and FACS analysis

Stable U937 cell clones expressing tetracycline-regulatable Flag-epitope tagged SET (FS) were generated and maintained as described previously.[21] Cells that were either treated or non-treated with vitamin D_3 (10^{-7} M) were cultured in the presence (FS-uninduced) or absence of tetracycline (FS-induced) after initial washing (tet[+] cells with tet[+] medium and tet[−] cells with tet[−] medium) and the expression of CD11b (Beckman Coulter Inc., Fullerton, CA, USA), CD14, CD86, CD83, CD80 and HLA-DR (all from BD Biosciences, Franklin Lakes, NJ, USA) were analyzed by FACS at day 4 of culture. For inhibition assays, cells were treated with specific inhibitors (CsA (2 μg/ml; Sigma, St Louis, MO, USA), W-7 (15 μM; Calbiochem, San Diego, CA, USA), KN-93 (3 μM; Calbiochem) SB202190 (5 μM; Upstate, Charlottesville, VA, USA), PD98059 (50 μM; Cell Signaling Technology, Beverly, MA, USA) or DMSO vehicle as a control. At day 4 of culture, each sample was divided into three aliquots, which were used for light microscopy, FACS and immunohistochemical analysis. For light microscopy, $1x10^6$ cells were resuspended in 2 ml serum-free RPMI, transferred onto polylysine-coated six well plates (Becton Dickinson Labware, MA, USA) and cultured for 1 additional hour. Cells were photographed with an Olympus IX-70 inverted microscope. All the inhibitors were first tested for toxicity and optimized nontoxic doses were used in the further experiments.

Microarray and data analysis

Total RNA was isolated from parental U937 and FS-induced cells at 8, 24 or 48 h of culture using TRIzol (Life Technologies,

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Gaithersburg, MD, USA). The RNA was processed and hybridized to human genome U95Av2 gene chips, following the manufacturer's protocols (Affymetrix, Santa Clara, CA, USA). Chips were scanned and analyzed using Microarray software (Affymetrix) as described previously.[22] Upregulation of a gene in the FS-induced cells was determined by comparing its level of expression with that in the control U937 cells.

Real-time RT-PCR

Quantitative real-time RT-PCR (TaqMan) analysis was performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). The amplification mix included 100 ng RNA, 0.2 μM of each primer, 0.2 μM of each probe and TaqMan one-step RT-PCR master mix reagent in a total reaction volume of 40 μl. As an internal control, amplification of GAPDH was performed in the same reaction mix and detected with an alternatively labeled probe. Triplicates of each standard and sample were assessed. Primer sequences are available upon request.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% PBS TritonX-100 and incubated with mouse anti-flag antibody (Sigma, St Louis, MO, USA) and Alexa-488 conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Cells were covered with mounting medium containing 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) and then analyzed with an Olympus BX-51 fluorescence microscope.

Western blot analysis

Total cell lysates were separated on 10% SDS-PAGE gels and transferred to polyvinylidene diflouride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with a solution of 5% low-fat milk in tris-buffered saline and 0.05% Tween 20 for 1 h. Samples were incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized with chemiluminescence (Amersham Biosciences, Little Chalfont, UK). To control for equal loading, membranes were either stained with fast-green before blocking or stripped with a solution containing 50 mM glycine and 150 mM sodium chloride (pH 1.8) and reprobed with anti-Actin antibody. The following antibodies were used for Western blotting: Actin, ERK1, ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p38/MAPK, phospho-p38/MAPK (Cell Signaling Technology), active-MAPK (pTEpY) (Promega, Madison, WI, USA) and Flag (Sigma, St Louis, MO, USA).

STAT1 DNA-binding assay

DNA-binding activity of STAT1 was detected using the TransAM method following the manufacturer's protocols (Active Motif, Carlsbad, CA, USA). Briefly, active STAT1 complexes in nuclear extracts from FS-uninduced and FS-induced cells were captured by binding them to a consensus oligonucleotide that was immobilized in wells of a 96-well plate. A colorimetric reaction using anti-STAT1 primary antibody and HRP-conjugated secondary antibody was used to identify the bound protein. The colorimetric readout was quantified by spectrophotometry. Each sample and positive control was analyzed in triplicate.

Results

SET and vitamin D_3 stimulate the expression of different markers on U937 cells

We recently reported that tetracycline (tet)-regulatable overexpression of flag-tagged SET (FS) in U937 cells blocked G_1/S transition and stimulated differentiation as detected by upregulation of the cell surface marker CD11b.[21] Vitamin D_3 stimulates monocytic differentiation in U937 cells via vitamin D_3 receptor-dependent expression of surface markers CD11b and CD14.[23] To determine whether overexpression of SET mimics other cellular responses to vitamin D_3, we assessed CD14 expression in FS-induced (tet [−]) cells. A 4-day induction of FS stimulated the expression of CD11b in 76% of the cells but CD14 expression remained undetectable (Figure 1a), whereas addition of vitamin D_3 activated the expression of CD14 and CD11b in over 75% of the cells, regardless of whether the cells were maintained in the presence (FS-uninduced) or absence of tet (FS-induced) (Figure 1b). These results suggest that overexpression of SET induces differentiation of U937 cells along a pathway distinct from that induced by vitamin D_3.

Ectopic SET stimulates the expression of calcium-responsive genes and dendritic cell (DC) markers

To determine the comprehensive profile of genes whose expression is induced by SET overexpression, we performed microarray analyses. We identified 18 genes that were significantly upregulated (two-fold or greater increase in expression) and eight genes that were significantly downregulated (two-fold or greater decrease in expression). Interestingly, six out of 18 upregulated genes (*GTP-cyclohydroxylase I, protein kinase JNK2*, myocyte specific enhancer factor 2A (*MEF2A*), Ins(1,3,4,5)P4-binding protein (*IP4BP*), lymphocyte-specific protein 1 (*LSP1*) and type 3 inositol 1,4,5-trisphosphate receptor (*IP3R3*) (Table 1)) are directly or indirectly regulated by calcium.[24–29]

Expression levels of four of these six upregulated genes were confirmed using real-time RT-PCR analysis. At 8 and 24 h of FS induction, the level of *SET* expression in the cells in the absence of tet increased 2.9- and 5.5-fold, respectively, compared with that in parental U937 cells (Figure 2a). Although the microarray analysis showed that in these cells only *LSP1* was upregulated more than two-fold after 24 h, real-time RT-PCR analysis showed that *GTP-cyclohydroxylase I* (Figure 2b) and *IP4BP* were upregulated more than two-fold after 8 h, and *MEF2A* was upregulated to that level at 24 h (Table 1).

Given that SET stimulated the expression of Ca^{2+}-regulated genes and elevation of the intracellular calcium level is involved in DC differentiation of human promyelocytic cells, peripheral blood monocytes, and chronic myelogenous leukemia progenitor cells,[30,31] we monitored the appearance of the DC markers CD80, CD83, CD86, and HLA-DR after FS induction by using FACS analysis. In addition to CD11b, 60% of FS-induced cells expressed CD86 at day 4 (Figure 1a) but failed to express other DC markers (data not shown). Nonetheless, the cells acquired a DC-like morphology (Figure 1c). Thus, the marker analysis suggested that the induction of the DC differentiation program was only partial. Moreover, vitamin D_3 treatment overruled these effects of FS and drove the differentiation program toward

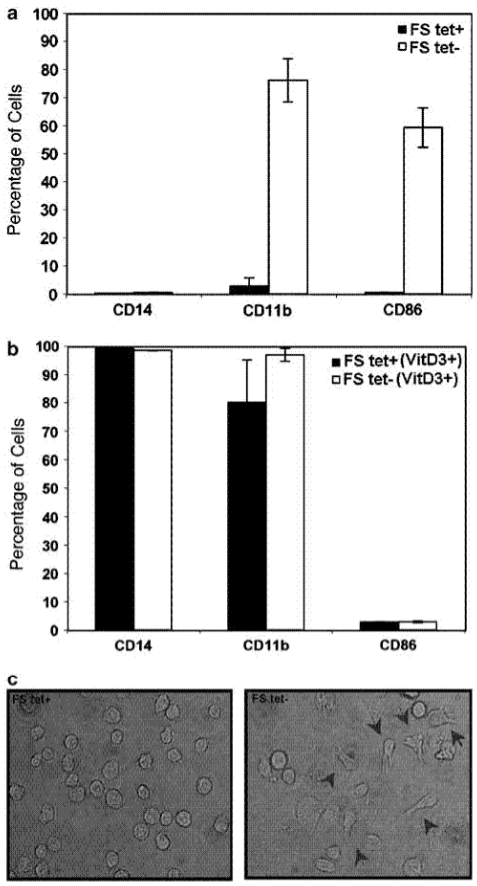

Figure 1 SET overexpression in U937 cells upregulates CD11b and CD86 and causes dendritic cell-like morphologic changes. (a) FS-expressing cells cultured for 4 days with (FS tet+) or without tetracycline (FS tet−) were analyzed by FACS for the expression of the cell surface markers CD14, CD11b and CD86. (b) In a parallel experiment, the same analysis was performed on cells cultured for 4 days with or without tetracycline in the presence of vitamin D_3. Data in (a) and (b) represent the mean (±s.d.) from three experiments. (c) Phase contrast images showing the morphological differences between FS-uninduced (FS tet+) and FS-induced (FS tet−) cells. The FS-induced cells show formation of pseudo-pods (arrows), whereas the FS-uninduced cells remain round.

a monocytic fate, as indicated by the expression of CD11b and CD14 but not CD86 in FS-induced cells (Figure 1b).

Inhibitors of calmodulin (CaM) and calmodulin-dependent kinase II (CaMKII) block SET-induced surface marker expression and morphologic changes

The Ca^{2+}/CaM complex regulates calcium signaling through activation of target kinases such as CaMKII and phosphatases such as calcineurin. To assess the role of Ca^{2+}-dependent signaling pathways in FS-stimulated DC differentiation, we treated FS-expressing cells with the Ca^{2+}/CaM antagonist W-7, the calcineurin inhibitor cyclosporin A (CsA), or the CaMKII inhibitor KN-93. Incubation with W-7 reduced the level of CD11b expression 2.6-fold and that of CD86 by 3.2 fold (Figure 3a) while KN-93 treatment decreased these levels two- and four-fold, respectively (Figure 3c). Both inhibitors also reversed the FS-induced morphologic changes (Figure 3b). In contrast, CsA only moderately altered the level of FS-stimulated expression of surface markers (Figure 3c). Together, these results indicated that Ca^{2+}/CaM and CaMKII activation is involved in FS-stimulated DC-like differentiation but probably there are also other contributing factors since the inhibition via Ca^{2+}/CaM and CaMKII was not complete.

The MAPK/ERK1/ERK2 pathway is required for SET-induced differentiation of U937 cells

Since CaMKII contributes to the FS-stimulated phenotype, it is noteworthy that CaMKII could provide a link between calcium signaling and MAPK/ERK activation through its regulation of the serine/threonine kinase RAF-1.[32 34] Also, SET overexpression in NIH3T3 cells activates the MAPK/ERK pathway by inhibiting PP2A.[17] In addition, both MAPK/ERK and p38/MAPK activation are involved in the maturation of human monocyte-derived DC.[35] Thus, we next explored whether MAPK/ERK and p38MAPK activation plays a role in SET-induced differentiation by using highly selective inhibitors of MEK1/ERK (PD98059) and p38/MAPK (SB202190), respectively. PD98059 (PD) treatment partially reversed SET-induced morphologic changes (Figure 3b) and decreased the expression of CD11b and CD86 by 1.7- and 4.4-fold, respectively, (Figure 3c). Western blot analysis supported results from the PD98059 experiment and showed transient higher levels of phospho-ERK1 at 12 h and a higher level of phospho-ERK2 at 12 and 14 h of FS induction (Figure 3d). In contrast, the p38/MAPK inhibitor SB202190 (SB) did not block SET-stimulated expression of CD11b or CD86 (Figure 3c), although SET overexpression transiently activated p38/MAPK at 12 h, as revealed by increased phospho-Thr180/Tyr182p38MAPK (Figure 3d). The transient increase in phosphorylation of ERK1 and p38/MAPK followed a transient increase in protein levels (Figure 3d) in FS-induced cells, while phospho-ERK2 initially also followed the increase in ERK2 protein but became dephosphorylated after 16 h of induction. The increase in FS background levels (compare all tet[+] lanes in Figure 3d) are due to necessary washing of the cells at the beginning of the experiment (not shown; see Materials and methods). None of the inhibitors affected FS expression or the viability of the cells (data not shown). These results suggest that in addition to Ca^{2+} signaling and activation of CaMKII, SET-induced DC-like differentiation also requires activation of MAPK/ERK1/2.

SET overexpression partially mimics but does not substitute interferon-γ signaling

The changes induced by ectopic SET expression mentioned above share similarities to those found in IFN-γ-treated cells.[36 41] Since the signal transducing activator of transcription-1 (STAT1) moderates IFN-γ-induced gene expression,[42] we analyzed whether ectopic expression of SET activates STAT1 DNA binding, which is a function of its phosphorylation at tyrosine

Table 1 Overexpression of SET upregulates several genes that are regulated by calcium

		Affymetrix			Real-time RT–PCR		
Gene name		*8*	*24*	*48*	*8*	*24*	*48*
37944-at	*GTP cyclohydrolase 1*	1.1	1.2	4	2.3	2	5.5
1238_at	*Protein kinase (JNK2)*	1.1	0.8	2.5	0.9	0.8	1.1
32862_at	Ins(1,3,4,5)P4-binding protein	1.4	1.4	2.5	2.1	1.6	3.5
36493_at	*Lymphocyte-specific protein 1 (LSP1)*	1.8	2.2	2.4	ND	ND	ND
41747_s_at	*Myocyte-specific enhancer factor 2A (MEF2A)*	1.3	1.6	2.3	1.2	2	2
182_at	*Type 3 inositol 1,4,5-trisphosphate receptor (IP3R3)*	1.7	1.1	2.3	ND	ND	ND

The level of expression of indicated genes in FS-induced (tet [−]) cells was compared with that in control U937 cells. Change after given periods (8, 24, 48 h) in culture are expressed as fold increases. ND, not determined.

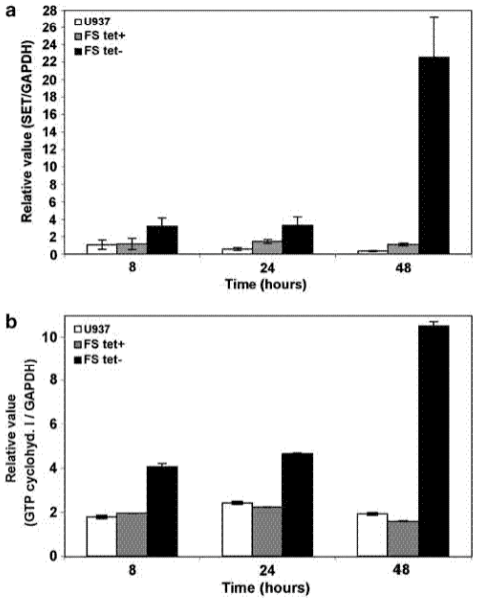

Figure 2 SET overexpression upregulates GTP cyclohydrolase I mRNA. Real time RT-PCR analysis performed to detect the levels of GTP cyclohydrolase I and SET mRNA using total RNA from FS-uninduced (FS tet +), FS-induced (FS tet−), and parental U937 cells at 8, 24 and 48 h of culture. All samples were analyzed in triplicate and histograms show the average amount of (a) SET mRNA and (b) GTP cyclohydrolase I. Levels of transcription were normalized by using GAPDH mRNA as an internal control. Data represent the mean of three experiments (±s.d.).

(Y701). Western blot analysis after 8, 11, 12, 14, 16 and 24 h of FS induction showed no Y701 phosphorylation of STAT1 and ELISA-based transcription factor-binding assays showed no difference in the DNA-binding activity of STAT1 after FS induction at the same time points (data not shown). Thus, SET induction does not fully mimic IFN-γ signaling.

Finally, we analyzed whether endogenous SET expression is affected by IFN-γ stimulation. Real-time RT-PCR analysis of *SET* mRNA in parental U937 cells showed that IFN-γ treatment upregulated *SET* transcription in a dose-dependent manner (Figure 4a). Timecourse experiments showed that the amount of *SET* mRNA returned to basal levels 2 h after IFN-γ treatment (Figure 4b). This suggests that *SET* expression might be subject to IFN-γ signaling.

Discussion

Ectopic expression of SET in U937 cells induces differentiation towards a DC phenotype and SET-stimulated acquisition of DC-like features requires calcium signaling, CaMKII and MAPK/ERK1/ERK2 activation. U937 cells that overexpress SET undergo a number of changes similar to cells treated with IFN-γ, that is, they exit the cell cycle;[36] upregulate the expression of *GTP cyclohydrolase I*,[37] CD11b[39] and CD86;[38] and activate the calcium signaling/CaMKII[40] and MAPK/ERK pathways,[41] but fail to activate STAT1, the main mediator of IFN-γ signaling.[42]

IFN-γ signaling orchestrates different cellular programs, including antimicrobial and antitumor mechanisms as well as cell proliferation and differentiation, through transcriptional regulation of target genes.[43] In response to IFN-γ, STAT1 becomes phosphorylated on Y701, and STAT1 dimers migrate into the nucleus and activate the expression of genes that contain STAT1-binding sites.[44] Given the lack of Y701 phosphorylation and the failure to induce STAT1 DNA binding, we conclude that, despite the many similarities, SET induction cannot fully mimic IFN-γ stimulation. Therefore, the upregulation of *CD86* expression, a direct transcriptional target of STAT1,[38] cannot be mediated by STAT1 in our SET-overexpressing cells. However, *CD86* is also a direct target of NFκB,[38] a transcription factor that is activated by Ca^{2+} flux[45], and therefore, a good candidate to upregulate this gene. Active NFκB is also necessary for *CD11b* expression.[46] Despite the differences in STAT1 activation after IFN-γ treatment or SET overexpression, it is reasonable to speculate that SET induction is part of the IFN-γ response, given the transient upregulation of *SET* after treatment of U937 cells with IFN-γ (Figure 4b).

The well-known inhibitory effect of *SET* on PP2A[10,12,14] should at least in part account for the observed activation of MAPK/ERK and CaMKII since PP2A negatively affects MAPK/ERK and CaMKII activation by dephosphorylation.[17,47] Therefore, the differentiation response of FS-induced U937 cells could be caused by inhibition of PP2A and activated calcium signaling which is induced via many stimuli such as stress or receptor activation.[48]

Currently, we do not know how SET expression activates calcium signaling. However, an integral role of SET in the stress response, as indicated by transient activation of p38/MAPK, may provide some clues, despite the observation that SB202190, the inhibitor of p38/MAPK, had no effect on differentiation. Also, there are many similarities between stress responses and the

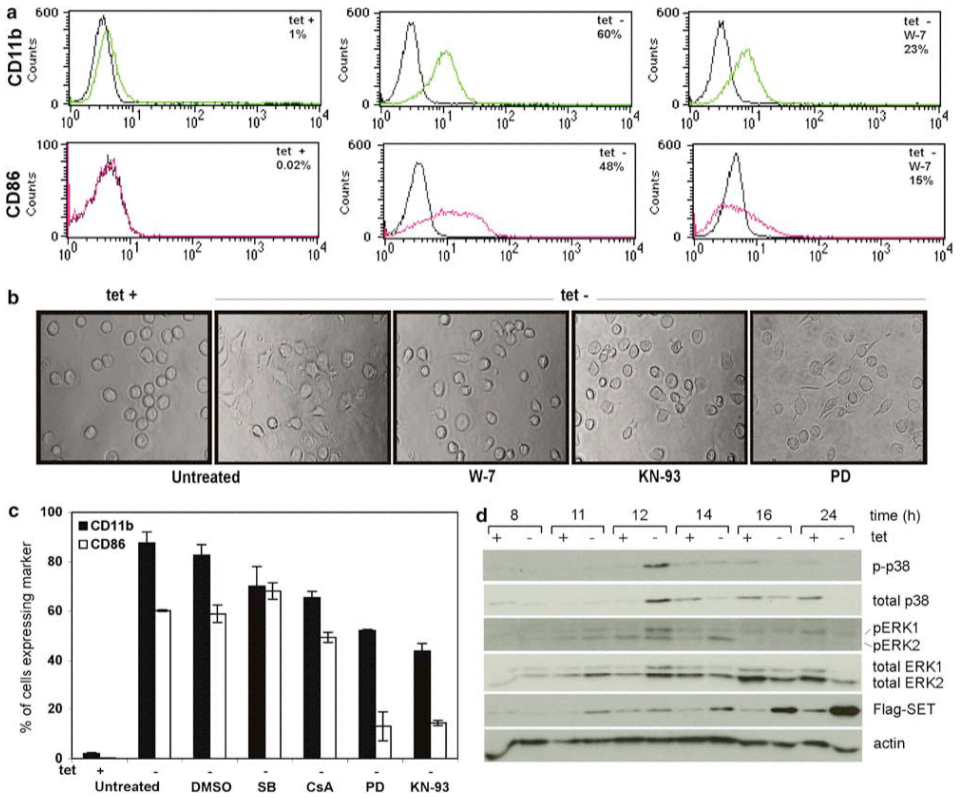

Figure 3 Inhibitors of, Ca^{2+}/CaM, CaMKII or MAPK/ERK pathways inhibit FS-induced immunophenotypic and morphologic changes. (a) Ca^{2+}/CaM inhibitor W-7 blocked FS-induced expression of CD11b (upper right panel, green) and CD 86 (lower right panel, red). The upper and lower left panels show expression of these markers in FS-uninduced (tet+) cells and middle panels in FS-induced (tet−) cells in absence of inhibitors. The percentage of cells expressing the marker is indicated in each panel. (b) Morphologic changes induced by FS overexpression were reversed by W-7, KN-93 (CaMKII inhibitor) and PD98059 ([PD] MEK1/ERK1,2 inhibitor). (c) PD98059 (PD) and KN-93 substantially reduced FS-induced expression of CD11b and CD86, whereas cyclosporine A (CsA) and SB202190 (SB), a specific inhibitor of p38, only moderately inhibited the expression of the markers. Data represent the average (±s.d.) of triplicate experiments. (d) The total amount and the active p38/MAPK (p-p38) and MAPK/ERK1/ERK2 (pERK1 and pERK2) were determined by using Western blot analysis at indicated time points in FS expressing cells that were cultured with (tet+) or without (tet−) tetracycline. FS expression was detected with anti-Flag antibody, and equal loading was verified by anti-Actin antibody.

response to IFN-γ treatment such as induction of calcium flux and MAPK/ERK activation.[41]

In FS-induced U937 cells, the activation of p38 and ERK/MAPKs are triggered soon after SET induction, when SET is only moderately upregulated. In fact, high levels of SET appear to inhibit p38 and ERK/MAPKs phosphorylation as well as protein levels (Figure 3d). This finding supports the physiological significance of the response and indicates that it is not simply a side effect of massive overexpression of SET. Also during IFN-γ stimulation, endogenous *SET* is induced to only a moderate increase. A slightly confounding observation is that the level of FS also increases in cells that remain under tet repression. This is caused by the obligatory washing of the cells at the beginning of the experiment to ensure that both tet[+] and tet[−] cells were handled in the same way. We believe that this upregulation remains below a level that induces differentiation and phosphorylation of ERK1,2 and p38/MAPK, whereas FS induction in tet[−] cells reaches a level high enough to induce these changes. Indeed, we never detected any signs of differentiation in tet[+] cells.

Recently, it was reported that SET downregulation rather than upregulation was associated with the induction of differentiation in the bladder carcinoma cell line TSU-Pr1.[49] Although these data seem contradictory to our findings the two data sets are difficult to compare because the authors did not assess the effect of SET overexpression in TSU-Pr1 cells. In addition, the effects of SET might be different in different cell types.

In addition to calcium signaling-mediated transcriptional effects, SET overexpression might also affect the activity of the HuR complex of which it is a component.[9] HuR regulates

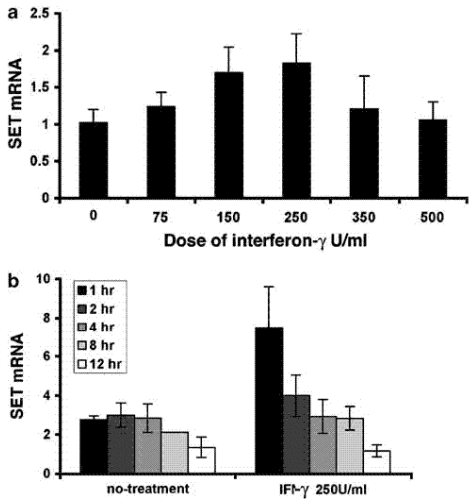

Figure 4 Interferon-γ treatment of U937 cells transiently induces expression of endogenous *SET*. (a) The amount of *SET* mRNA in RNA samples from U937 cells treated for 1 h with the indicated concentrations of human recombinant INF-γ (InterMune, Inc., Brisbane, CA) was determined by real-time RT-PCR. (b) Time course of *SET* mRNA expression, as determined by real-time RT-PCR analysis in untreated cells and during treatment with the optimum dose of INF-γ (250 U/ml). Levels of transcription in (a) and (b) were normalized by using *GAPDH* mRNA as an internal control.

stabilization of mRNAs containing AU-rich elements (ARE) in their 3′UTRs,[50,51] which targets them for rapid degradation.[52,53] Elevated SET expression might, therefore, increase the activity of the HuR complex and inhibit degradation of short-lived mRNAs, which may encode proteins important for differentiation. Future studies will address this possibility. To assess this and the role of SET in stress responses, we are beginning to analyze the effects of a *SET*-knockout mutation in mice.

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Chapter 4

SET-CAN, the product of the t(9;9) in acute undifferentiated leukemia, causes expansion of early hematopoietic progenitors and hyperproliferation of stomach mucosa in transgenic mice

Submitted

SET-CAN, THE PRODUCT OF THE t(9;9) IN ACUTE UNDIFFERENTIATED LEUKEMIA, CAUSES EXPANSION OF EARLY HEMATOPOIETIC PROGENITORS AND HYPERPROLIFERATION OF STOMACH MUCOSA IN TRANSGENIC MICE.

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SUMMARY

CAN/NUP214, an essential nucleoporin, is one of the targets of recurrent leukemia-specific chromosome translocations and fuses with alternative partners involving *DEK*, *ABL1* and *SET*. Although *DEK-CAN* and *CAN-ABL1* have been associated with acute myeloid leukemia (AML) and T-cell acute lymphoblastic leukemia (T-ALL), respectively, the *SET-CAN* fusion gene was identified in a patient with acute undifferentiated leukemia (AUL). Recently we have shown that the ectopic expression of SET-CAN partially inhibited vitamin-D_3 induced differentiation of the human promonocytic cell line U937,

while ectopic SET expression stimulated their differentiation. In the present study, we addressed the leukemogenic potential of SET-CAN when expressed in the hematopoietic system of a transgenic mouse model. Even though SET-CAN mice developed hematopoietic abnormalities, including expansion of early progenitor cells and partial depletion of lymphocytes, they were not leukemia-prone and did not show shortening of disease latency after retroviral tagging. Surprisingly, all SET-CAN mice developed spontaneous hyperplasia of the stomach mucosa. Our results suggest that the presence of SET-CAN in AUL might determine the primitive phenotype of the hematopoietic disease, possibly via a block in differentiation, while secondary genetic lesions, not present in our mouse model, are necessary for disease development.

INTRODUCTION

One of the characteristics of leukemias is the frequent presence of recurrent chromosome translocations. These often lead to the formation of chimeric genes encoding fusion proteins, which are generated by fusion via breakpoints in introns of the fusion partners. Many of these translocations result in the expression of altered transcription factors that are thought to induce aberrant expression of crucial target genes and thereby contribute to the dysregulated growth of hematopoietic progenitors (26). Translocations are associated with specific leukemia subtypes suggesting that the *in vivo* oncogenicity of these genes is not only restricted to the hematopoietic system but also shows high specificity within the hematopoietic system.

The translocation t(6;9)(p23;q34) is the hallmark of a specific subtype of acute myeloid leukemia M2/M4, rarely M1, and characterized by a poor prognosis and a young age of onset(27, 31). On chromosome 9, breakpoints occur in a specific intron, icb-9, of the nucleoporin *CAN/NUP214,* a protein essential for nucleocytoplasmic transport and cell cycle progression (34, 36).

CAN/NUP214 is also targeted by the t(9;9)(q32;q34) in a case of acute undifferentiated leukemia (AUL) forming a fusion gene with *SET*. The chimeric gene consists of nearly the entire *SET* coding sequence fused to the C-terminal two-third of *CAN/NUP214* and encodes a SET-CAN fusion protein of 155kDa (37). The *SET* gene, (also known as template-activating factor I beta [TAF-Iβ]), encodes a highly conserved, ubiquitously expressed, mainly nuclear phosphoprotein(1). SET physically interacts with several protein complexes, which suggests that it has diverse functions including Granzyme A–induced apoptosis(4, 10), chromosome remodeling(24, 29), transcriptional regulation(9), mRNA stabilization(6) cell cycle regulation(7) and differentiation(20). In addition to its participation in the SET-CAN fusion in AUL, SET also associates with the AT-hook region of MLL, a protein that is frequently translocated in acute leukemias, and forms a PP2A-SET-MLL complex, which suggests an important role for SET in MLL-mediated transcription and possibly chromatin maintenance (2, 30).

Recently we have shown that ectopic expression of SET-CAN in human U937 promonocytic cells blocks their vitamin D_3-induced differentiation whereas SET overexpression in these cells induces their differentiation(20). To further explore the causal role of SET-CAN in leukemogenesis, we present here a transgenic mice model, which expresses SET-CAN in hematopoietic progenitor cells.

MATERIALS AND METHODS

Generation of SET-CAN transgenic mice

A SET-CAN minigene was constructed by placing a 150 bp hybrid intron consisting of the splice donor and 5' sequences of intron 7-8 of *SET* and 3' sequences and splice acceptor of intron 17-18 of *CAN/NUP214* between the *SET* and *CAN/NUP214* sequences in the fusion cDNA (Fig. 1A). This minigene fragment was cloned into the *Cla*I site of the first exon of the mouse

Ly-6E.1 expression cassette, which directs expression of the transgene to early hematopoietic cells (23). Transgenic FVB NJ mice were generated using microinjection of gel-purified linear Ly-6E.1/SET-CAN fragment free of plasmid sequences into the pronucleus of fertilized eggs. Four founder mice were obtained (2945, 2956, 2969, and 2971). Initially, founders and their progeny were genotyped by Southern blotting of tail DNA digested with BamHI, fractionated on a 0.8% agarose gel, transferred to Hybond nylon membrane (Amersham Life Science), and hybridized with ^{32}P labeled 4 kb 3' *CAN/NUP214* cDNA probe. Later SET-CAN mice were genotyped by PCR analysis using (*SET*-5'SE) 5'GACCATTCTGATGCAGGTC3' and (*CAN/NUP214*-3'AS) 5'GATGTGAATGATGTTCTAGACTTG primers. All lines were kept as heterozygotes whereas line 2956 was lost at the F1 generation. Due to prohibitive costs, tumorigenesis experiments were done with the 2969 line only.

Fluorescence-activated cell sorting (FACS) analysis

Bone marrow (BM) cells isolated from the femur and tibia of mice were stained with directly conjugated fluorescent antibodies to CD4, CD8, B220, Mac-1, Gr-1, Ter 119, Sca-1, Thy 1.1, c-Kit, CD34, CD3, CD43 (Pharmingen, San Diego, CA) and analyzed on a FACS Calibur or a LSRII (BD Immunocytometry Systems) as described(8).

Hoechst 33342 SP cell analysis

Hoechst 33342 staining and analysis of SP cells were done as described (15). In short, BM cells resuspended at 1x10^6 cells/ml in DMEM plus 10 mM HEPES and 2% FBS. Cells were incubated at 37^{o}C for 90 minutes followed by addition of 5 mg/ml Hoechst 33342 (Fisher Scientific, Pittsburgh, PA). Cells were centrifuged and resuspended in ice cold HBSS plus 10 mM HEPES and 2% FBS at a concentration of 10^7 cells/ml. For flow cytometric

analysis the FACS Vantage flow cytometer (Beckon Dickinson, San Jose, CA) was configured for dual emission wavelength analysis(15). Cells were gated based on forward and side light scatter to exclude debris. The SP cell gate was defined based on wild type FVB mouse BM.

RT-PCR and RPA analysis of BM RNA of SET-CAN transgenic and wild type FVB mice

BM RNA of SET-CAN mice and wild type mice were reverse transcribed using random hexamer primers. A SET-CAN cDNA fragment containing the translocation breakpoint was amplified using (*SET*-5'SE) 5'GACCATTCTGATGCAGGTC3' and (*CAN/NUP214*-3'AS) 5'GATGTGAATGATGTTCTAGACTTG primers, producing a 294 bp fragment. RPA analysis was performed with a *SET-CAN* cDNA plasmid containing 151 bp of SET sequence fused to 85 bp of CAN/NUP214 sequence cloned in the EcoRI site of pBluescript. After linearizing the plasmid with *Cla*I ^{32}P-labeled c-RNA consisting of 57nt of *pBluescript*, 151 nt *SET* and 85 nt of *CAN/NUP214* was produced using T7 polymerase following the manufacturer`s protocol (Ambion, Inc.). After hybridization with RNA and digestion with RnaseA, 3 protected fragments were generated: 236 bp (*SET-CAN*), 151 bp (*Set*) and 85 bp (*Can/Nup214*). As a positive control for the reaction we used RNA of HeLa cells transiently transfected with a pCS-TOP-HA-tagged *SET-CAN* expression construct(12) and as a negative control wild type FVB BM RNA and yeast tRNA. Quantification of the bands was performed using The Image Processing Tool Kit software (Reindeer Graphics, Inc., NC).

Colony Assays

BM cells were plated in methylcellulose (StemCell Technologies, Vancouver, Canada) with the following growth factors: 3 U/ml erythropoietin (Epo), 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 ng/ml

interleukin-3 (IL-3), 50 ng/ml stem cell factor (SCF), and 50 ng/ml Flt-3 ligand. After 14 days of incubation at 37 ^{o}C in 5% CO_2, colonies were counted. The pre-B lymphoid progenitor assays were done in the same manner using IL-7 as the only growth factor.

Day 12 CFU-S assays

BM cells were harvested from the femur of adult SET-CAN mice and wild type FVB NJ littermates. Recipient FVB NJ mice were lethally irradiated (800-850 cGy, x-ray) and then injected with 10^5 unfractionated marrow cells per mouse. Irradiated mice that were not transplanted served as controls in each experiment for endogenous CFU-S and they showed no colonies. Twelve days after injection animals were sacrificed, and the number of macroscopic colonies on the spleen were evaluated after fixation in Telleyesniczky's solution.

Statistical Analysis

We used the method of one-, two-, and three-way Multivariate Analysis of Variance to analyze data including simultaneous measurements on the fourteen different blood count values for each of the 3 transgenic lines and wild-type mice.

Retroviral tagging of SET-CAN and wild type FVB mice

MOL4070LTR virus was produced as previously described(39). Newborn pups of heterozygous *SET-CAN* x wild type FVB crosses were injected subcutaneously with 0.1 ml viral supernatant (~10^5 pfu). The SET-CAN mice in these litters were identified by genotyping and mice were maintained until moribund. Tissues were harvested, fixed in 10% formalin, processed for paraffin sectioning and stained with H&E(8). Malignant cells were detected by

immunohistochemical staining with CD3, TdT, (DAKO, Carpinteria, CA) and CD45, (Pharmingen, San Diego, CA) as described previously(18).

Immunohistochemistry

Cryosections of 9 months-old wild type FVB and SET-CAN mice stomach were stained with anti-CAN(11), anti Ki67 (Vector Laboratories, Burlingame, CA) and anti b-catenin (Sigma, St. Louis, MO) antibodies as described previously(22). To detect apoptotic cells in stomach paraffin sections, TUNEL assays were performed using the ApopTag® kit (Chemicon International, Temecula,CA) following the manufacturer's protocol.

RESULTS

Generation of SET-CAN transgenic mice

Because SET-CAN was found in a patient with AUL, we reasoned that targeting expression of the *SET-CAN* transgene to primitive mouse hematopoietic cells might faithfully mimic the pathogenesis of the human disease. We generated transgenic FVB mice in which SET-CAN is expressed under the control of the Ly-6E.1 minigene, an expression cassette that directs expression of transgenes to primitive mouse hematopoietic progenitors (23) (Fig. 1A). We obtained 4 independent transgenic lines, 2945, 2956, 2969, and 2971, respectively.

Analysis of the expression of SET-CAN in transgenic lines

Expression of the *SET-CAN* transgene was detected by RT-PCR of RNA isolated from the bone marrow (BM) of these 4 strains (Fig. 1B). To confirm these results and to quantify expression of *SET-CAN* we performed RNA protection assays (RPA) on the same BM samples. As a probe we used radio labeled *SET-CAN* cRNA, which detects *SET-CAN* RNA, but also endogenous mouse *Set* and *Can/Nup214* RNA. RNA of BM from wild type

mice and RNA of HeLa cells transiently transfected with a *SET-CAN* expression plasmid were used as a negative and positive control, respectively (Fig. 1C).

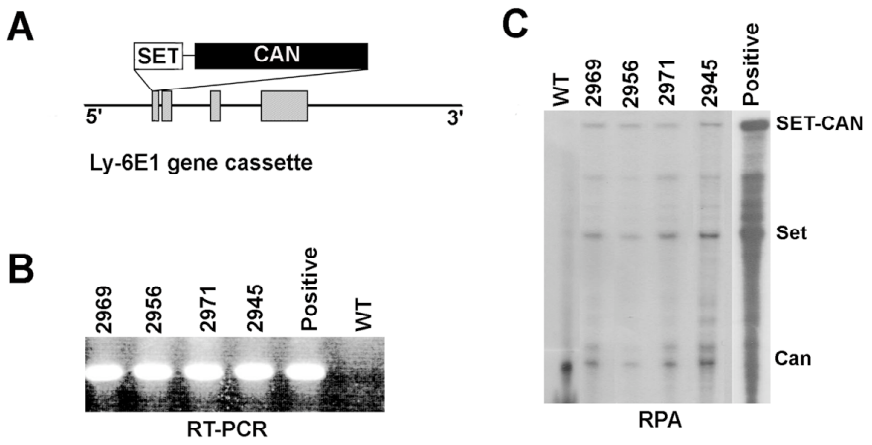

Figure 1. Generation of Ly-6E.1-*SET-CAN* constructs and characterization of transgenic mouse lines. (A) The *SET-CAN* mini gene containing a small artificial intron between *SET* and *CAN/NUP214* cDNA sequences was cloned into a *Cla*I site of the first exon of the mouse Ly-6E.1 expression cassette. (B) Bone marrow (BM) RNA of each of the *SET-CAN* transgenic lines (2969, 2956, 2971 and 2945) was analyzed for *SET-CAN* mRNA expression using RT-PCR. RNA of HeLa cells transiently transfected with a pSCTOP-*SET-CAN* expression construct and BM RNA of wild type FVB mice were used as a positive (Positive) and negative (WT) controls, respectively. (C) RPA analysis was performed with a *SET-CAN* cRNA probe containing 56 nt of pBluescript sequence, 151 nt of *SET* sequence fused to 85 nt of *CAN/NUP214* sequence. After hybridization with BM RNA and digestion with RNaseA, 3 protected fragments were generated in SET-CAN transgenic mice: *SET-CAN* (236 bp), *Set* (151 bp) and *Can/Nup214* (85 bp). Quantification of the bands was done with the Image Processing Tool Kit software (Reindeer Graphics).

Using an image processing tool kit (Reindeer Graphics, Inc., NC), we determined that the expression of *SET-CAN* in mouse line 2956 was the highest (equivalent to 100% of endogenous *Set* expression, which is used as an internal standard), whereas expression in the other 3 lines (2945, 2969, 2971) was about equal (equivalent to 62%-74% of endogenous *Set* expression). We suspect that the higher expression in 2956 line might have been the reason that the F1 generation stopped breeding and the line could not be propagated further. Hence, besides peripheral blood counts no data are available describing the hematopoietic system of the 2956 line.

SET-CAN transgenic mice have a reduced number of peripheral blood lymphocytes

First we analyzed the peripheral blood counts of lines 2945, 2969 and 2971 and compared these with that of wild type mice. The differential counts of approximately 25 individual mice of each line were obtained and subjected to statistical analysis. All three lines showed a significant decrease (1.7 to 2.3-fold) in the number of lymphocytes (P=0.0001), and a slight increase in neutrophils, which in lines 2945 and 2969 was not statistically significant. There was an overall but moderate decrease in total white blood cell counts in all three lines, which were significant for lines 2945 and 2969 (P=0.002). SET-CAN mice showed normal red blood cell counts, hematocrit, and platelet counts (Table 1).

SET-CAN transgenic BM contains a higher number of primitive hematopoietic cells

We next determined whether the disparity in differential peripheral blood counts of transgenic mice were also reflected in the composition of their BM. We isolated BM of 10 3-6 month-old transgenic (all 3 lines) and wild type mice and determined the composition of the BM using FACS analysis of cell

surface markers. Although there was considerable variability in the percentages of cells positive for each marker among individual mice of the same genotype some consistent differences emerged, including a 13% to 44% reduction in $B220^+$ cells, and an 2-3.4 fold increase in the numbers of more mature (Sca1+) and primitive progenitor cells ($Sca1^+/c\text{-}Kit^+$)(Table 2). We next determined if this increase in primitive progenitors was also reflected in the size of the side-population (SP) fraction after dual color Hoechst 33342 staining and FACS analysis. The SP fraction is highly enriched in primitive, long-term repopulating cells as has been shown by transplantation of SP cells into lethally irradiated recipients(15). This analysis was done on 5 individual animals as well as on 3 individual pools of 3 BM samples of each of the strains. In agreement with the cell surface marker analysis, the average size of the SP fraction in transgenic animals was 2-3 fold larger than that in wild type mice (Fig. 2).

Table 1. Peripheral blood counts of 3 SET-CAN transgenic lines and FVB control mice

Cell Type	SC line 2945 (n = 25) [a]	SC line 2969 (n = 27) [a]	SC Line 2971 (n = 24) [a]	FVB (n = 28) [a]
Red blood cells	7,060,000	8,332,962	8,051.666	7,787,500
Hematocrit (%)	40,3	46,1	44,0	44,3
Platelets	399,040	507,481	452,916	547,821
White blood cells	4,859*	5,073*	6,234	7,541
Lymphocytes	2,650*	3,529*	3,571*	6,094
Neutrophils	1,796	1,264	2,206*	1,097

[a] mean/ul

* P<0.002 *v* wild-type FVB and SETCAN transgenic

Table 2. FACS analysis of BM samples of SET-CAN and wild-type FVB mice

Gated %	WT	2945	2969	2971
CD4	1.71	1.99	2.55	2.18
CD8	1	1.03	0.9	1.45
B220	36.13	20.27	29.45	31.43
Mac-1	53.08	59.47	57.77	51.04
Gr-1	49.74	61.42	56.45	52.14
Thy1.1	10.82	13.98	9.35	11.56
Ter119	5.36	5.74	3.4	4.12
Sca-1	7.84	26.53	22.77	19.37
c-KIT	3.13	6.65	8.03	5.9
CD34	18.79	20.17	20.17	21.74
Hoechst 33342 (SP)	0.33	0.86	0.68	0.51

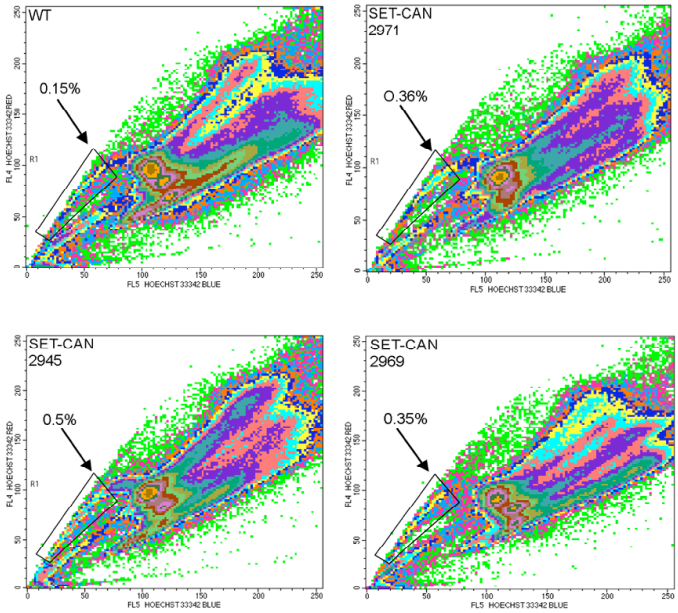

Figure 2. FACS profile of side-population (SP) cells in FVB and SET-CAN transgenic mice. BM cells of wild type FVB and SET-CAN transgenic mice were stained with Hoechst-33342 and sorted by FACS for Hoechst 33342 blue and red fluorescence. SP fractions of BM cells are shown in the control wild type (WT, 015%) and three transgenic SET-CAN lines (2971, 0.36%; 2945, 0.5%; 2969, 0.35%). The SET-CAN transgenic mice have an increased SP fraction.

SET-CAN bone marrow shows a slight increase in colony forming activity in methylcellulose cultures

We performed methylcellulose cultures to test whether this apparent increase in primitive cells would also produce increased numbers of colonies under myeloid culture conditions. We counted colonies after 2 weeks of culture and in total we analyzed individual BM and pools of BM of 26 mice of each strain in 12 independent experiments. BM of transgenic lines produced roughly 1.3-1.7-fold as many colonies as wild type BM (Fig. 3A). Upon replating 10^3 cells from the first methylcellulose culture into a subsequent culture, the difference in the number of colonies between normal and transgenic BM was lost and additional replating into subsequent methylcellulose cultures showed no difference in the number of colonies (not shown). This suggested that an increased number of committed progenitors was present in the transgenic BM but they did not have an increased self-renewal capacity in methylcellulose cultures. We also tested which growth factors caused this difference in methylcellulose colony numbers and determined that both IL-3 and GM-CSF recapitulated the effects seen with a full growth factor complement (not shown).

SET-CAN bone marrow does not show increased colony forming activity in long-term culture initiating cell (LTC-IC) assays

To further investigate the increased colony forming activity of SET-CAN transgenic BM in methylcellulose cultures, we also determined the number of LTC-IC present in transgenic BM compared with those in wild type BM. LTC-IC cultures were performed for 4,5, and 6 weeks and the number of colony forming cells present at each time point were assayed in methylcellulose cultures. No differences in colony numbers were detected between the BM of different transgenic lines and wild type mice (not shown). Thus, the LTC-IC

cultures did not recapitulate the situation in the BM, possibly due to the lack of factors stimulating the growth of very primitive progenitors.

SET-CAN bone marrow shows a very moderate increased activity in day 12 spleen colony forming unit (CFU-S) assays

As an alternative for measuring the number of early multilineage progenitors, we performed day 12 CFU-S assays with SET-CAN and wild type BM samples. In 3 separate experiments using pools of BM from 10 mice of 2 transgenic lines and wild type, we found a small increase in the number of CFU-S in mice transplanted with SET-CAN BM compared to those transplanted with wild type BM (Fig. 3B). This small increase is consistent with the results of the MC assays.

SET-CAN bone marrow shows a reduced capacity to form Pre-B cell colonies in methylcellulose

Because the numbers of $B220^+$ cells were reduced in SET-CAN BM (Table 2) and the number of lymphocytes were reduced in the peripheral blood of SET-CAN mice (Table 1), we tested whether the BM contained fewer pre-B-cell colony forming units than wild type BM by plating the cells in methylcellulose cultures supplemented with only IL-7. We analyzed pools of BM cells of 8 mice each of wild type, 2945 and 2969 transgenic mice in 5 independent experiments (Fig. 3C), which established that the number of pre-B CFU was reduced at least 2-fold in these mice. This suggested a partial defect at an early step of lymphoid differentiation in SET-CAN BM.

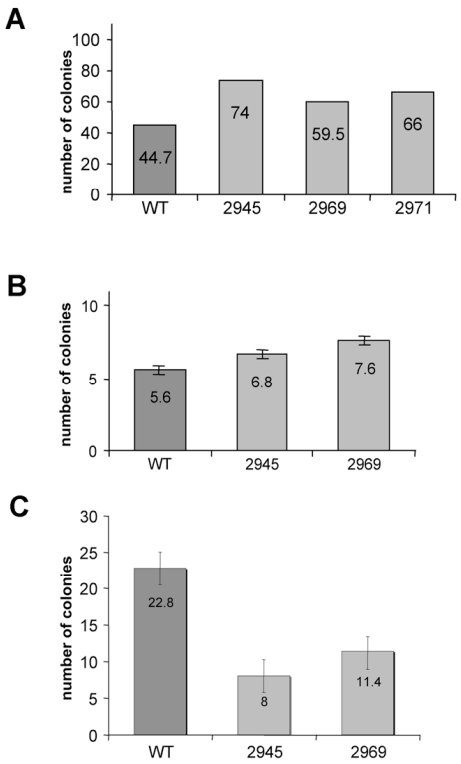

Figure 3. Effects of SET-CAN on BM colony formation in methylcellulose and CFU-S assays. (A) BM cells (10^3) from wild type FVB (WT) and three transgenic SET-CAN lines (2971, 2945 and 2969) were plated into methylcellulose cultures in the presence of Epo, IL-3, SCF, GM-SCF and FLT-3 ligand and colonies were counted after 14 days of culture. The data depict one representative experiment. (B) BM cells of FVB mice (WT) and two SET-CAN transgenic lines were transplanted into lethally irradiated FVB recipients. 12 days after transplantation macroscopic colonies on the spleen of recipient mice (3 per group) were counted. Each bar shows the average number of colonies in each group, error bars show the variation between experiments. (C) Methylcellulose assays of BM of FVB (WT) and SET-CAN mice (2945, 2969) were performed in the presence of IL-7 only to score for pre-B cell colonies. Each bar shows the mean value of triplicate experiments. Error bars show the variation between experiments.

Retroviral tagging of SET-CAN transgenic mice

Given the increase in primitive progenitors in SET-CAN transgenic mice, but their failure to generate hematopoietic malignancy, we tested whether they were more prone to develop leukemia upon retroviral tagging(35). Newborn wild type (n=12) and 2969 SET-CAN (n=10) animals were infected with MOL4070ALTR hybrid virus, which was shown to produce both myeloid and lymphoid malignancies in FVB mice(39). Mice were sacrificed when moribund, but there was no acceleration of disease onset in SET-CAN transgenic mice as compared to wild type virus-infected mice (Fig. 4A). Ly-6E.1 transgenes were reported to express in BM, kidney and liver(23). Using RT-PCR we ensured that SET-CAN was expressed in these tissues of the transgenic animals used in this experiment, (Fig 4B). The most prominent disease in both wild type and SET-CAN mice was T cell lymphoma (Table 3). Malignant cells invaded the thymus, lungs, kidney, liver, lymph nodes and intestines. As an example, a lymph node of a SET-CAN animal is shown in which all cells stained positive for the T cell marker CD3 and negative for the B cell marker CD45 (Fig 4C). However, 4 of 10 male SET-CAN animals showed lymphoid hyperplasia rather than T cell lymphoma, and suffered from glomerulonephritis. One SET-CAN mouse showed severe multi-focal glomerulonephritis and global glomerulopathy without T cell lymphoma or lymphoid hyperplasia (Fig 4D, Table 3). The kidney damage might have been severe enough to cause the death of this animal. We conclude that expression of SET-CAN did not accelerate leukemogenesis after retroviral tagging.

Since the 4 males of the 10 virus infected SET-CAN animals developed kidney abnormalities we also analyzed the kidney of age-matched, 9 months old uninfected SET-CAN mice (n=9). Again similar kidney lesions were detected in the 2 males but not the 7 females in this group. This suggested that the kidney lesions are gender-specific. In addition, 8 out of 9

SET-CAN mice had developed mucosal hyperplasia of the stomach and moderate hyperplasia of the colonic mucosa, whereas none of the age-matched wild type littermates (n=6) showed such lesions. One representative example of stomach hyperplasia is shown in FIG. 5A, B.

Table 3. Disease incidence and phenotype of wild type and SET-CAN mice infected with MOL4070A retrovirus.

Mouse ID	Diagnosis	Incidence*
Wild Type	T-cell lymphoma	9/12
	B- cell lymphoma	1/12
	Myeloproliferation	2/12
SET-CAN	T-cell lymphoma	4/10
	B- cell lymphoma	1/10
	Lymphoid proliferation 4/10	
	Kidney lesions only	1/10

* Number of mice with disease / total number of MOL4070A infected mice.

Immunohistological analysis with a C-terminal CAN antibody showed bright staining of nuclear SET-CAN in the hyperplastic mucosa, whereas mucosa of wild type mice showed weak mainly nuclear envelope and cytoplasmic staining of endogenous CAN with this antibody (FIG 5A). To assess whether there was increased proliferation in the hyperplastic SET-CAN mucosa, we stained stomach sections of SET-CAN and age-matched wild type littermates with an antibody against the S-phase-specific antigen Ki67. This showed increased numbers of $Ki67^+$ cells in the SET-CAN mucosa (FIG. 5B), in agreement with increased proliferation.

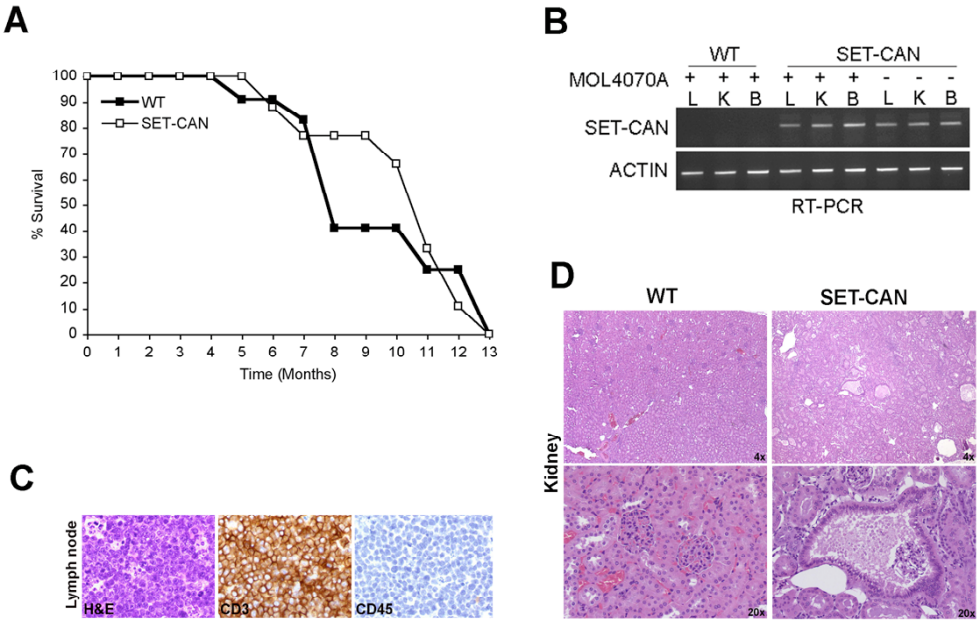

Figure 4. SET-CAN expression does not accelerate or increase the leukemia incidence in MOL4070A virus infected FVB mice. (A) Survival curve of wild type (WT, black squares) and SET-CAN mice (open squares) after infection with MOL4070A retrovirus. Both groups of mice died between 5 and 13 months after virus infection. (B) SET-CAN expression is shown using RT-PCR in the liver [L], kidney [K] and BM [B] of transgenic animals infected (MOL4070A +) or not infected (MOL4070A -) with retrovirus. Actin expression was used as an internal control for cDNA synthesis and the PCR reaction. RNA samples of wild type FVB mice were used as a negative control. (C) Morphology and immunohistochemical features of cells in lymph node sections stained with H&E, CD3 antibody and CD45 antibody of a SET-CAN mouse that developed T cell lymphoma. Neoplastic cells invaded the lymph nodes and stained positive for CD3 but negative for CD45. (D) Low (40x) and high power (200x) view of H&E stained sections of kidney of one SET-CAN mouse that developed global glomerulopathy after retrovirus infection.

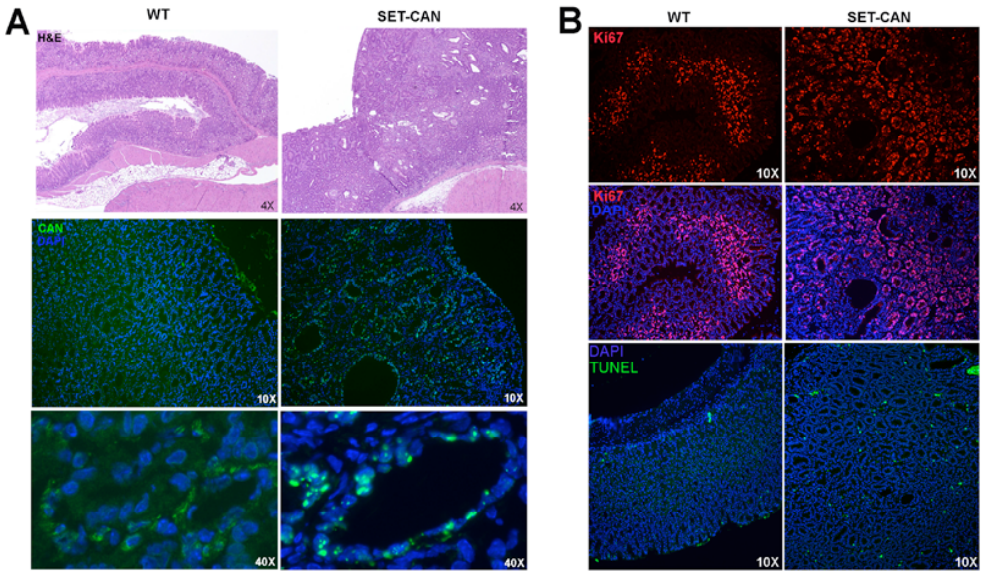

Figure 5. SET-CAN mice develop hyperplasia of the stomach mucosa. (A) Upper panels: H&E stained tissue paraffin sections of stomach of an 9 months-old wild type FVB (WT, left panels) and SET-CAN transgenic mouse (SET-CAN, right panels), showing hyperplasia of the stomach mucosa in the SET-CAN transgenic animal. Middle and lower panels: Immunofluorescence analysis of Can/Nup214 and SET-CAN expression in cryosections of the same mice with a C-terminal CAN/NUP214 antibody. Cells in the SET-CAN section show a bright punctate staining (green) in the nucleus, whereas sections of the wild type mouse show staining of endogenous Can/Nup214 (green) at the nuclear envelope and in the cytoplasm. The gain in brightness of the micrographs on the left is 5-fold higher than that of the micrographs on the right. The nuclei in these sections were stained with DAPI (blue). (B) Upper panels: Cryosections of the same mice shown in A stained with a Ki67 antibody (red). SET-CAN cells show increased numbers of $Ki67^{+}$ cells. Middle panels: Same as upper panels but with nuclei stained with DAPI (blue). Lower panels: Paraffin sections of the same mice showing TUNEL staining (green) with a DAPI counter stain (blue). SET-CAN mucosa shows an increased number of apoptotic cells.

Because the mucosa is hyperplastic but not transformed we also analyzed the apoptotic index using TUNEL staining in SET-CAN and wild type stomach sections. This revealed a significantly increased number of apoptotic cells in the hyperplastic SET-CAN mucosa.

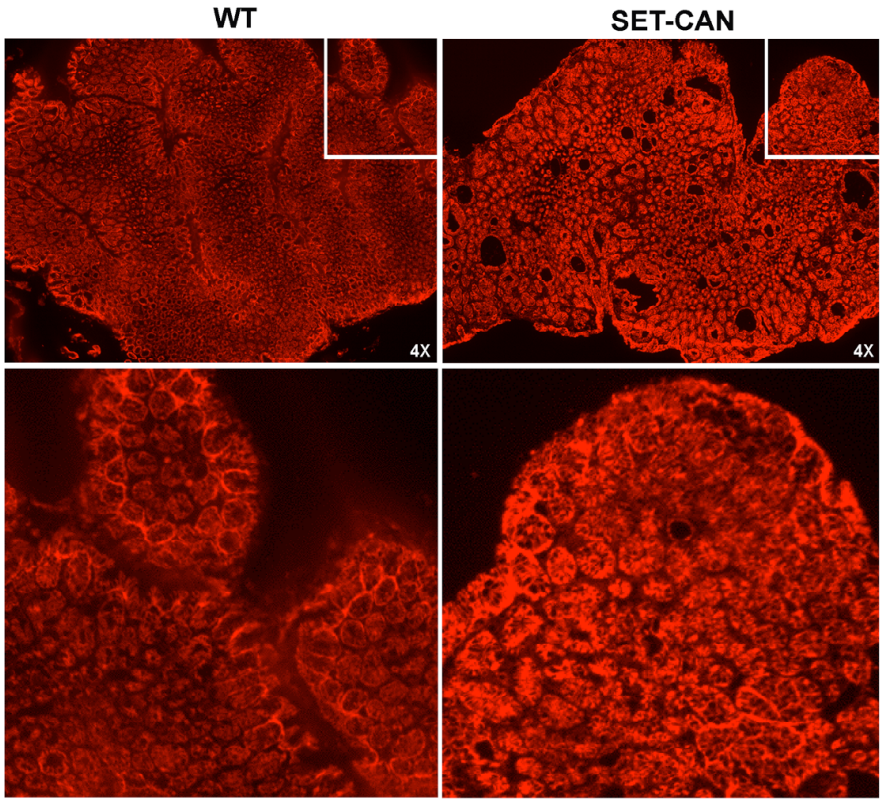

Figure 6. Stomach mucosa of 9 months old SET-CAN transgenic mice express an increased amount of b-catenin. Upper panels: Cryosections of the stomach of a 9 months old wild type littermate (left panels) and SET-CAN (right panels) transgenic mouse stained with a b-catenin antibody (red). Lower panels: Blow-ups of the boxed areas of the upper panels to provide a more detailed view of the b-catenin staining. Both pictures were taken with the same exposure times and gain. SET-CAN mucosa shows increased b-catenin staining.

Given that Wnt-signaling, which stabilizes and increases the concentration of b-catenin (reviewed in ref. (25)), regulates growth of epithelial cells of the stomach and gut mucosa (reviewed in ref.(28)), we also stained the stomach sections with an antibody against b-catenin. This showed a clear increase in the amount of b-catenin in the SET-CAN stomach mucosa (FIG. 6), suggesting that hyperplasia might be due to increased Wnt signaling.

DISCUSSION

In this study we tested the in vivo leukemogenicity of the SET-CAN fusion protein in a transgenic mouse model. The translocation encoding this fusion protein was found in a single patient with AUL. *CAN/NUP214* is usually the target of the t(6;9) in AML, producing a DEK-CAN fusion gene or of a chromosome 9 deletion/amplification in T ALL, generating a *CAN/NUP214-ABL* fusion gene (16). Given the primitive phenotype of the SET-CAN leukemia, we decided to use a transgenic expression construct, which would ensure SET-CAN expression in at least primitive hematopoietic cells. We chose the Ly-6E.1 minigene, which directs expression to both primitive and more differentiated cells (23). Our expression analysis showed SET-CAN mRNA in the BM (Fig. 1B, C), spleen and thymus (not shown) of all 4 transgenic lines. For one transgenic line, 2969, we also verified expression in the liver and kidney as was reported for Ly-6E.1-driven transgenes (23) (Fig. 4B). We were surprised to find that it also directed expression of SET-CAN in the mucosa of the stomach of this transgenic line. We do not know whether SET-CAN was also expressed in the stomach mucosa of the other transgenic lines because they were no longer available for analysis. Since no expression of Ly6E.1/Sca1 was reported in studies that analyzed the presence of this protein in other mouse tissues(21, 33), it is possible that the mucosal expression in line 2969 is ectopic and is the result of the transgene integration site.

The line with the highest SET-CAN expression, based on RPA analysis (Fig. 1C), failed to thrive and the F2 generation could not be obtained. The reason for this remains unknown but high expression of DEK-CAN, SET-CAN and C-terminal CAN/NUP214 in cell lines is toxic(5, 20). C-terminal CAN/NUP214 interacts with CRM1-cargo-RanGTP nuclear export complexes at the outer nuclear pore (13) and is supposedly involved in export complex disassembly and/or CRM1 recycling (3). However, the CAN/NUP214

sequences of the nuclear DEK-CAN and SET-CAN proteins, associate with CRM1 and prevent its exit from the nucleus thereby inhibiting nucleo-cytoplasmic trafficking of CRM1 and cargo(5). We suspect that this might be the reason for the loss of strain 2956, possibly due to increased expression of the transgene in the F1 offspring. In the same vein, we were unable to obtain homozygous transgenic mice for the other 3 lines. Because 2969 appeared the second highest SET-CAN expressing line, which produced normal numbers of pups, we used this line for our tumorigenesis experiments.

Despite expression of SET-CAN in the BM none of the transgenic lines was leukemia-prone. The 3 transgenic lines analyzed showed similar subtle hematopoietic abnormalities as compared to wild type mice. The major difference was an enlargement of a primitive hematopoietic compartment in the BM of these mice as determined by FACS analysis of cell surface markers (Sca1, cKit) and a similar increase in size of the Hoechst 33342 SP fraction (Table 2 and Fig. 2). In support of this finding, SET-CAN BM also produced increased numbers of methylcellulose colonies under conditions promoting growth of myeloid cells. However this difference was lost in secondary methylcellulose cultures, suggesting that the increased number of colonies in the MC1 reflected the accumulation of progenitors in SET-CAN BM but these cells possessed no increased self-renewal activity. To verify these findings in a biological assay we also performed CFU-S assays. Although the 2 transgenic lines gave slightly higher numbers of day 12 spleen colonies, the difference was very moderate. These data are most compatible with a model in which SET-CAN partially inhibits differentiation of primitive progenitors rather than stimulate their proliferation. This interpretation is also in agreement with in vitro experiments in which SET-CAN expression partially inhibited vitamin-D_3 induced monocytic differentiation of the human promonocytic cell line U937(20) and inhibited their proliferation. Moreover, experiments testing whether SET-CAN BM showed an advantage over wild

type BM in competitive repopulation assays after transplantation into lethally irradiated mice were negative. This is again in agreement with the notion that SET-CAN partially inhibited differentiation of primitive progenitors rather than stimulating their proliferation. We believe that the increased pool of primitive progenitors in part compensates for the reduced differentiation capacity of these cells, resulting in relatively small differences in peripheral blood cell counts. Nonetheless, this compensation was incomplete since the peripheral blood of SET-CAN mice showed a slight increase in neutrophils and a 1.7-2.3-fold decrease in lymphocytes, indicating an overall mild skewing of hematopoietic differentiation. The number of B220 cells in the BM of the mice was reduced to a lesser extent than that of peripheral blood, indicating an additional loss of B cells during later stages of differentiation, possibly through increased apoptosis. The decreased capability of SET-CAN BM to produce pre-B cells was also reflected in the reduced numbers of colonies produced in methylcellulose cultures in the presence of IL-7 (Fig. 3C).

The suggestion that SET-CAN might not promote cell proliferation in hematopoietic cells is further supported by the fact that SET-CAN transgenic mice are not leukemia-prone, neither alone, or after retroviral tagging. MOL4070ALTR hybrid virus was shown to cause both myeloid and lymphoid disease in FVB mice(39), although in our wild type FVB mice it appeared to mainly accelerate T cell lymphomagenesis, with only 2 mice showing myeloproliferation. MOL4070ALTR virus infected SET-CAN mice died at the same rate as their non-transgenic littermates. If at all, the effect of SET-CAN appeared to be mitigating viral leukemogenesis rather than accelerating it, since half of the virus infected mice suffered from a lymphoproliferative disease instead of T cell lymphoma. If SET-CAN inhibits proliferation of hematopoietic progenitors, as it does in hematopoietic cell lines(20), it might reduce their rate of infection by the MOL4070ALTR retrovirus, resulting in a

lower level of gene tagging, which would explain the reduced frequency of leukemia in SET-CAN mice.

Nonetheless, our results may reflect the role of SET-CAN in the patient with AUL, i.e. forcing the primitive phenotype of the leukemia. The reason this phenotype is not reproduced in the SET-CAN transgenic lines could be several. First, because we used a standard transgenic approach and the mice have a normal diploid gene dose of *Set* and *Can/Nup214* in their hematopoietic cells, unlike the patient whose leukemic cells are haploid for both genes. Haploinsufficiency for either one or both genes might be essential for leukemia development for reasons currently not understood. Second, the main tumorigenic effect of *SET-CAN* gene might be to reduce the gene dose of *SET* and *CAN/NUP214*, while the presence of the fusion protein itself only affects the phenotype of the leukemia. To test these possibilities, we would need more sophisticated mouse models, in which the *SET-CAN* transgene is bred onto a *Can/Nup214*$^{+/-}$*/Set*$^{+/-}$ background. Currently we are in the process of obtaining such mice. In addition, the necessary secondary mutations might not easily occur in mice despite the use of retroviral tagging. To suppress the development of lymphoid malignancy, the SET-CAN transgenic mice might have to be bred onto a Rag-null background, thereby promoting development of non-lymphoid hematopoietic malignancies. An additional problem in the development of a SET-CAN leukemia mouse model is that we no longer have cells available of the SET-CAN AUL patient. Thus, it is impossible to establish which secondary mutations would be appropriate to combine with SET-CAN by using expression profiling or proteomics of patient BM.

It was unexpected that the 2969 transgenic mice all developed extensive hyperplasia of the stomach mucosa later in life with epithelial cells expressing abundant amounts of SET-CAN (FIG. 5A). It is highly surprising that stomach mucosal cells can handle that high a level of SET-CAN expression, which is lethal in U937 cells, HeLa cells, and Cos cells(5, 20).

The SET-CAN mucosa showed both increased proliferation and apoptosis, with proliferation outpacing apoptosis given that the mucosa became hyperplastic. Currently, we have no explanation for the increased growth of the mucosal epithelial cells but they overexpress b-catenin (FIG. 6). This indicates increased Wnt signaling or alterations of other effectors of the β-catenin pathway, which could be accomplished in many different ways(17, 38). The most surprising aspect of our work is that the effects of SET-CAN are cell type specific. One possible explanation for the stomach phenotype is that SET-CAN-associated Crm1(14) might interact with Axin(38), a negative regulator of the Wnt/b-catenin pathway(40) and APC. Unlike b-catenin, the export of these proteins from the nucleus is CRM1-dependent(38)(reviewed in ref.(17)). Interaction with the SET-CAN/CRM1 complex would reduce export of Axin scaffold protein and APC to the cytoplasm, which would negatively impact on b-catenin phosphorylation(19), thereby activating its transcriptional activity. Why a similar mechanism would not stimulate the growth of primitive hematopoietic cells, which are also dependent on Wnt signaling for growth (reviewed in ref.(32), is unclear. It is possible that Wnt/b-catenin activation does occur in SET-CAN hematopoietic cells but compounding effects of the fusion protein on nuclear export of other proteins (whose amounts are limiting in hematopoietic cells) might overrule growth stimulation. Clearly, additional studies will be necessary to address SET-CAN's effects on the b-catenin pathway and it will be interesting to determine whether the SET-CAN fusion plays a role in gastro-intestinal carcinomas.

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Chapter 5

SUMMARY and DISCUSSION

SUMMARY and DISCUSSION

Recurrent chromosomal translocations are the hallmark of many leukemias. Usually, the fusion of two genes, which give rise to chimeric proteins, often define the subtype of leukemia and are used as distinctive diagnostic markers. Cloning of the genes at the chromosomal break points led to the identification of many known, as well as unknown, genes. Characterization of the normal function of these genes and further understanding of their altered functions in the leukemic fusion genes has not only given insights into the mechanisms underlying the transformation process but also provided leads for better and more specific therapeutic intervention. The best example for the latter is the therapeutic use a small molecule inhibitor, imatinib mesylate, which specifically inactivates the constitutively activated protein tyrosine kinase BCR-ABL in chronic myeloid leukemia and acute lymphocytic leukemia (reviewed in ref.[1]).

The DEK-CAN fusion, the product of the t(6;9), is one of the markers of a specific subtype of AML and is associated with poor prognosis[2]. Screening of patients for the presence of t(6;9) led the identification of a novel gene, *SET*. In a patient with AUL *CAN* was found to be fused to the *SET* gene instead of *DEK*[3]. *CAN* encodes a 214-kDa nucleoporin that is part of the nuclear envelope and it is essential for the nucleo-cytoplasmic transport[4,5]. *SET* encodes a ubiquitously expressed, mainly nuclear phosphoprotein with an acidic tail at its C-terminus[6]. Although the available data suggest involvement of SET in many different functions, such as chromatin remodeling[7,8], transcriptional regulation[9], cell cycle regulation[10] and apoptosis[11], its function in hematopoiesis and effect of SET-CAN on that function remain elusive.

The studies in thesis first focused on the role of SET in survival, proliferation and differentiation of hematopoietic cells using the human U937 promonocytic cell line as a model system. **Chapter 2** showed that ectopic

expression of SET inhibits proliferation and induces cell cycle arrest at G_0/G_1, an effect lost in a SET mutant lacking the 50-amino acids acidic tail located at the C-terminus, which mediates histone interaction and binding to several other proteins[7,12]. Growth arrest in SET overexpressing cells is accompanied by their differentiation as judged by increasing levels of the cell surface marker CD11b. In contrast, ectopic expression of SET-CAN partially inhibited vitamin D_3-stimulated differentiation, and growth arrest induced by SET-CAN was associated with increased cell death, indicating that fusion of SET to CAN creates a protein with a distinct function. Previously Boer et. al showed that overexpression of CAN and any CAN mutant containing the C-terminal hCRM1 interacting domain (preserved in SET-CAN fusion) induced cell cycle arrest due to interference with nucleo-cytoplasmic transport[13]. This is caused by nuclear sequestration of the leucine-rich NES-binding export protein hCRM1. Therefore, forced SET-CAN expression in U937 cells is likely to cause growth arrest and enhanced cell death through this type of interference. However, ectopic expression of CAN-deletion mutant (Kandilci A and Grosveld G, unpublished data) and DEK-CAN, which both possess the same portion of CAN as SET-CAN, did not inhibit the differentiation of U937 cells[13], suggesting that suppression of differentiation by SET-CAN might be due to an altered SET-related rather than a CAN-related function of the fusion protein.

In chapter 3, the studies sought to obtain further insight into the mechanism underlying SET-induced differentiation of U937 cells, and first used micro array analysis to identify the genes whose expression is affected by SET overexpression. The finding that one third of the up-regulated genes [GTP cyclohydrolase I*,* protein kinase *JNK2,* myocyte specific enhancer factor 2A (*MEF2A*), Ins(1,3,4,5)P4-binding protein (*IP4BP*), lymphocyte-specific protein 1 (*LSP1*) and type 3 1,4,5-triphosphate receptor (*IP3R3*)] are directly or indirectly targeted by calcium signaling pathways[14,15 16-19] prompted us to

investigate the involvement of calcium signaling in SET-induced differentiation. Since calcium signaling is involved in dendritic cell (DC) differentiation of human monocytes[20], we analyzed the presence of DC-related cell surface markers including CD11b, CD86, CD83, CD80 and HLA-DR in SET overexpressing U937 cells. This analysis showed that SET overexpression forced partial dendritic differentiation since it up-regulated only two of the DC-related markers, CD11b and CD86, but induced a DC-like morphological appearance of the cells. SET-induced cell surface marker up-regulation, as well as DC-like morphological changes, were suppressed by addition of Ca^{2+}/Calmodulin (CaM) or calmodulin dependent kinase II (CaMKII)-specific inhibitors which target two different branches of the calcium signaling pathway. In addition, specific inhibitors of the MAPK/ERK pathway, a target of calcium signaling and of protein phosphatase 2A activity[21,22], also inhibited SET-induced differentiation. Similar phenotypic changes were induced by interferon-γ (IFN-γ) treatment of U937 cells[23,24] with the distinction that IFN-γ signaling activated the DNA-binding activity of STAT1 whereas SET overexpression did not. In addition, and distinct from IFN-γ signaling, SET also stimulated stress induced p38/MAPK activity. Interestingly, the endogenous SET level was upregulated via IFN-γ treatment. Taken together, these data suggested that SET expression might be subject to IFN-γ signaling and SET might have a role in both IFN-γ and stress induced cellular responses.

Currently it is not known how SET activates calcium signaling. Considering that SET interacts with HuR[25], a protein involved in stabilization of mRNAs with AU-rich elements (AREs) such as cytokine mRNAs, it is possible that SET affects the activity of the HuR complex, which increases the half-life of certain mRNAs that as a result activate calcium signaling. In addition, the well-known inhibitory effect of SET on PP2A[12,26] should at least in part account for the observed activation of CaMKII and MAPK/ERK

pathways, since both kinases are negatively regulated via dephosphorylation by PP2A. Future experiments aimed at detecting the effect of SET on mRNA half-life using a genome wide approach such as expression profiling using transcriptional inhibitors, and the generation and analysis of Set-knockout mice, will help to address these possibilities.

In **Chapter 4**, the leukemogenicity of the SET-CAN fusion in FVB mice was tested by generating transgenic mice in which transgene expression was driven by the Ly6E.1 gene expression cassette. This directs transgene expression to mainly primitive hematopoietic cells[27]. We first analyzed the effect of SET-CAN on adult hematopoiesis by comparing the hematopoietic systems of transgenic mice with that of wild type littermates and found that SET-CAN decreased the number of B220 positive B-cells and enhanced the number of the Sca1/c-kit and Sca1 positive primitive progenitors in the bone marrow. These results were in agreement with the increased size of the Hoechst 33342 side-population fraction (SP) in the bone marrow of SET-CAN mice, which is highly enriched in HSCs. Also, in support of this finding, we observed that SET-CAN BM produced increased numbers of methylcellulose colonies under myeloid growth conditions and decreased numbers of colonies under conditions that support B-cell growth. Differences in the BM cell population of SET-CAN mice were also reflected in the peripheral blood. The mice showed a slight increase in neutrophils and an overall 2-fold decrease in lymphocytes. Given that SET-CAN inhibited the growth and vitamin D_3 induced differentiation of U937 cells, the data are more compatible with a model in which SET-CAN partially blocks differentiation of primitive cells rather than promoting their proliferation. This notion is supported by the fact that the transgenic mice are not leukemia-prone, neither alone nor after retroviral tagging with MOL4070ALTR hybrid retrovirus, which causes both myeloid and lymphoid tumors in FVB mice[28].

There is accumulating evidence that impaired nucleo-cytoplasmic transport could contribute to tumorigenesis via deregulated trafficking of tumor suppressor and onocogene mRNAs and proteins (reviewed in ref.[29]). CAN is essential for CRM1 dependent NES-mediated nucleocytoplasmic shuttling of target proteins and SET-CAN causes sequestration of CRM1 in the nucleus[30], possibly affecting the trafficking of target cargo proteins such as tumor suppressors p53[31] and FOXO1[32] as well as of oncogenes, which may in turn impair their function.

Altogether, the opposite effects of SET and SET-CAN on the differentiation of hematopoietic cells, suggests that the deletion of one allele of SET and CAN in the patient might contribute to circumstances which are essential for oncogenic conversion of hematopoietic cells, a condition not met in our current transgenic SET-CAN model. It is likely that the main tumorigenic effect of SET-CAN might be to reduce the dose of both SET and CAN, whereas the presence of the fusion protein itself only affects the phenotype of leukemia. This possibility can be readily tested in $Can^{+/-}/Set^{+/-}$ /SET-CAN triple mutant mice.

Interestingly, uninfected SET-CAN mice are prone to develop hyperplasia of gastric mucosal cells. We showed that this was caused by high expression of SET-CAN in stomach mucosa resulting in both increased proliferation and apoptosis, with proliferation outpacing apoptosis. Given that high expression of SET-CAN in cell lines causes apoptosis rather than increased growth, we are puzzled by the fact that stomach epithelial cells cope with this high level of SET-CAN expression. As mentioned, SET-CAN captures CRM1 in the nucleus and blocks nucleo-cytoplasmic transport. One of the most reasonable explanations for their resistance is that stomach epithelial cells express large enough amounts of CRM1 to escape the nuclear export block imposed by SET-CAN. Although this explanation seems reasonable, we have not yet determined the amount of CRM1 expression in

these cells and therefore do not know whether this explanation is correct or not.

We are very excited by the observation that the SET-CAN gastric mucosal cells express increased amounts of β-catenin. Since this discovery was made towards the end of my thesis work, I have not been able to study this observation further. However, it is interesting to speculate how SET-CAN might cause this upregulation of b-catenin. The level of b-catenin is regulated by Wnt signaling (reviewed in ref. [33]). β-catenin is continuously degraded by the proteosome as a result of its phosphorylation by GSK3b. This occurs in the cytoplasm in a complex consisting of Axin, APC, b-catenin and GSK3b. [34] Wnt signaling reduces the phosphorylation of b-catenin and the stabilized protein translocates to the nucleus where it forms an active transcription factor through association with TCF. Active b-catenin/TCF complexes in turn upregulate the expression of cMyc and Cyclin D1, which directly stimulates cell proliferation (reviewed in ref. [35]). We hypothesize that SET-CAN expression somehow interferes with b-catenin turnover, resulting in larger amount of the protein and thus, stimulation of cell growth. b-catenin stabilization/activation can also be caused by other events than Wnt signaling. For instance Axin, which functions as a scaffold for the Axin/APC/b-catenin/GSK3b complex positively impacts on the phosphorylation of b-catenin[36] and is a mainly cytoplasmic localized protein that nonetheless shuttles to the nucleus[37]. Unlike b-catenin, nuclear export of Axin and its binding partner APC is CRM1 dependent [37](reviewed in ref.[38]) and the possible reduced export of these 2 proteins in SET-CAN expressing cells would result in increased amounts of nuclear b-catenin. More extensive analysis of the hyperproliferative SET-CAN stomach mucosa would be necessary to test this hypothesis, which is currently possible with the available reagents.

Another interesting question is why does SET-CAN fail to show this growth stimulating effect in hematopoietic cells? It has been well documented that HSC and lymphoid cells are dependent on Wnt signaling for growth (reviewed in ref.[39]). It would be reasonable to speculate that other effects of SET-CAN expression, which do not occur in stomach epithelial cells, could be responsible for suppression of the growth stimulatory effects of increased b-catenin expression in hematopoietic cells. However, it has yet to be verified whether the levels of β-catenin are indeed increased. The nature of these effects are difficult to predict at this point in time, but reduced nuclear export of critical factors would be a good possibility. Together, our observation of the effects of SET-CAN on proliferation of gastric mucosa points to a possible involvement of SET-CAN in gastrointestinal tumorigenesis, an exciting prospect that we are planning to test in the near future.

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Samenvatting

Veel leukemieën worden gekarateriseerd door specifieke chromosoom translokaties. Het kloneren van de chromosomale breukpunten heeft geleid tot identificatie van veel bekende en onbekende genen. Karakterisatie van de normale funktie van deze genen en het verder begrijpen van hun veranderde funktie als onderdeel van leukemie-specifieke fusiegenen heeft niet alleen inzicht verschaft over de mechanismen die transformatie veroorzaken maar heeft ook geleid tot betere en meer specifieke behandelings methoden.

De t(6;9)(p23;q34) translokatie, die meestal voorkomt bij akute myeloide leukemie (AML) van het M2-, of het M4-subtype, koppelt de *DEK* en *CAN* genen die respectievelijk op chromosoom 6 en 9 liggen aan elkaar, wat leidt tot de expressie van een DEK-CAN fusie-eiwit. Bij een andere chromosomale verandering bij een patient die leed aan een acute ongedifferencieerde leukemia (AUL), was het *CAN* gen gekoppeld aan een ander partner-gen, *SET* geheten, dat op hetzelfde chromosoom ligt als *CAN*. In dit proefschrift heb ik geprobeerd de funkties van het SET gen in bloedcellen te ontrafelen om te kunnen begrijpen hoe deze functies in het SET-CAN fusie-gen veranderd zijn.

In **hoofdstuk 2** laten we zien dat overexpressie van SET de groei van de myeloide cellijn U937 vertraagt en differentiatie induceert. De cellen stoppen in de G_0/G_1 fase van de celcyclus, een funktie die verloren gaat als we een gemuteerd SET eiwit tot expressie brengen waarvan de 50 aminozuren-lange staart van zure residuen zijn verwijderd. Deze staart gaat interaktie aan met histonen en verschillende andere eiwitten. In tegenstelling hiermee veroorzaakt expressie van SET-CAN in U937 cellen een remming van de vitamine D3-reïnduceerde differentiatie en ook een verhoogde geprogrammeerde celdood, wat aangeeft dat de fusie van SET met CAN een eiwit met een veranderde funktie creëert. Overexpressie van CAN of van CAN mutanten, die het interaktie domain met hCRM1 bevatten (zoals SET-

CAN), stopt de voortgang van de cel-cyclus and induceert celdood door het remmen van het transport van macromoleculen van de kern naar het cytoplasma. Dit komt door verankering van het leucine-rijke NES (nucleaire export sequentie)-bevattende export eiwit hCRM1 in de kern door SET-CAN. Het is daarom aannemelijk dat hetzelfde mechanisme verantwoordelijk is voor het stoppen van de de cel-cyclus en het induceren van celdood in U937 cellen die SET-CAN overexpresseren. Echter, soortgelijke overexpressie van DEK-CAN, een eiwit dat dezelfde CAN sequenties bevat als SET-CAN, heeft geen effect op de differentiatie van U937 cellen, wat erop duidt dat het differentiatie-remmende effect van SET-CAN eerder veroorzaakt zou worden door een SET-geassocieerde dan een CAN-geassocieerde funktie.

In **hoodstuk 3**, hebben we geprobeerd verder inzicht te verkrijgen in het differentiatie proces dat SET induceert in U937 cellen. D.m.v. microarray analyse hebben we kunnen vaststellen dat expressie verhoogd wordt van verscheidene genen die normaal via calcium signaal transductie worden geïnduceerd. Omdat calcium signaal transductie betrokken is bij de differentiatie van humane monocyten in dendritische (DC) cellen, hebben we gekeken of SET over-expresserende cellen dendritische cel-oppervlakte moleculen tot expressie brengen. Dit bleek inderdaad het geval; zowel CD11b als CD88 worden door SET geinduceerd en de cellen krijgen een dendriet-achtige morfologie. Het optreden van deze veranderingen wordt gestopt door de cellen te incuberen met substanties die de Ca^{2+}/Calmodulin (CaM) en de calmodulin afhankelijke kinase II (CaMKII) remmen, of die MAPK/ERK kinases remmen, die gereguleerd worden door calcium signaal transductie, of die fosfatase 2A remmen. Soortgelijke fenotypische veranderingen worden waargenomen als U937 cellen met interferon-γ geincubeerd worden met het verschil dat interferon-γ signaal transductie de DNA bindings aktiviteit van STAT1 aktiveert, terwijl overexpressie van SET dat niet doet. Een ander verschil is dat SET stress-geinduceerde p38/MAPK aktiveert en interferon-γ

niet. Bovendien is het interessant dat endogeen SET geinduceerd wordt door interferon-γ signaling. Tesamen duiden deze resultaten erop dat SET gereguleerd wordt door interferon-γ signal transduktie en dat SET zelf een rol speelt bij zowel de interferon-γ als de stres respons. Op het ogenblik weten we niet hoe SET de calcium signaal transductie aktiveert. Omdat SET bindt aan HuR, een eiwit dat een rol speelt bij de stabilisatie van mRNAs die AU-rijke elementen (AREs) bevatten zoals bij mRNAs die koderen voor cytokines, is het mogelijk dat SET het HuR complex aktiveert. Dit zou de levensduur van bepaalde mRNAs kunnen verlengen, met als gevolg dat de calcium signaal transductie geaktiveerd wordt. Bovendien zou het wel-bekende remmende effect van SET op PP2A tenminste gedeeltelijk verantwoordelijk moeten zijn voor de aktivatie van de CaMKII en MAP/ERK signaal transductie, omdat beide kinases geremd worden via defosforylatie door PP2A. Toekomstige experimenten zullen er dan ook op gericht zijn het effect van SET op de levensduur van mRNAs te bestuderen, door gebruik te maken van expressie profilerings proeven in de aanwezigheid van transcriptie-remmers als ook de analyse van $Set^{-/-}$ muizen. We hopen dat zulke proeven een definitief onderscheid zullen maken tussen de verschillende mogelijkheden.

In **hoodfstuk 4** onderzoeken we de leukemogene aktiviteit van het SET-CAN fusie eiwit door gebruik te maken van transgene muizen die *SET-CAN* voornamelijk tot expressie brengen in primitieve hematopoïetische cellen onder de kontrole van een Ly6E.1 expressie-cassette. Hoewel *SET-CAN*^{tg} muizen afwijkingen in het hematopoïetische systeem ontwikkelen, zoals expansie van een primitief hematopoietisch compartiment en depletie van lymfocyten door remming van de B-cel differentiatie, vertonen de muizen geen hogere incidentie van leukemie en geen verkorting in de ziekte-latentie na infectie met langzaam transformerende retrovirussen. Dit suggereert dat de expressie van SET-CAN in AUL het primitieve fenotype van de hematopoietische ziekte zou kunnen bepalen, mogelijkerwijs via remming van

de differentiatie, terwijl secundaire veranderingen, zoals haplo-insufficientie van *Set* en *Can/Nup214* nodig zijn om leukemie te veroorzaken, mutaties die niet in ons muis model optreden. Op het ogenblik zijn we bezig *Can/Nup214*$^{+/-}$ */Set*$^{+/-}$/*SET-CAN*^{tg} muizen te genereren om deze mogelijkheid te testen. Geheel onverwacht ontwikkelen alle SET-CAN muizen spontane hyperplasie van de maag-mucosa, die een verhoogde hoeveelheid b-catenine tot expressie brengt. Een van de mogelijkheden waarmee dit mee verklaard zou kunnen worden is dat het SET-CAN-gebonden Crm1 in de kern Axin en APC bindt, twee negatieve regulators van de Wnt/b-catenine aktiviteit. In tegenstelling tot b-catenine is transport van deze eiwitten de kern uit afhankelijk van Crm1. Interaktie met het SET-CAN/Crm1 complex zou de export van Axin en APC uit de nucleus verminderen met als gevolg een verminderde fosforylatie van cytoplasmatisch b-catenine, wat de transcriptie aktiviteit van b-catenine zou vergroten. Het is duidelijk dat additionele studies nodig zijn om het effect van SET-CAN op de b-catenine aktivatie beter te bestuderen en het lijkt ons tevens interessant om te bepalen of het SET-CAN fusie-eiwit wellicht een rol speelt bij gastro-intestinale carcinomen.

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PUBLICATIONS

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