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Brief Report

HEMATOPOIESIS AND STEM CELLS

Wnt signaling induces transcription, spatial proximity, and translocation of fusion gene partners in human hematopoietic cells

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Key Points

- Wnt/β-catenin signaling increases *ETO* and Runx1 transcription in human hematopoietic progenitors.
- Wnt/β-catenin signaling enhances spatial proximity of *ETO* and *RUNX1* genes and induces the generation of a recurrent translocation event.

Chromosomal translocations are frequently associated with a wide variety of cancers, particularly hematologic malignancies. A recurrent chromosomal abnormality in acute myeloid leukemia is the reciprocal translocation t(8;21) that fuses *RUNX1* and *ETO* genes. We report here that Wnt/β-catenin signaling increases the expression of *ETO* and *RUNX1* genes in human hematopoietic progenitors. We found that β-catenin is rapidly recruited into RNA polymerase II transcription factories (RNAPII-Ser5) and that *ETO* and *RUNX1* genes are brought into close spatial proximity upon Wnt3a induction. Notably, long-term treatment of cells with Wnt3a induces the generation a frequent *RUNX1-ETO* translocation event. Thus, Wnt/β-catenin signaling induces transcription and translocation of *RUNX1* and *ETO* fusion gene partners, opening a novel window to understand the onset/development of leukemia. (*Blood*. 2015;126(15):1785-1789)

Introduction

One of the most common chromosomal abnormalities in acute myeloid leukemia (AML) is the reciprocal translocation t(8;21)(q22;q22), which involves the *RUNX1* (*AML1*) and the *ETO* (*RUNX1T1*) genes and produces a Runx1-ETO fusion transcript that inhibits Runx1-dependent transcriptional regulation.[1,2] Although *RUNX1-ETO* translocations could act as initiating events in hematopoietic stem cells (HSCs), after which leukemia clonally evolves through the acquisition of secondary mutations,[3,4] the molecular mechanism and cellular signals that drive the generation of the t(8;21) translocation remain to be elucidated.

Although much has been learned in recent years about the onset or development of AML from studies examining Runx1 expression or function,[5-8] we are still far from a complete understanding of the cellular mechanisms controlling the transcription of its translocation partner, *ETO*.[9-11] Considering that Wnt/β-catenin plays essential roles during the proliferation or differentiation of HSCs and that reactivation of β-catenin signaling is important for self-renewal of leukemia stem cells,[12-14] we studied whether Wnt/β-catenin signaling was involved in *ETO* expression and *RUNX1-ETO* fusion.

Methods

Human CD34$^+$ cells and cell lines

CD34$^+$ cells were isolated from umbilical cord blood samples after normal full-term delivery from the Maternity Service at Complejo Asistencial Barros Luco (Santiago, Chile). Informed consent was obtained in accordance with the Declaration of Helsinki and the ethics committee of the Universidad Andres Bello. KG1, HEK293, and L cells secreting Wnt3a were obtained from ATCC (Rockville, MD).

Cell culture conditions, Wnt/β-catenin signaling activity, semiquantitative and quantitative polymerase chain reaction, *ETO* promoter activity, plasmids and site-directed mutagenesis, β-catenin and RNAPII immunofluorescence, RNA and DNA fluorescent in situ hybridization (FISH) experiments, image acquisition and processing, and statistical analyses are described in supplemental Methods (available on the *Blood* Web site).

Results and discussion

According to the current assembly of the human genome (GRCh38), the *ETO* gene consists of 17 exons distributed over 148 kb (Figure 1A),

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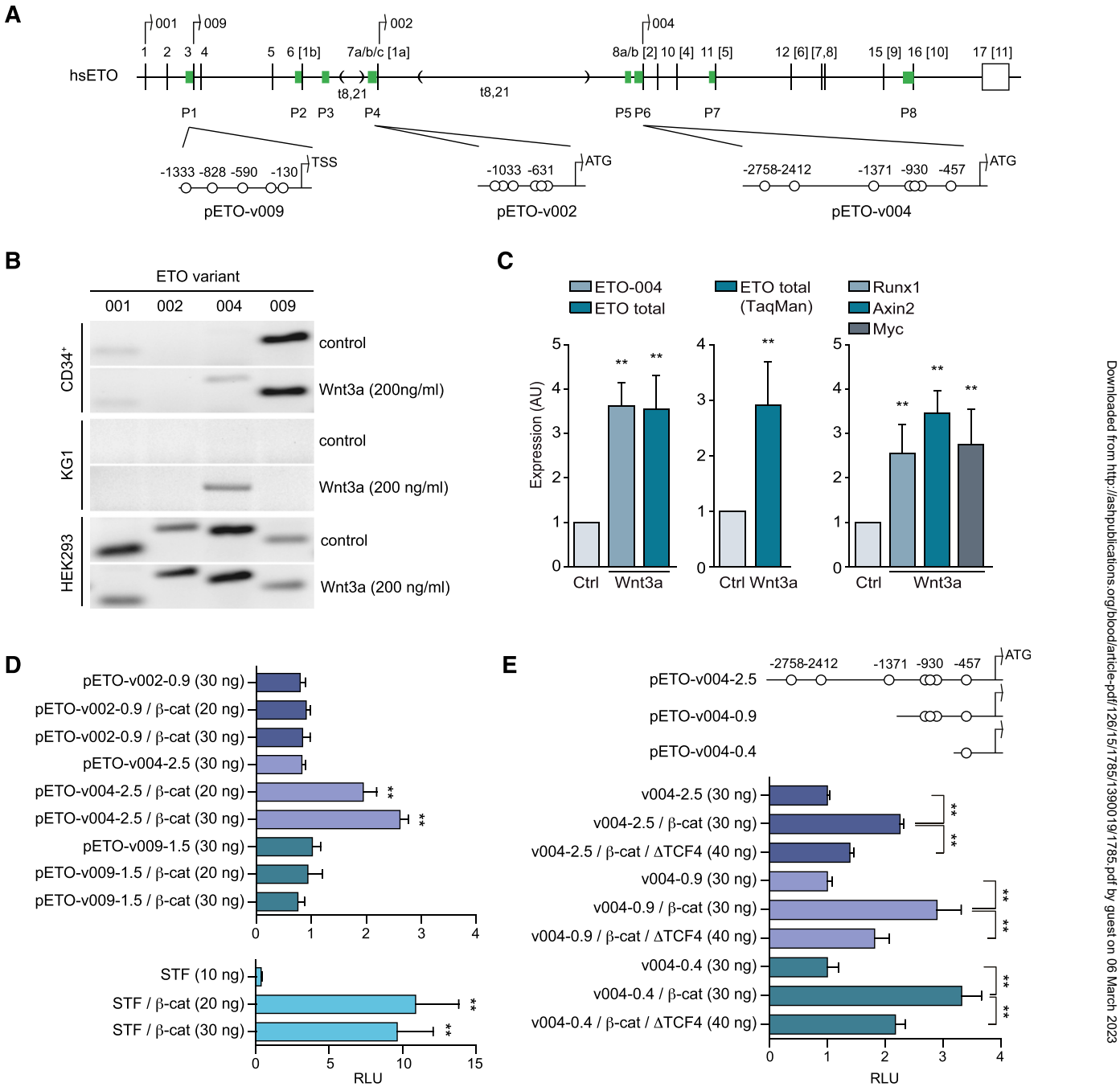


Figure 1. Wnt/β-catenin signaling modulates *ETO* transcriptional activity. (A) Genomic context of the human *ETO* gene on chromosome 8. Selected *ETO* mRNA variants (001: ENST00000523629; 002: ENST00000396218; 004: ENST00000422361; and 009: ENST00000360348) (top). Exons are depicted as bars numbered according to NCBI RefSeq: NC_000008.11. Numbers within square brackets indicate numbering as it has been reported.[9] Green boxes (P1-P8) represent predicted genomic regions containing putative promoters.[11] t(8,21): Chromosomal translocation sites *RUNX1/ETO*. The location of predicted *TCF/LEF*-binding elements (TBE; open circles) in the promoter region of variants 002, 004, and 009 (bottom). (B) Detection of alternative *ETO* mRNA variants 001, 002, 004, and 009 in $CD34^+$ (top), KG1 (middle), and HEK293 cells (bottom) under control conditions or in the same cells treated with purified Wnt3a (200 ng/mL) for 4 hours. (C) Quantitative determination of mRNA levels for *ETO*-004 and *ETO* total (using primers designed in conserved exons 8 and 9 or determined by TaqMan assays; left and middle, respectively), as well as for Runx1 and Wnt/β-catenin target genes Axin2 and Myc (right). Cells were incubated under control conditions or stimulated with 200 ng/mL purified Wnt3a for 4 hours. Quantitative polymerase chain reaction results were normalized using *GAPDH* mRNA levels as a reference gene and are expressed as relative expression. Each figure corresponds to at least 5 independent experiments (with replicates). Ctrl, control conditions. Statistical significance was determined by a Mann-Whitney *U* test. (D) Dose-dependent effects of constitutively active β-catenin S33Y on the transcriptional activity of *ETO*-luciferase promoter constructs in HEK293 cells (24 hours). SuperTOPFLASH (STF) was measured as positive Wnt/β-catenin signaling readout. Each figure corresponds to at least 3 independent experiments. Statistical significance was determined by an ANOVA test (**$P < .01$). (E) Effect of β-catenin S33Y and increasing doses of dominant-negative ΔTCF4 on the promoter activity of pETO-004 deletion constructs, determined as in panel D. RLU, relative luciferase units.

and its expression generates multiple messenger RNA (mRNA) isoforms by alternative splicing or the use of different promoters.[9-11] We searched for core *TCF/LEF*-binding elements (TBE: CTTTG) upstream and downstream of the proposed transcriptional start sites of the human gene and found multiple TBE sites in these genomic regions. We designed primers to detect common alternative mRNA transcripts (HGNC: *ETO*-001, 002, 004, and 009; supplemental Table 1). Although endogenous *ETO* mRNA levels were readily detected in

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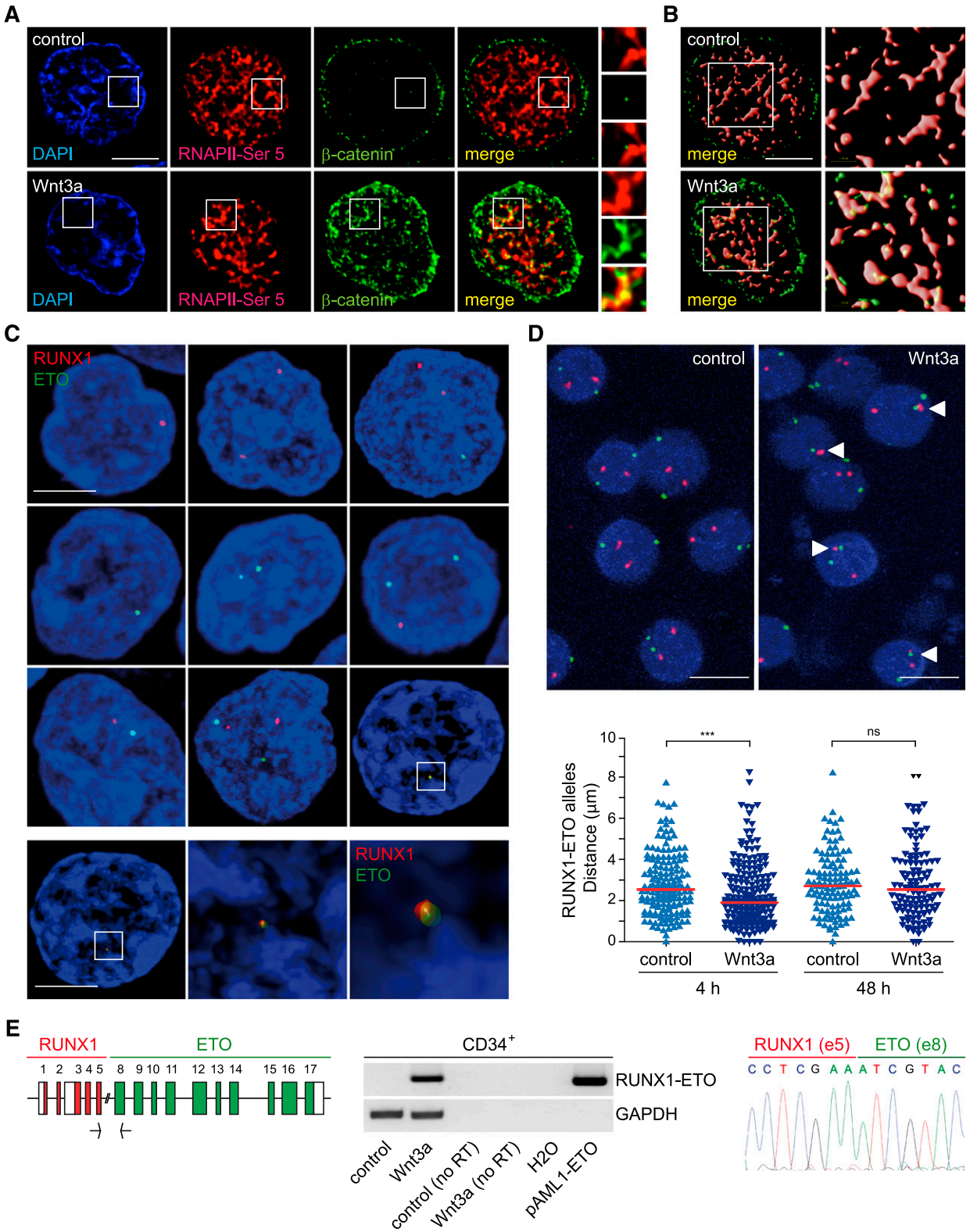


Figure 2. Wnt3a induces spatial proximity and translocation of *RUNX1* and *ETO* in human hematopoietic progenitors. (A) Localization of β-catenin into active RNAPII transcription factories. Immunodetection of β-catenin and active RNAPII-Ser5P through confocal microscopy in CD34$^+$ cells incubated under control conditions or in the presence of purified Wnt3a (200 ng/mL; 4 hours). White boxes indicate selected regions of interest that are shown at higher magnification at the right panel. Scale bar represents 5 μm. (B) Surface-rendered CD34$^+$ cells (as depicted in panel A) and zoom in (4×; white boxes) showing colocalization of β-catenin and RNAPII-Ser5P in Wnt3a-treated cells. (C) Representative confocal images of RNA-FISH experiments in CD34$^+$ cells upon 4-hour treatment with 200 ng/mL purified Wnt3a (number of cells and other parameters are given in supplemental Table 2). *RUNX1* alleles are shown in red and *ETO* alleles in green. Scale bar represents 4 μm. Selected region of interest (white box) is shown at higher magnification below as a maximum intensity projection reconstruction for 4,6 diamidino-2-phenylindole (DAPI) and signal rendering for *RUNX1* (red) and *ETO* (green). Scale bar represents 4 μm. Zoom in (4× and 8×; middle and right, respectively) corresponds to region of interest defined above. (D) Representative confocal images of DNA-FISH experiments in control and Wnt3a-treated CD34$^+$ cells (4 hours) (top). White arrowheads in Wnt3a-treated cells indicate *RUNX1* (red) and *ETO* (green) alleles

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isolated $CD34^+$ cells under control conditions,[6] we observed that a differential enhancement in the transcription of *ETO*-004 variant occurred in $CD34^+$ cells after short-term treatment (4 hours) with purified Wnt3a (Figure 1B). Similarly, whereas endogenous levels of *ETO* mRNA were barely detected in the KG1 cells[15] under control conditions, a clear increase in the expression of *ETO*-004 variant occurred in these cells upon Wnt3a treatment. *ETO* variants were also detected in HEK293 cells under control or Wnt3a conditions, suggesting that this embryonic cell line could be a useful model to study alternative *ETO* expression.

Quantitative determination of mRNA levels in $CD34^+$ cells showed that Wnt/β-catenin induced a rapid (4-hour) and significant increase in *ETO*-004 variant (3.7-fold, $P = .0028$, n = 6) or total *ETO* mRNA levels (3.6-fold, $P = .0028$, n = 6; Figure 1C). Wnt3a effects on total *ETO* levels were further confirmed by TaqMan analyses (2.9-fold, $P = .0011$, n = 7; Figure 1C and supplemental Figure 1). Finally, the effect of the signaling cascade on *ETO* expression was paralleled by enhanced transcription of Runx1[16,17] (2.6-fold, $P = .0075$, n = 5) and classical target genes Axin2 and Myc (3.5- and 2.8-fold, respectively; $P = .0075$, n = 5).[18,19]

Putative promoters for *ETO*-002 and -004 variants are located downstream of common *RUNX1-ETO* translocation hotspots (Figure 1A). To study if these promoters contained functional TBE sites, we PCR-cloned 890 and 2459 nucleotides of the 5′ upstream/exon 1 sequences of *ETO*-002 and -004, containing 6 and 7 potential TBE sites, respectively (Figure 1D). We also analyzed 1535 nucleotides with 5 TBE sites upstream of the transcriptional start sites described for variant *ETO*-009.[10] We observed a significant increase in the promoter activity of pETO-004 (2.5 kb) in HEK293 cells cotransfected for 24 hours with increasing concentrations of constitutive active β-catenin (S33Y) (Figure 1D). Although basal transcription was observed for pETO-002 and pETO-009 promoters, no significant effects were found in cells cotransfected with these constructs and β-catenin S33Y (24 hours). Then, we serially deleted potential TBE sites in pETO-004 (pETO-004-0.9 and pETO-004-04, respectively) and observed that these constructs responded accordingly to β-catenin stabilization or its inhibition by a dominant-negative ΔTCF4 construct (Figure 1E). Next, because pETO-004-0.4 contains a single TBE site (−457 nucleotides upstream of the ATG of *ETO*-004 variant), we mutated the core consensus sequence (CTTTG → CCTCG) and found that the activity of the mutant promoter was decreased to basal levels (supplemental Figure 2). Collectively, our findings indicate that Wnt/β-catenin signaling rapidly induces *ETO*-004 variant and that this effect is probably accomplished through *ETO*-004 TBE site I.

Translocation-prone genes are preferentially recruited into transcription factories with active RNA polymerase II (RNAPII-Ser5)[20,21] and need to be positioned in close spatial proximity relative to each other prior to translocation.[22,23] Because β-catenin participates in recruiting basal transcription components at the promoter of its target genes, we studied if activation of the cascade induced β-catenin relocalization into these transcriptional structures. We found that transcription factories labeled with active RNAPII-Ser5 were clearly distinguished in resting or Wnt3a-treated $CD34^+$ cells (4 hours) (Figure 2A-B). Under control conditions, the number and diameter of RNAPII speckles within the nucleus were in agreement with previous observations[22] (32.1 ± 1.13 and 130.7 ± 3.5 nm, respectively; supplemental Figure 3A). Remarkably, Wnt3a induced a significant increase in the number of endogenous β-catenin speckles within the nucleus of $CD34^+$ cells (control: 1.5 ± 0.3; Wnt3a: 19.5 ± 2.7; $P < .001$; supplemental Figure 3A), some of which clearly colocalized with active RNAPII foci (supplemental Figure 3B-C). Similar effects of β-catenin colocalization with RNAPII-Ser5 were observed in Wnt3a-treated KG1 cells (supplemental Figure 4).

Our data indicate that β-catenin rapidly associates with RNAPII transcription factories in response to Wnt/β-catenin activation, further implying that expression of *ETO* and *RUNX1* translocation partners could occur in these chromosomal structures. To address this idea, we performed RNA-FISH experiments in $CD34^+$ cells using fluorescent probes targeting nascent sequences within *ETO* and *RUNX1* genes (introns 11 and 4, respectively) (Figure 2C). We noted that the total number of *ETO* and *RUNX1* alleles expressed in control $CD34^+$ cells increased from 25 to 54 after 4-hour Wnt3a-treatment (supplemental Figure 5 and supplemental Table 2). Wnt/β-catenin induction was similarly observed for both genes (*ETO* alleles: from 9 to 21; *RUNX1* alleles: from 16 to 33) and turned on the expression of a second allele in a greater number of cells. We determined the distance between *RUNX1* and *ETO* alleles being simultaneously transcribed and observed that from a total of 105 Wnt3a-treated cells, 7 cells had distances <300 nm between alleles (there were no events in the control setting that showed a proximity <300 nm) and that the RNA signals in 3 of these $CD34^+$ cells localized into the same chromosomal foci following activation of the cascade (Figure 2C and supplemental Figure 5). Altogether, these observations confirm that Wnt/β-catenin enhances *ETO* and Runx1 expression in hematopoietic precursors and indicate that transcription of these genes is likely occurring into specialized nuclear factories. To further examine the hypothesis that Wnt signaling facilitates genomic proximity, we performed DNA-FISH experiments in $CD34^+$ cells and observed that *RUNX1* and *ETO* genes are indeed brought up in close proximity (<1 μm) after short-term Wnt3a treatment (4 hours) (23% vs 10%; Wnt3a vs control conditions, respectively; Figure 2D and supplemental Table 3). No significant differences in proximity were found between *RUNX1* and *ETO* at 48 hours, suggesting that an early transcriptional program commanded by the signaling cascade is involved in chromosome spatial organization and gene regulation.

Recent experiments indicate that β-catenin promotes genomic instability and T-cell transformation in mice by compromising DNA repair and enhancing illegitimate recombination.[24] Because most *RUNX1-ETO* translocations occur within *RUNX1* intron 5 and *ETO* intron 7 we designed primers in *RUNX1* exon 5 and *ETO* exon 8 to detect the product of the fusion of these genes. Notably, in $CD34^+$ cells incubated with Wnt3a for 48 hours, we observed amplification of the expected *RUNX1-ETO* chimeric product (Figure 2E and supplemental Figure 6). Finally, Wnt/β-catenin effects were further detected in HEK293 cells transfected for 48 hours with increasing doses of β-catenin S33Y (supplemental Figure 7). Our data thus indicate that sustained transcriptional activation induced by the signaling cascade represents an early event that triggers the translocation of *RUNX1* and *ETO* genes.

Deregulation of Wnt/β-catenin signaling as a causative factor in leukemogenesis has become increasingly apparent.[12-14] Here, we

Figure 2 (continued) with a distance of less than 1 μm. Scale bar represents 10 μm. Scatterplot showing shortest distances between *RUNX1* and *ETO* alleles in control and Wnt3a-treated cells for 4 and 48 hours (bottom) (number of cells and other parameters are given in supplemental Table 3). The median in each group is depicted as a red line. Statistical significance was determined by a Mann-Whitney *U* test (***$P < .0001$; NS, nonsignificant). (E) Wnt/β-catenin signaling induces a common RUNX1-ETO translocation event. Diagram representing a recurrent RUNX1-ETO translocation event (left). Detection of *RUNX1-ETO* fusion gene and *GAPDH* products in $CD34^+$ cells following long-term treatment with Wnt3a for 48 hours (middle). Chromatogram of the band shown for $CD34^+$ cells treated with Wnt3a (48 hours) (right). no RT, no reverse transcription; pAML1-*ETO*, amplification of *RUNX1-ETO* from chimeric plasmid pCMV5-*AML1-ETO*.

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studied the transcriptional program directed by Wnt/β-catenin signaling in $CD34^+$ and KG1 cells because they resemble normal human HSCs[15] and it has been suggested that immature cells, rather than committed progenitors, are the targets for leukemic transformation.[25] We observed that gain of function of Wnt/β-catenin signaling enhances transcription of *ETO* and *RUNX1* translocation partners in genome organization centers within the nucleus, thereby increasing the potential for a chromosomal translocation event. To understand the role of Wnt/β-catenin signaling in other recurrent translocations remains a goal of further investigations.

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Authorship

Contribution: G.V.D.F. and G.D.U. designed the study; G.D.U., M.F.V., M.A.M., P.L., and D.N. performed experiments, analysis, and interpretation of the data; S.E.G., A.A.E., R.T.M., and A.L. helped in drafting the manuscript and contributed cell or molecular tools; and G.D.U., M.F.V., M.A.M., and G.V.D.F. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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