Tumorigenesis and Neoplastic Progression

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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Leukemia-specific chromosome translocations involving the nucleoporin CAN/NUP214 lead to expression of different fusion genes including *DEK-CAN*, *CAN-ABL*, and *SET-CAN*. *DEK-CAN* and *CAN-ABL* are associated with acute myeloid leukemia and T-cell acute lymphoblastic leukemia, respectively, whereas *SET-CAN* was identified in a patient with acute undifferentiated leukemia. In addition, *SET* is overexpressed in solid tumors of the breast, uterus, stomach, and rectum. Ectopic expression of SET-CAN inhibits vitamin-D₃-induced differentiation of the human promonocytic U937 cells, whereas ectopic SET expression induces differentiation. Here, we assessed the leukemogenic potential of SET-CAN in the hematopoietic system of transgenic mice. Although SET-CAN mice showed expansion of an early progenitor cell pool and partial depletion of lymphocytes, the animals were not leukemia-prone and did not show shortening of disease latency after retroviral tagging. This suggests that SET-CAN expression in acute undifferentiated leukemia might determine the primitive phenotype of the disease, whereas secondary genetic lesions are necessary for disease development. Surprisingly, SET-CAN mice developed spontaneous hyperplasia of the stomach mucosa, which coincided with overexpression of β -catenin and vastly increased numbers of proliferating gastric mucosa

cells, suggesting a role of SET-CAN in proliferation of certain epithelial cells. (Am J Pathol 2007, 171:654–666; DOI: 10.2353/ajpath.2007.060934)

Leukemia-specific chromosome translocations frequently lead to the formation of chimeric genes encoding fusion proteins. 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.¹ Translocations are associated with specific leukemia subtypes, suggesting that the *in vivo* oncogenicity of the encoded fusion proteins shows high specificity within the hematopoietic system.

The translocation t(6;9)(p23;q34) is mostly associated with the M2/M4 subtype of acute myeloid leukemia and is characterized by a poor prognosis and a young age of onset.^{2,3} 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.^{4,5} *CAN/NUP214* is also targeted by the t(9;9)(q32;q34) in a case of acute undifferentiated leukemia (AUL), generating a fusion gene with *SET*. Recently, the same fusion was reported in a patient with acute myeloid leukemia.⁶ This chimeric gene consists of nearly the entire *SET* coding sequence fused to the C-terminal two thirds of *CAN/NUP214* and encodes a protein of 155 kd.⁷ The *SET* gene (also known as template-activat-

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ing factor-I β , TAF-I β), encodes a highly conserved, ubiquitously expressed, mainly nuclear phosphoprotein.⁸ SET physically interacts with several protein complexes, which suggests that it has diverse functions including granzyme A-induced apoptosis,^{9,10} chromosome remodeling,^{11,12} transcriptional regulation,¹³ mRNA stabilization,¹⁴ cell-cycle regulation,¹⁵ and differentiation.¹⁶ 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.^{17,18} Interestingly, SET is up-regulated in multiple solid tumors,¹⁹ and the chromosome 9q32–34 region, which contains *SET* and *NUP214/CAN*, is most frequently deleted in human bladder cancers,²⁰ suggesting a role of these genes in tumorigenesis.

Recently, we have shown that ectopic expression of SET-CAN in human U937 promonocytic cells blocks their vitamin D₃-induced differentiation whereas SET overexpression in these cells induces their differentiation.¹⁶ To explore further the causal role of SET-CAN in leukemogenesis, we present here a transgenic mice model that 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 to 8 of *SET* and 3' sequences and splice acceptor of intron 17 to 18 of *CAN/NUP214* between the *SET* and *CAN/NUP214* sequences in the fusion cDNA (see Figure 1A). This minigene fragment was cloned into the *Clal* site of the first exon of the mouse *Ly-6E.1* expression cassette, which directs expression of the transgene to early hematopoietic cells.²¹ 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 *Bam*HI, fractionated on a 0.8% agarose gel, transferred to Hybond nylon membrane (Amersham Life Sciences, Buckinghamshire, UK), and hybridized with ³²P-labeled 4-kb 3' *CAN/NUP214* cDNA probe. Later, SET-CAN mice were genotyped by polymerase chain reaction (PCR) analysis using (*SET*-5'SE) 5'-GACCATTCTGATGCAGTTC-3' and (*CAN/NUP214*-3'AS) 5'-GATGTGAATGATGTTCTAGACTTG-3' primers. All lines were kept as heterozygotes, whereas line 2956 was lost at the F1 generation. Because of prohibitive costs, tumorigenesis experiments were done with the 2969 line only.

Fluorescence-Activated Cell Sorting (FACS) Analysis and BrdU Treatment

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, and BrdU (Pharmin-gen, San Diego, CA) and analyzed on a FACSCalibur or a LSRII (BD Immunocytometry Systems, Franklin Lakes, NJ) as described.²² BM cells of wild-type mice and the SET-CAN line 2696 were also analyzed for CD43 expression. For BrdU analysis, 3-month-old wild-type FVB and SET-CAN mice were given each an intraperitoneal injection of 1.5 mg of BrdU (Sigma, St. Louis, MO) in 0.9% saline. After 2 hours, BM cells were isolated for FACS analysis.

Hoechst 33342 SP Cell Analysis

Hoechst 33342 staining and analysis of SP cells were done as described.²³ In short, BM cells were resuspended at 1×10^6 cells/ml in Dulbecco's modified Eagle's medium plus 10 mmol/L HEPES and 2% fetal bovine serum. Cells were incubated at 37°C for 90 minutes followed by the addition of 5 μ g/ml Hoechst 33342 (Fisher Scientific, Pittsburgh, PA). Cells were centrifuged and resuspended in ice-cold Hanks' balanced salt solution plus 10 mmol/L HEPES and 2% fetal bovine serum 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.²³ 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.

Reverse Transcriptase (RT)-PCR and RNA Protection Assays (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'-GACCATTCTGATGCAGTTC-3' and (*CAN/NUP214*-3'AS) 5'-GATGTGAATGATGTTCTAGACTTG-3' 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 *Eco*RI site of pBluescript. After linearizing the plasmid with *Clal* ³²P-labeled cRNA consisting of 57 nucleotides of pBluescript, 151 nucleotides of *SET* and 85 nucleotides of *CAN/NUP214* was produced using T7 polymerase following the manufacturer's protocol (Ambion, Inc., Austin, TX). After hybridization with RNA and digestion with RNaseA, three 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 pSC-TOP-HA-tagged *SET-CAN* expression construct²⁴

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., Asheville, NC). *mSet*-exon5-F 5'-TGA-GAGTGGTGACCCGTCTTC-3', *mSet*-exon8-R 5'-ATCATC-CTCGCCTTCGTCTC-3' and *mCan*-exon1-F 5'-GCAC-GATGGGAGACGAGATG-3', *mCan*-R 5'-GGTGATGGACT-GGGAATTC-3' primers were used to analyze endogenous mouse *Set* and *Can* expression by RT-PCR, respectively.

Colony Assays

BM cells were plated in methylcellulose (StemCell Technologies, Vancouver, BC, 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°C in 5% CO₂, 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 to 850 cGy, X-ray) and then injected with 10⁵ 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 was 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 14 different blood count values for each of the three transgenic lines and wild-type mice.

Retroviral Tagging of SET-CAN and Wild-Type FVB Mice

MOL4070LTR virus was produced as previously described.²⁵ Newborn pups of heterozygous SET-CAN × wild-type FVB crosses were injected subcutaneously with 0.1 ml of viral supernatant (~10⁵ 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.²² Malignant cells were detected by immunohistochemical staining with CD3, TdT (DAKO, Carpinteria, CA), and CD45 (Pharmingen) as described previously.²⁶

Immunohistochemistry

Cryosections or paraffin-embedded tissue sections of stomachs of 9-month-old wild-type FVB and SET-CAN transgenic mice were stained with anti-CAN,²⁷ anti-Ki67 (Vector Laboratories, Burlingame, CA) and anti- β -catenin (Sigma) antibodies as described previously.²⁸ To detect apoptotic cells in stomach paraffin sections, terminal dUTP nick-end labeling assays were performed using the ApopTag kit (Chemicon International, Temecula, CA) following the manufacturer's protocol.

Cell Lines and Western Blots

293T cells (2×10^6) were seeded into 10-cm dishes and maintained in Dulbecco's modified Eagle's medium complemented with 10% fetal calf serum, penicillin, and streptomycin. The next day, cells were transiently transfected either with 4 μ g of construct expressing HA-tagged SET-CAN or with empty vector (pSCTOP)²⁴ as a control. Cells were harvested 48 hours later and were lysed in 1.5 ml of 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (15 mmol/L Tris, pH 6.8, 5% sodium dodecyl sulfate, 40% glycerol, 0.005% bromophenol blue, and 8% β -mercaptoethanol). Cells transfected with empty vector and treated with 20 mmol/L LiCl for 6 hours served as a positive control for both the level of activated β -catenin and total amount of β -catenin expression in 293T cells, as described previously.²⁹ After denaturation by heating at 98°C for 5 minutes, cells were centrifuged for 5 minutes at 21,000 × *g* and 10 μ l of each sample were separated on 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). The membrane was first immunoblotted with an anti-active β -catenin antibody (1:250, anti-ABC; Upstate Technology, Lake Placid, NY) and subsequently with anti-total β -catenin (1:1000; Sigma), anti-HA (1:500; Sigma), and anti-SET (1:4000; a gift from Dr. T. Copeland, National Cancer Institute, Bethesda, MD) antibodies followed by a horseradish-peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham Biosciences).

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 progenitors²¹ (Figure 1A). We obtained four independent transgenic lines, 2945, 2956, 2969, and 2971, respectively.

Figure 1. Generation and characterization of Ly-6E.1-*SET-CAN* transgenic mouse lines. **A:** The *SET-CAN* minigene containing a small artificial intron between *SET* and *CAN/NUP214* cDNA sequences was cloned into a *Clal* site of the first exon of the mouse Ly-6E.1 expression cassette. **B:** 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 positive (positive) and negative (WT) controls, respectively. **C:** RPA analysis was performed with a *SET-CAN* cRNA probe containing 56 nucleotides of pBluescript sequence, 151 nucleotides of *SET* sequence fused to 85 nucleotides of *CAN/NUP214* sequence. After hybridization with BM RNA and digestion with RNaseA, three 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).

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 BM of these four strains (Figure 1B). To confirm these results and to quantify expression of *SET-CAN*, we performed RPA on the same BM samples. As a probe we used radiolabeled *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 negative and positive controls, respectively (Figure 1C). Using an image processing tool kit (Reindeer Graphics, Inc.), 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 three lines (2945, 2969, and 2971) was approximately equal (equivalent to 62 to 74% of endogenous *Set* expression). We suspect that the higher expression in the 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.

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
CD43*	14.4	ND	8.8	ND
Hoechst 33342 (SP)	0.33	0.86	0.68	0.51

ND, not determined.

*CD43 expression was only determined in wild-type and 2969 transgenic mice.

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 ~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 was 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 was also reflected in the composition of their BM. We isolated BM of 10 3- to 6-month-old transgenic (all three lines) and wild-type mice and determined the composition of the BM using cell surface marker analysis by FACS. 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

Table 1. Peripheral Blood Counts of Three *SET-CAN* Transgenic Lines and FVB Control Mice

Cell type	SC line 2945 ($n = 25$)*	SC line 2969 ($n = 27$)*	SC line 2971 ($n = 24$)*	FVB ($n = 28$)*
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	4859 [†]	5073 [†]	6234	7541
Lymphocytes	2650 [†]	3529 [†]	3571 [†]	6094
Neutrophils	1796	1264	2206 [†]	1097

*Mean/ μ L.

[†] $P < 0.002$ versus wild-type FVB and *SET-CAN* transgenics.

Figure 2. BrdU labeling and cell-cycle analysis of pro/pre-B cells. **A** and **B**: Three-month-old wild-type (wt) and SET-CAN mice (three mice in each group) were injected with 1.5 mg of BrdU (intraperitoneally), and BM was harvested 2 hours later. Cells within each group were pooled, stained with anti-CD43 and anti-B220 antibodies, and B220⁺/CD43^{low} (P1) and B220⁺/CD43^{high} (P2) fractions were sorted. After anti-BrdU and 7-amino-actinomycin D staining, cells were analyzed by FACS to determine their cell cycle profile. **C**: In a parallel experiment, RNA was isolated from the sorted cells and subjected to semiquantitative RT-PCR analysis to determine *SET-CAN*, *Actin*, *Set*, and *Can* expression. **D**: Histogram representing expression of *Set* and *Can* normalized for *Actin* expression using the histogram tool of Adobe Photoshop CS2 software (Adobe Systems, Mountain View, CA).

and a 2- to 3.4-fold increase in the numbers of more mature (*Scal*⁺) and primitive progenitor cells (*Scal*⁺/*c-Kit*⁺) (Table 2). To determine the cell-cycle status of these progenitor cell compartments, three 3-month-old wild-type and three SET-CAN mice (line 2969) received an injection with BrdU (intraperitoneally). BM cells were harvested 2 hours later, pooled, and sorted by FACS for CD43^{low}/B220⁺ and CD43^{high}/B220⁺ cells (P1 and P2 fractions, Figure 2A), which represent pre-B and pro-B cell populations, respectively.³⁰ Comparison of the cell-cycle status of these two fractions showed that SET-CAN expression caused accumulation of cells in S phase of the cell cycle (Figure 2, A and B) but a similar (P1) and a reduced (P2) amount of cells in the G₂/M phase. Endogenous *Set* and *Can* expression in each fraction was not affected (Figure 2C), suggesting that the S-phase effects are caused by SET-CAN and not by altered expression of *Set* and/or *Can*. Similarly, and in agreement with the fact that SET-CAN expression is controlled by the *Ly6/Scal* cassette, both *Scal*⁺ cells and *c-Kit*⁺/*Scal*⁺ cells, but not *c-Kit*⁺ cells and *c-Kit*⁺/*Scal*[−] cells, in SET-CAN BM accumulated in the S phase of the cell cycle (Figure 3, A–C). In addition, the BrdU-labeling experiments showed a threefold increase in pro- and pre-B cells with a sub-genomic DNA content (sub-G₀/G₁ fraction) in BM of SET-CAN mice, representing dying and dead cells (data not shown). This might explain the reduction in the total number of pro- and pre-B cells found in SET-CAN mice (two-

fold reduced in pooled BM of three mice, data not shown), resulting in a reduced number of lymphocytes in the peripheral blood. Surprisingly, increased cell death was not noticeable in *c-Kit*⁺/*Scal*⁺ cells of SET-CAN mice. *Scal*⁺ cells on the other hand showed a twofold increased cell death but their number was 7.7-fold larger than that in normal BM (Figure 4). Thus, these observations support the observed decrease of lymphocytes and increase of myeloid cells in the peripheral blood of SET-CAN mice (Table 1).

We next determined if this increase in primitive progenitors in SET-CAN mice 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.²³ We compared the size of the SP fraction in BM of the three transgenic lines with that of wild-type mice. This analysis was done with five individual animals as well as with three individual pools of three BM samples of each of the strains (data not shown). In agreement with the cell-surface marker analysis, the average size of the SP fraction of transgenic animals was twofold to sixfold larger than that of wild-type mice. One representative SP analysis of wild-type (SP = 0.08%) and SET-CAN BM (line 2969; SP = 0.49%) is shown in Figure 4, A and B, representing a sixfold difference. Additional marker

Figure 3. BrdU labeling and cell-cycle analysis of *Sca1*⁺ and c-Kit⁺/*Sca1*⁺ populations. **A:** BM cells of wild-type (WT-BM) and SET-CAN (SET-CAN-BM) mice were isolated 2 hours after BrdU injection and were stained with PE-labeled *Sca1*, APC-labeled c-Kit, and FITC-labeled BrdU antibodies and the DNA binding dye 7-amino-actinomycin D. The cell cycle profile of wild-type (**A** and **B**) and SET-CAN (**A** and **C**) c-Kit⁻/*Sca1*⁻, c-Kit⁺/*Sca1*⁺, c-Kit⁻/*Sca1*⁺, and c-Kit⁺/*Sca1*⁺ cells were determined by FACS.

analysis of these SP fractions showed that the percentage of *Sca1*⁺ cells remained similar but the percentage of c-Kit⁺ and *Sca1*⁺/c-Kit⁺ cells was lower in the SET-

CAN SP fraction (8.4- and 3.2-fold reduced, respectively; Figure 4, A and B). Given that the SP fraction in SET-CAN BM is sixfold larger than in WT BM, the actual number of c-Kit⁺ cells in the SET-CAN SP fraction was only slightly lower (0.7-fold) than in the WT SP fraction, whereas the number of *Sca1*⁺ and *Sca1*⁺/c-Kit⁺ cells was 7.7- and 1.9-fold higher, respectively.

Figure 4. Identification of side-population (SP) cells in BM of wild-type and SET-CAN transgenic mice by FACS. BM cells of wild-type FVB and SET-CAN transgenic mice were stained with APC-labeled c-Kit and PE-labeled *Sca1* antibodies and Hoechst-33342 dye. The labeled cells were analyzed by FACS for Hoechst 33342 blue and red fluorescence and for the indicated cell surface markers. The SP fraction of BM cells of a wild-type (WT-BM, 0.08%; **A**) and a transgenic SET-CAN line (2969, 0.49%; **B**) are outlined. Although the percentages of both c-Kit⁺ and c-Kit⁺/*Sca1*⁺ cells are lower in the SET-CAN SP fraction than in the wild-type SP fraction, their absolute numbers are higher.

SET-CAN BM 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 ~1.3- to 1.7-fold as many colonies as wild-type BM (Figure 5A). On replating 10³ 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

Figure 5. 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. Twelve days after transplantation, macroscopic colonies on the spleens of recipient mice (three 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.

numbers and determined that both IL-3 and GM-CSF recapitulated the effects seen with a full growth factor complement (not shown).

SET-CAN BM Does Not Show Increased Colony-Forming Activity in Long-Term Culture-Initiating Cell (LTC-IC) Assays

To investigate further 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 attributable to the lack of factors stimulating the growth of very primitive progenitors.

SET-CAN BM Shows Minimally 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 three separate experiments using pools of BM from 10 mice of two transgenic lines and wild-type mice, we found a small increase in the number of CFU-S in mice transplanted with SET-CAN BM compared with those transplanted with wild-type BM (Figure 5B). This small increase is consistent with the results of the MC assays.

SET-CAN BM 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 Figure 2B) and the number of lymphocytes were reduced in the peripheral blood of SET-CAN mice (Table 1), we tested whether SET-CAN

BM contained fewer pre-B-cell colony-forming units than wild-type BM by plating the cells in methylcellulose cultures supplemented with IL-7 only. We analyzed pools of BM cells of eight mice each of wild-type, 2945, and 2969 transgenic mice in five independent experiments (Figure 5C), which established that the number of pre-B colony-forming units was reduced at least twofold in these mice. This result is in agreement with the S-phase arrest and the reduced number of pro-B and pre-B cells in SET-CAN BM.

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.³¹ Newborn wild-type ($n = 12$) and 2969 SET-CAN ($n = 10$) animals were infected with MOL4070ALTR hybrid virus, which produces both myeloid and lymphoid malignancies in FVB mice.²⁵ However, there was no acceleration of disease onset in SET-CAN transgenic mice as compared to wild-type virus-infected mice (Figure 6A). Ly-6E.1 transgenes were reported to express in BM, kidney, and liver.²¹ Using RT-PCR we ensured that SET-CAN was expressed in these tissues of the transgenic animals used in this experiment (Figure 6B). 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 (Figure 6C). 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 multifocal glomerulonephritis and global glomerulopathy without T-cell lymphoma or lymphoid hyperplasia (Figure 6D, 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.

Because the four males of the 10 virus-infected SET-CAN animals developed kidney abnormalities, we also

Figure 6. 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 (SET-CAN) 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 (WT) 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- and high-power view of H&E-stained sections of kidney of a wild-type FVB mouse and one SET-CAN mouse that developed global glomerulopathy after retrovirus infection. Original magnifications: $\times 40$ (D, top); $\times 200$ (D, bottom).

analyzed the kidney of age-matched, 9-month-old uninfected SET-CAN mice ($n = 9$). Again, similar kidney lesions were detected in the two males but not in the seven females in this group. This suggested that the kidney lesions are gender-specific. In addition, eight of nine SET-CAN mice 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. Figure 7 shows the comparison of the fundic gland region of the gastric mucosa of wild-type stomach (Figure 7, A and C) with that of SET-CAN stomach (Figure 7, B and D), in which

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.

the increase in thickness of the mucosa is easily observed. In SET-CAN mucosa, the overall architecture is disrupted, showing dilation of the glands and a decreased number of parietal and chief cells, which are replaced by low columnar to cuboidal epithelial cells (Figure 7D). Immunohistological analysis with a C-terminal CAN antibody showed bright staining of nuclear SET-CAN in epithelial cells lining the hyperplastic mucosal glands (Figure 7F), whereas mucosa of wild-type mice showed weak mainly nuclear envelope and cytoplasmic staining of endogenous CAN with this antibody (Figure 7E). 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 Ki67.³² This antigen showed labeling from the surface to the deep mucosa in SET-CAN stomach (Figure 8, B and E), whereas in wild-type mucosa there was only labeling of the middle zone of the gastric mucosa (isthmus) (Figure 8, A and D) where the progenitor cells reside.^{33,34} We determined that 45% of cells of the SET-CAN mucosa expressed Ki67, whereas only 21% of cells in the wild-type mucosa were positive (Figure 8C). This result suggests that SET-CAN mucosa contains an increased number of progenitor cells. Because the mucosa

Figure 7. SET-CAN mice develop hyperplasia of the stomach mucosa. H&E-stained sections of the fundic gland region of the gastric mucosa of a 9-month-old wild-type FVB mouse (**A** and **C**) and a SET-CAN transgenic mouse (**B** and **D**). At ×40 magnification, the marked difference in the thickness between wild-type (**A**) and SET-CAN (**B**) mucosa is easily observed. **C:** At ×400 magnification, the normal architecture of the fundic mucosa with chief cells (arrows) and parietal cells (arrowheads) is observed in wild-type stomach. In SET-CAN stomach (**D**), the normal architecture is disrupted and there are decreased numbers of parietal and chief cells. The proliferating cells are low columnar to cuboidal epithelial cells that are not differentiated to the parietal or chief cell lineages but occasionally produce mucous. There is dilation of the glands with sloughing of the lining epithelia into the gland lumen. Epithelial cells lining the glands are crowded and often bilayered consistent with hyperplasia. Immunofluorescence analysis (**E** and **F**) of Can/Nup214 and SET-CAN expression in stomach cryosections of the same mice with a C-terminal CAN/NUP214 antibody shows a bright punctate staining (green) in the nucleus in the SET-CAN section (**F**), whereas a section of the wild-type mucosa (**E**) shows staining of endogenous Can/Nup214 (green) at the nuclear envelope and in the cytoplasm. The gain in brightness of the green staining in the micrograph on the left is fivefold electronically enhanced compared with that of the micrograph on the right. The nuclei in these sections were stained with DAPI (blue).

is hyperplastic but not transformed, we determined whether cells showed an increased apoptotic index because of proliferative stress. Indeed, terminal dUTP nick-end labeling staining revealed an increased number of apoptotic cells in the hyperplastic SET-CAN mucosa (Figure 8, F and G). However, as judged by the Ki67 staining, there were far fewer apoptotic cells than proliferating cells in the SET-CAN mucosa.

Given that Wnt signaling stabilizes and increases the concentration of β -catenin³⁵ and regulates growth of epithelial cells of the stomach and gut mucosa,³⁶ we also stained the stomach sections with an antibody against β -catenin. Confocal microscopy showed a substantial increase in the amount of β -catenin in the SET-CAN stomach mucosa (Figure 9, B, D, and F) compared with age-matched wild-type mucosa (Figure 9, A, C, and E), although most staining was membranous rather than nuclear. Nonetheless, this comparison also revealed that some nuclei in the SET-CAN mucosa displayed a low level of β -catenin staining (Figure 9, D and F) suggesting an increase in active nuclear β -catenin. However, Figure 9, G–I, shows costaining of SET-CAN (determined with a C-terminal CAN antibody as punctate nuclear staining) and β -catenin in the stomach of SET-CAN mice. This revealed some, but by no means strict coincidence of β -catenin and SET-CAN expression, suggesting that SET-CAN may have both a cell autonomous and paracrine effect, opening the possibility that hyperplasia of the stomach mucosa might be attributable to direct or indirect SET-CAN-induced Wnt signaling. This possibility can be assessed by a co-culture experiment. Given that we only had the 2969 transgenic line left when we discovered the stomach phenotype, we wanted to further support this possibility by assessing the effect of SET-CAN in cultured epithelial cells and transfected 293T cells with a SET-CAN or an empty expression plasmid. We compared the levels of total and active β -catenin with that of 293T cells treated with LiCl (Figure 9, J and K). LiCl presumably mimics Wnt signaling by inhibiting GSK3 activity.³⁷ Indeed, SET-CAN-transfected cells showed an increased level of both types of β -catenin, suggesting that downstream effects of the fusion protein mimic Wnt signaling. This supports the possibility that the hyperproliferation of SET-CAN mucosa in the 2969 transgenic mice is caused by the same effect. We also analyzed if endogenous *Set* and *Can* expression is affected by SET-CAN expression but RT-PCR analysis of wild-type and SET-CAN stomach cells did not reveal any significant difference in the expression of both genes (Figure 9, L and M).

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 patient with AUL and recently in a patient with acute myeloid leukemia.⁶ *CAN/NUP214* is more frequently the target of the t(6;9) in acute myeloid leukemia, producing a *DEK-CAN* fusion gene or of a chromosome 9 deletion/amplification in T ALL, generating a *CAN/NUP214-ABL* fusion gene.³⁸ 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.²¹ Our expression analysis showed SET-CAN mRNA in the BM (Figure 1, B and

Figure 8. Immunohistochemical staining of proliferating and apoptotic cells in sections of wild-type and SET-CAN gastric mucosa. **A**, $\times 40$ magnification of the fundic gland region of the gastric mucosa of a 9-month-old wild-type FVB (**A**) and a SET-CAN (**B**) transgenic mouse after staining for the proliferation antigen Ki67 shows that in the wild-type mouse Ki67-labeled nuclei are restricted to the proliferating epithelial cells in the middle zone of the gastric mucosa, whereas in stomach of the SET-CAN mouse labeling extends from the surface to the deep mucosa. Enlargements ($\times 400$ magnification) of the boxed areas in **A** and **B** are shown in **D** and **E**, respectively. The Ki67 proliferation index (**C**) was determined by counting the number of Ki67-positive cells among a total of 500 cells in the gastric glands of two wild-type and two SET-CAN mice (cells in the lamina propria were not counted), and the average percentage of positive cells is represented in the graph. Paraffin sections of the same mice (wild type, **F**; SET-CAN, **G**) showing apoptotic cells (green) using terminal dUTP nick-end labeling staining with a DAPI counterstain of the nuclei (blue). Compared with wild-type mucosa, SET-CAN mucosa shows an increased number of apoptotic cells.

C), spleen, and thymus (not shown) of all four 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 (Figure 6B).²¹ 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. Because no expression of Ly6E.1/Scal was reported in studies that analyzed the presence of this protein in other mouse tissues,^{39,40} 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 (Figure 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.^{16,41} C-Terminal CAN/NUP214 interacts with CRM1-cargo-RanGTP nuclear export complexes at the outer nuclear pore⁴² and is supposedly involved in export complex disassembly and/or CRM1 recycling.⁴³ 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 nucleocytoplasmic trafficking of CRM1 and cargo.⁴¹ We suspect that this might be the reason for the loss of strain 2956, possibly attributable 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 three 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. Compared with wild-type mice, the three transgenic lines analyzed all showed similar subtle hematopoietic abnormalities. 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 (Scal, cKit) and a similar increase in the size of the Hoechst 33342 SP fraction (Table 2 and Figures 3A and 4). 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. We were unable to mimic the effects in BM in LTC-ICs because no accumulation of primitive cells occurred under these culture conditions. To verify these findings in a biological setting we also performed CFU-S assays. Although the two transgenic lines gave slightly higher numbers of day 12 spleen colonies, the difference was very moderate. We believe that the partial block of SET-CAN Scal⁺/cKit⁺ and Scal⁺ progenitor cells in S phase causes them to accumulate because there is

Figure 9. Stomach mucosa of 9-month-old SET-CAN transgenic mice express increased amounts of β -catenin. **A** and **B**: Cryosections of the stomach of a 9-month-old wild-type FVB mouse (**A**) and a SET-CAN transgenic littermate (**B**) stained with a β -catenin antibody (red). **C** and **D**: Confocal microscopy shows the greatly increased staining of mainly membrane-bound β -catenin in SET-CAN mucosa (**D**) compared to wild-type (**C**). Some nuclei in SET-CAN mucosa also showed light β -catenin staining (arrows), whereas other nuclei are devoid of staining (arrowhead). **D** and **F**: The same picture as in **C** and **D** but with the nuclei counterstained with DAPI. **G–I**: Staining of sections with SET-CAN and DAPI (**G**), β -catenin and DAPI (**H**), and SET-CAN and β -catenin (**I**). All pictures were taken with the same exposure times and electronic gain. **J**: A Western blot containing protein of 293T cells expressing ectopic HA-SET-CAN (SET-CAN) was incubated with antibodies recognizing activated and total β -catenin, HA, and actin. This showed increased levels of active and total β -catenin similar to vector-transfected 293T cells treated with LiCl, which mimics Wnt signaling. The levels of β -catenin in empty vector-transfected cells (vector) were lower. In the left three lanes, 10 μ l of total cell lysate was loaded, whereas in the right lane 5 μ l of total lysate was loaded. **K**: Graphic representation of the β -catenin band intensities (**J**) normalized for actin as measured by a ChemiDoc EQ gel documentation system (Bio-Rad, Hercules, CA). **L**: RNA was isolated from the stomach of both wild-type (wt) and SET-CAN (sc) mice and subjected to semi-quantitative RT-PCR for *Actin*, *SET-CAN*, endogenous *Set*, and *Can*. Histogram represents expression values of each gene normalized for *Actin* expression using the histogram tool of Adobe Photoshop CS2 software (**M**). Original magnifications: $\times 40$ (**A** and **B**); $\times 400$ (**C** and **D**); $\times 200$ (**G–I**).

insufficient cell death to keep their numbers in check. This apparently is not the case in pro-B and pre-B cells in which S phase accumulation is accompanied by a level of cell death sufficient to reduce their total numbers. Clearly, all these progenitors proliferate slower. This interpretation is also in agreement with *in vitro* experiments in the human promonocytic cell line U937¹⁶ which also showed accumulation in S phase and inhibition of prolif-

eration. Moreover, competitive transplantation experiments in lethally irradiated mice comparing the repopulation activity of wild-type BM with that of SET-CAN BM showed no difference. This is again in agreement with the notion that SET-CAN is slowing down cell-cycle traverse of primitive progenitors rather than stimulating their proliferation, which results in enlargement of this cell pool. We believe that this increased pool of primitive progeni-

tors in part compensates for the total number of cells progressing development to fully differentiated cells, resulting in relatively small differences in peripheral blood cell counts. Nonetheless, this compensation was incomplete because the peripheral blood of SET-CAN mice showed a slight increase in neutrophils and a 1.7- to 2.3-fold decrease in lymphocytes, indicating an overall mild skewing of hematopoietic differentiation.

The observation that SET-CAN does not promote proliferation of hematopoietic cells is further supported by the fact that SET-CAN transgenic mice are not leukemia-prone, neither alone nor after retroviral tagging. MOL4070ALTR hybrid virus was shown to cause both myeloid and lymphoid disease in FVB mice,²⁵ although in our wild-type FVB mice it appeared mainly to accelerate T-cell lymphomagenesis, with only two mice showing myeloproliferation. MOL4070ALTR virus infected SET-CAN mice died at the same rate as their nontransgenic littermates. If at all, the effect of SET-CAN appeared to be mitigating viral leukemogenesis rather than accelerating it, because half of the virus-infected mice suffered from a lymphoproliferative disease instead of T-cell lymphoma. It is possible that inhibition of proliferation of hematopoietic progenitors by SET-CAN, as observed in hematopoietic cell lines,¹⁶ might reduce their rate of infection by the MOL4070ALTR retrovirus, resulting in a lower level of gene tagging, which might explain the reduced frequency of leukemia in SET-CAN mice.

Nonetheless, our results might reflect the role of SET-CAN in the patient with AUL, ie, it may enforce the primitive phenotype of the leukemia. The reason that the phenotype is not reproduced in the SET-CAN transgenic lines could be as follows. Our SET-CAN transgenic mice have a normal diploid gene dose of *Set* and *Can/Nup214* in their hematopoietic cells, whereas the patient's leukemic cells are haploid for both genes. Haploinsufficiency for either one or both genes might contribute to leukemia development for reasons currently not understood. 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 nonlymphoid 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 this patient's BM.

It was unexpected that all 2969 transgenic mice developed extensive hyperplasia of the stomach mucosa later in life with epithelial cells expressing abundant amounts of SET-CAN (Figure 7F). The phenotype segregated with the transgene as transgenic mice form different backcrosses with wild-type FVB mice showed the same phenotype. In addition, it was surprising that stom-

ach mucosal cells can cope with the high levels of SET-CAN expression, given that high levels of the fusion protein are lethal in U937, HeLa, and Cos cells because of SET-CAN's ability to block nucleocytoplasmic transport by retaining the leucine-rich nuclear export factor CRM1 in the nucleus.^{16,41} Currently, we have no clear-cut explanation for the increased growth of the stomach mucosa but the overexpression of β -catenin is intriguing (Figure 9). Moreover, forced expression of SET-CAN in 293T epithelial cells also increased the amount of total and active β -catenin, suggesting that the observed up-regulation of β -catenin in the stomach mucosa of our 2696 mice is a bona fide downstream effect of SET-CAN. This latter observation also argues against the possibility that the stomach phenotype is attributable to an integration effect rather than a result of the transgene itself. How SET-CAN affects Wnt signaling in the stomach mucosa remains to be determined, and it will be worthwhile to study the fate of Wnt signaling or other effectors of the β -catenin pathway, which occurs in many different ways.^{44,45} Clearly, additional studies will be necessary to address SET-CAN's effects on the β -catenin pathway and it will be interesting to determine whether the SET-CAN fusion plays a role in gastrointestinal carcinomas in humans.

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