

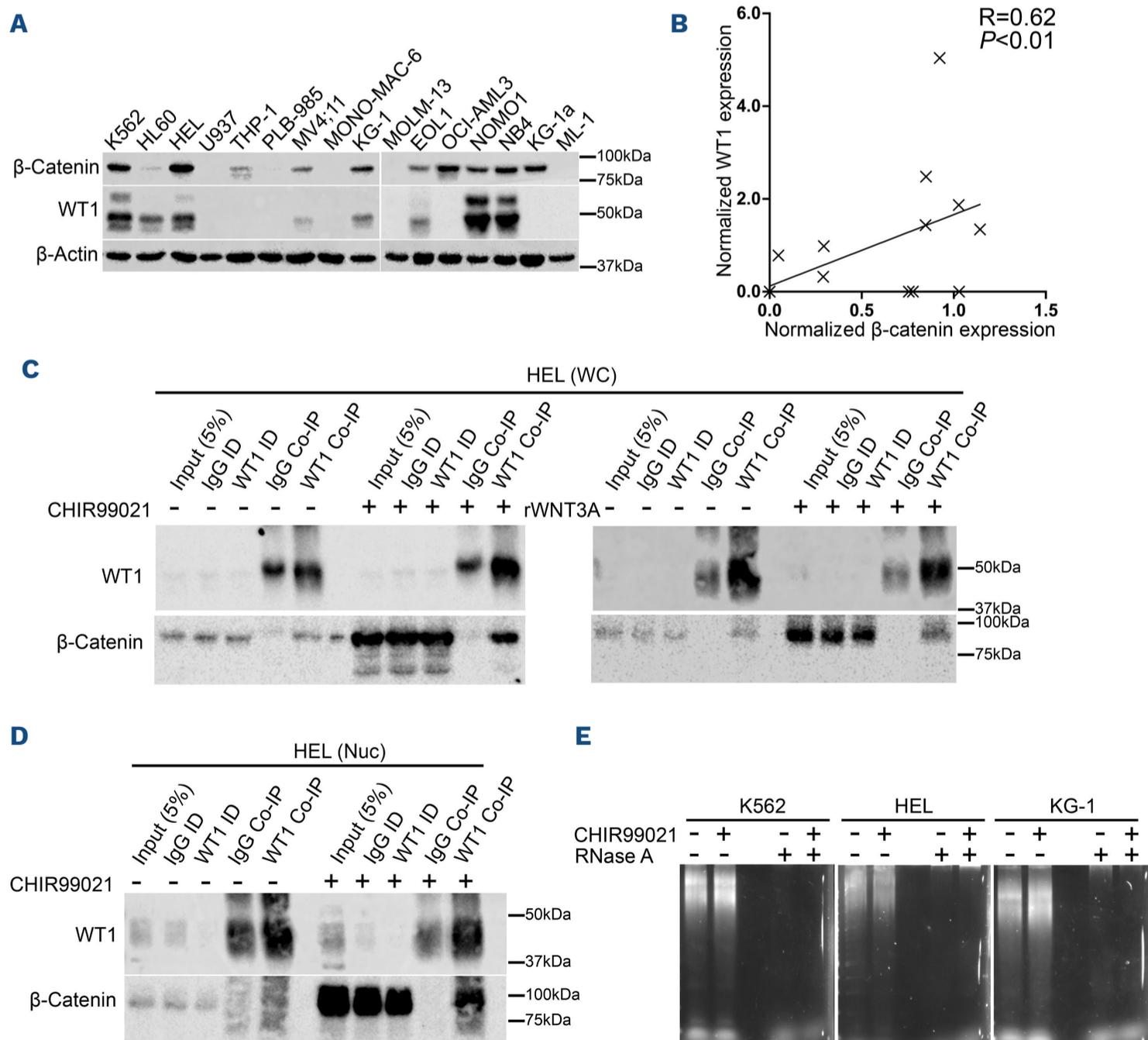
Crosstalk between β -catenin and WT1 signaling activity in acute myeloid leukemia

Acute myeloid leukemia (AML) affects around 3,200 people annually in the UK (Cancer Research UK statistics, accessed May 2021) and is a significant health burden. New targeted therapies in AML are showing promising efficacy but further novel treatments are required which target specific molecular aberrations, reduce toxicity, and induce long-lasting remissions. One such molecular target of considerable interest given its frequent dysregulation in AML is Wnt/ β -catenin signaling.

β -Catenin is the central mediator of Wnt signaling and is frequently overexpressed in AML in which it is associated with poor prognosis.¹ Wnt/ β -catenin is also known to drive the emergence and maintenance of leukemia stem cells in AML.² Protein interactions are critical to the stability, localization and activity of β -catenin, and we recently performed the first proteomic analyses of the β -catenin interactome in myeloid cells.³ This study identified Wilms

tumor protein (WT1) as a putative novel interaction partner in myeloid cells.³ WT1 is also overexpressed and mutated in AML, in which it confers inferior survival,^{4,5} yet the interplay between these two signaling proteins has not been examined previously within a hematopoietic context.

In order to identify appropriate cell lines in which to study β -catenin:WT1 interplay we first performed a screen of myeloid cell lines to examine β -catenin and WT1 protein expression. We observed a statistically significant correlation between β -catenin and WT1 expression across 16 myeloid cell lines, with 50% (8/16) co-expressing β -catenin and WT1 to varying degrees (Figure 1A, B). There was no particular association of this correlation with cell line morphology or genotype. To validate the interaction between β -catenin and WT1 we performed the reciprocal WT1 co-immunoprecipitation (Co-IP) in one of the β -



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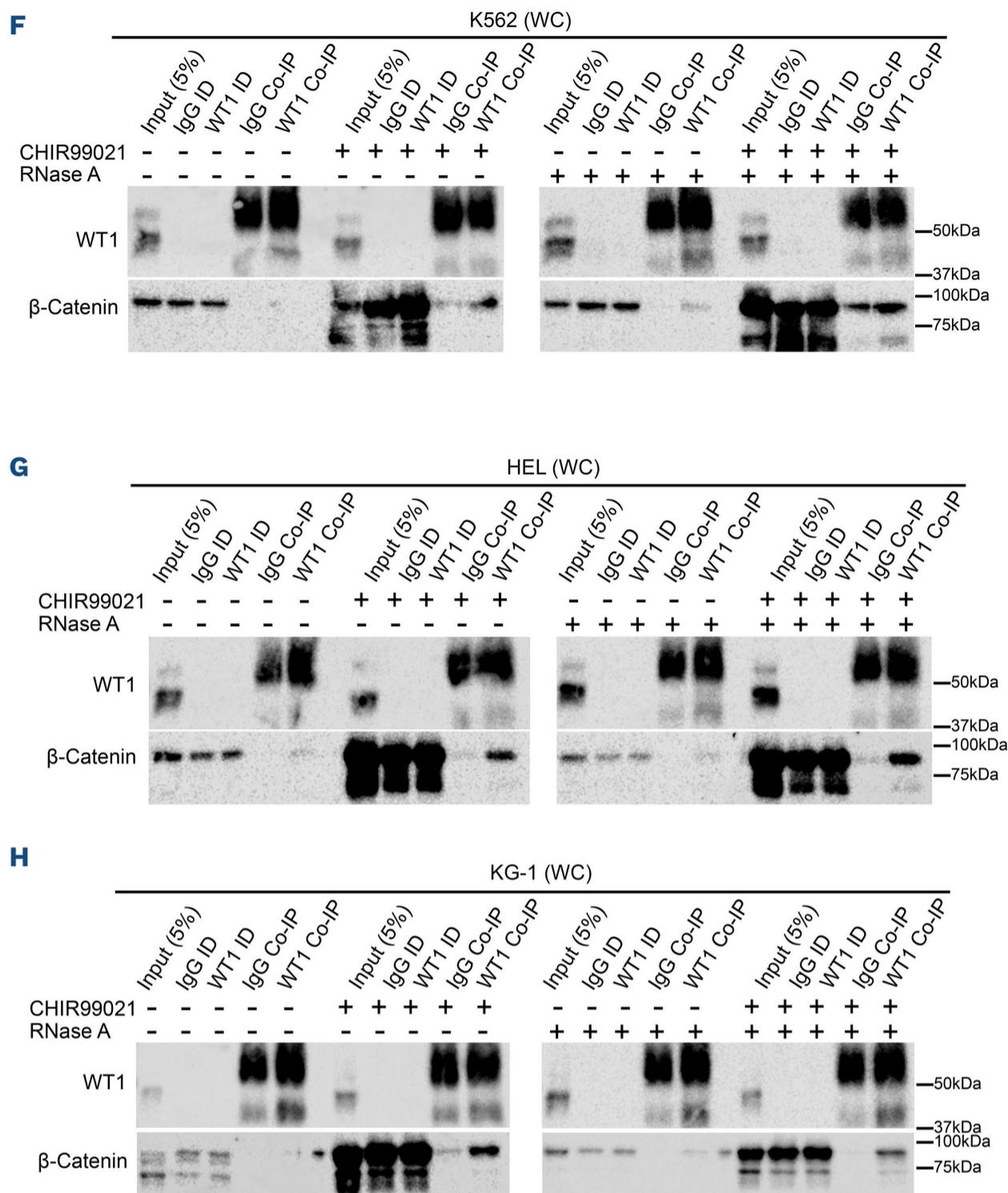


Figure 1. Association between β -catenin and WT1 in myeloid cell lines. (A) Immunoblot of myeloid leukemia cell lines showing the relative level of β -catenin (~92 kDa) and WT1 (~50 kDa) protein, with β -actin (42 kDa) used to assess protein loading. (B) Summary scatter plot showing the correlation (Spearman rank $R=0.62$, $P<0.01$) between relative β -catenin and WT1 protein expression in myeloid cell lines (normalized to β -actin expression within the cell line). (C) Immunoblots showing the level of β -catenin protein present in WT1 co-immunoprecipitation (Co-IP) derived from HEL whole cell lysate under basal (DMSO), pharmacologically induced (5 μ M CHIR99021) and naturally induced (1 μ g/mL rWNT3A) Wnt signaling conditions. (D) Immunoblot showing the level of β -catenin protein present in WT1 Co-IP derived from HEL nuclear lysate under basal (DMSO) *versus* induced (5 μ M CHIR99021) Wnt signaling conditions. (E) Agarose gel electrophoresis showing the stability of total RNA in K562, HEL and KG-1 cell lysates \pm 5 μ M CHIR99021 treated overnight \pm 20 μ g/mL RNaseA prior to WT1 Co-IP analysis. Immunoblots showing the level of β -catenin protein present in WT1 Co-IP derived from (F) K562, (G) HEL and (H) KG-1 cells \pm 5 μ M CHIR99021 and \pm 20 μ g/mL RNaseA. All immunoblots shown are representative of 3 independent biological replicates. WC: whole cell; ID: immunodepleted lysate; Nuc: nuclear; DMSO: dimethylsulfoxide.

catenin/WT1 co-expressing cell lines (HEL) and confirmed the interaction under basal conditions (with dimethylsulfoxide [DMSO] or phosphate-buffered saline/bovine serum albumin), pharmacologically activated (with CHIR99021; a GSK3 β inhibitor), and naturally activated (with recombinant rWNT3A) Wnt signaling conditions (Figure 1C). We further validated the interaction in two other myeloid cell lines (KG-1 and K562) (*Online Supplementary Figure S1A*)

and confirmed that this interaction also occurs in the nucleus (Figure 1D). WT1 is an RNA-binding protein,⁶ and β -catenin has also been shown to bind RNA,⁷ so to confirm this interaction was not indirect via RNA binding we repeated WT1 Co-IP (with or without CHIR99021) after first confirming complete digestion of RNA through RNase A pre-treatment of cell lysates (Figure 1E). As shown in Figure 1F-H, the β -catenin:WT1 interaction remained in K562,

HEL and KG-1 cells under both basal (DMSO) and stimulated (CHIR99021) Wnt signaling. We further wanted to ascertain whether this protein interaction was direct using recombinant versions of purified β -catenin and WT1 protein but failed to detect any association (*Online Supplementary Figure S1B, C*). This suggested that perhaps the interaction is mediated through a post-translational modification, common partners such as WTX,⁸ or cellular structures like DNA, given their well-documented roles in transcription.

To examine the subcellular location of the β -catenin:WT1 interaction we performed co-localization studies using confocal laser scanning microscopy. Using KG-1, K562 and HL60 cell lines, we observed that WT1 is mainly a nuclear protein with some co-localization with β -catenin (mainly cytosolic) during basal Wnt signaling. However, a significant increase in the co-localization of β -catenin and WT1 signal was observed in the nucleus during stimulated Wnt signaling in all three cell lines (Figure 2A, B) including NB4 and HEL cells (*Online Supplementary Figure S1D*). To evaluate the clinical relevance of this protein interaction we examined β -catenin and WT1 expression across a panel of primary AML patients' samples by immunoblotting. The patients' clinical details are provided in *Online Supplementary Table S1*. We observed that approximately one-third (9/30) of this cohort co-overexpressed both proteins relative to normal CD34⁺ cord blood-derived hematopoietic stem and progenitor cells (Figure 2C) – a similar frequency of co-expression to that observed in myeloid cell lines (Figure 1A). We detected β -catenin expression in hematopoietic stem and progenitor cells in keeping with the protein's self-renewal role in this context,⁹ but WT1 was undetectable, as expected, given that only 1.2% of the CD34⁺ HSPC pool are estimated to express this protein.¹⁰ Overall, we observed WT1 overexpression in around 47% (14/30) of our AML patients' blast screen, consistent with previous estimates of high WT1 overexpression in this disease.⁵ As with the cell line correlation, there was no obvious association of this co-expression with any particular clinical characteristic. From this screen we performed nuclear/cytosol fractionation on selected AML samples co-overexpressing both proteins and observed high levels of both proteins in the nucleus and cytoplasm, including the presence of the active (non-phosphorylated) form of β -catenin (Figure 2D). This would indicate that the β -catenin:WT1 interaction has the potential to be cytoplasmic or nuclear in primary AML patients' blasts as we previously showed in HEL cells.³ We further used an AML patient's sample (#4) which expressed high levels of both proteins (and for which ample viable cellular material was available) to perform WT1 Co-IP and confirmed the β -catenin:WT1 interaction by immunoblot (Figure 2E). Finally, we performed a single tandem mass tag-labeled mass spectrometric analysis of the β -catenin interactome (using β -catenin Co-IP vs. IgG Co-IP) in the highest β -catenin expressing AML patient's sample (#1) and revealed an approximately 70% enrichment of WT1 in the

nucleus (*Online Supplementary Table S2* – full interactome available via

<https://www.biorxiv.org/content/10.1101/2021.11.06.467095v2>). Taken together, these data demonstrate for the first time that β -catenin and WT1 interact in both myeloid cell lines and primary AML samples.

Given that both Wnt and WT1 signaling have been heavily implicated in AML and that crosstalk has been identified in other contexts,¹¹ we wanted to assess the potential signaling interplay between these two proteins in AML cells. Using KG-1 cells (an AML cell line expressing both proteins that could tolerate WT1 knockdown) we knocked down WT1 in KG-1 cells using two different shRNA sequences and observed a consistent reduction in the β -catenin nuclear localization capacity (Figure 3A). This corresponded with a significant reduction in basal, pharmacologically activated (with CHIR99021), and naturally activated (with rWNT3A) Wnt signaling output using the β -catenin activated reporter system (Figure 3B-D). This supports previous studies demonstrating cooperation between WT1 and Wnt signaling in other systems.¹²

WT1 mutations are frequent in AML, presenting in approximately 10% of cases but the impact on Wnt/ β -catenin signaling has not previously been investigated.⁵ Using doxycycline-inducible mutant WT1 expression constructs (a kind gift from Constanze Bonifer)⁴ we examined the impact of WT1 mutations on β -catenin expression and T-cell factor activity using frequently reported WT1 mutations. These variants include frameshift mutations to exons 8 and 9, which truncate the protein at different Zn²⁺-finger domains.⁵ The presence of these mutations has recently been found to significantly increase the growth and clonogenicity of AML cells as well as decrease apoptosis.⁴ Using KG-1 cells we confirmed the expression of the truncated WT1 mutant proteins following exposure to doxycycline, with the exon 8 mutant expressed more abundantly than the exon 9 mutant (Figure 3E). The presence of both mutations resulted in increased expression of the endogenous wild-type WT1 as reported previously,¹³ and also a concomitant increase in total β -catenin expression (Figure 3E). Examination of Wnt signaling output using the β -catenin activated reporter showed that both WT1 mutants significantly augmented T-cell factor activity (Figure 3F, G). These are the first studies to examine Wnt signaling in the context of WT1 mutations in AML and suggest that the presence of WT1 mutations in AML cells can augment Wnt signaling activation.

To complement these studies, we also performed the reciprocal experiments to examine the effect of β -catenin knockdown on WT1 expression and signaling activity. Using both shRNA and CRISPR/Cas9 we successfully reduced β -catenin in AML cell lines able to tolerate its loss; i.e., both KG-1 and NB4 (Figure 3H). Only a short-term shRNA approach was possible in HEL cells, due to the lethality of β -catenin loss in these cells. In all cell lines we observed a dramatic decrease in total WT1 protein level (Figure 3H) in

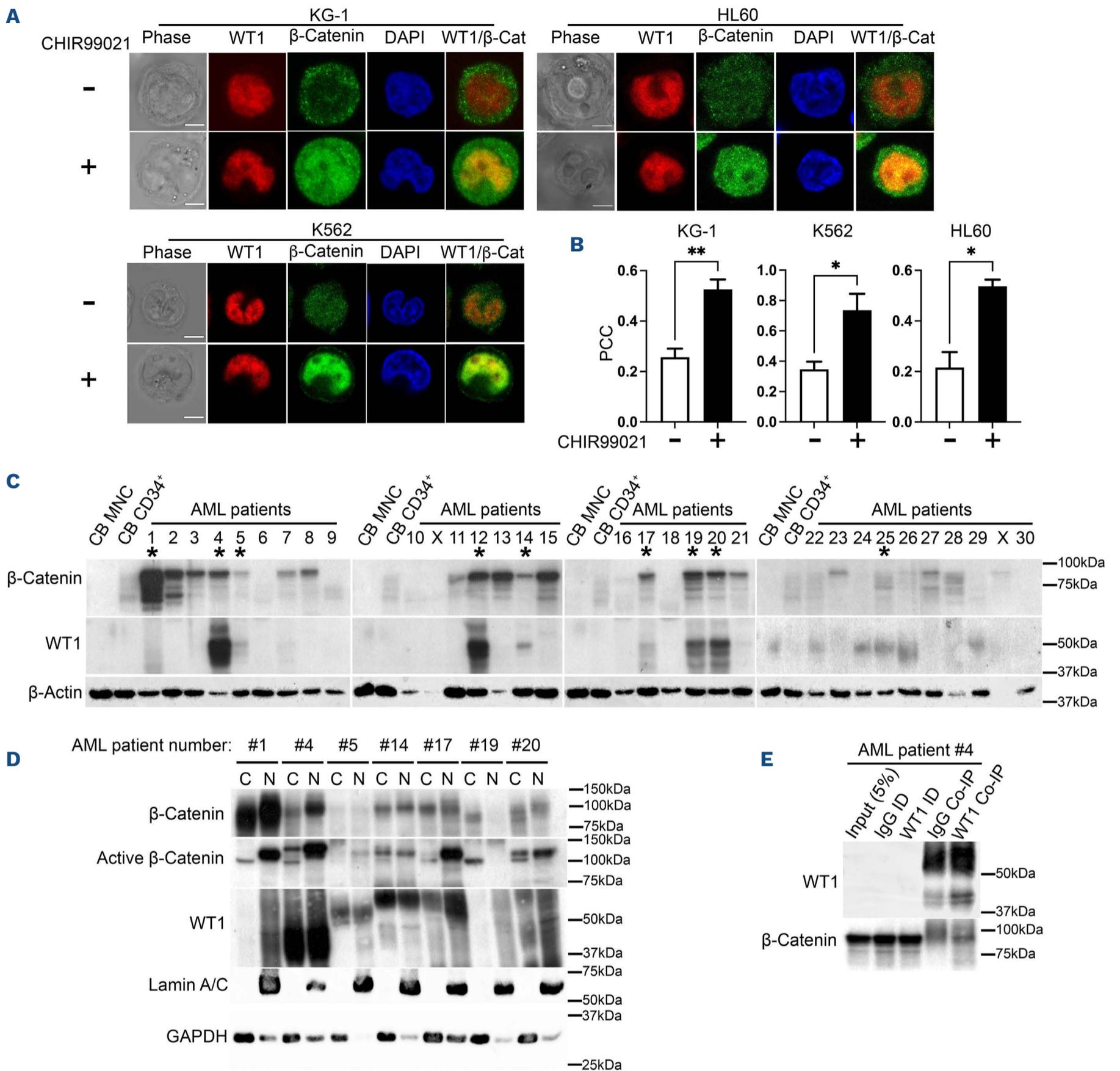
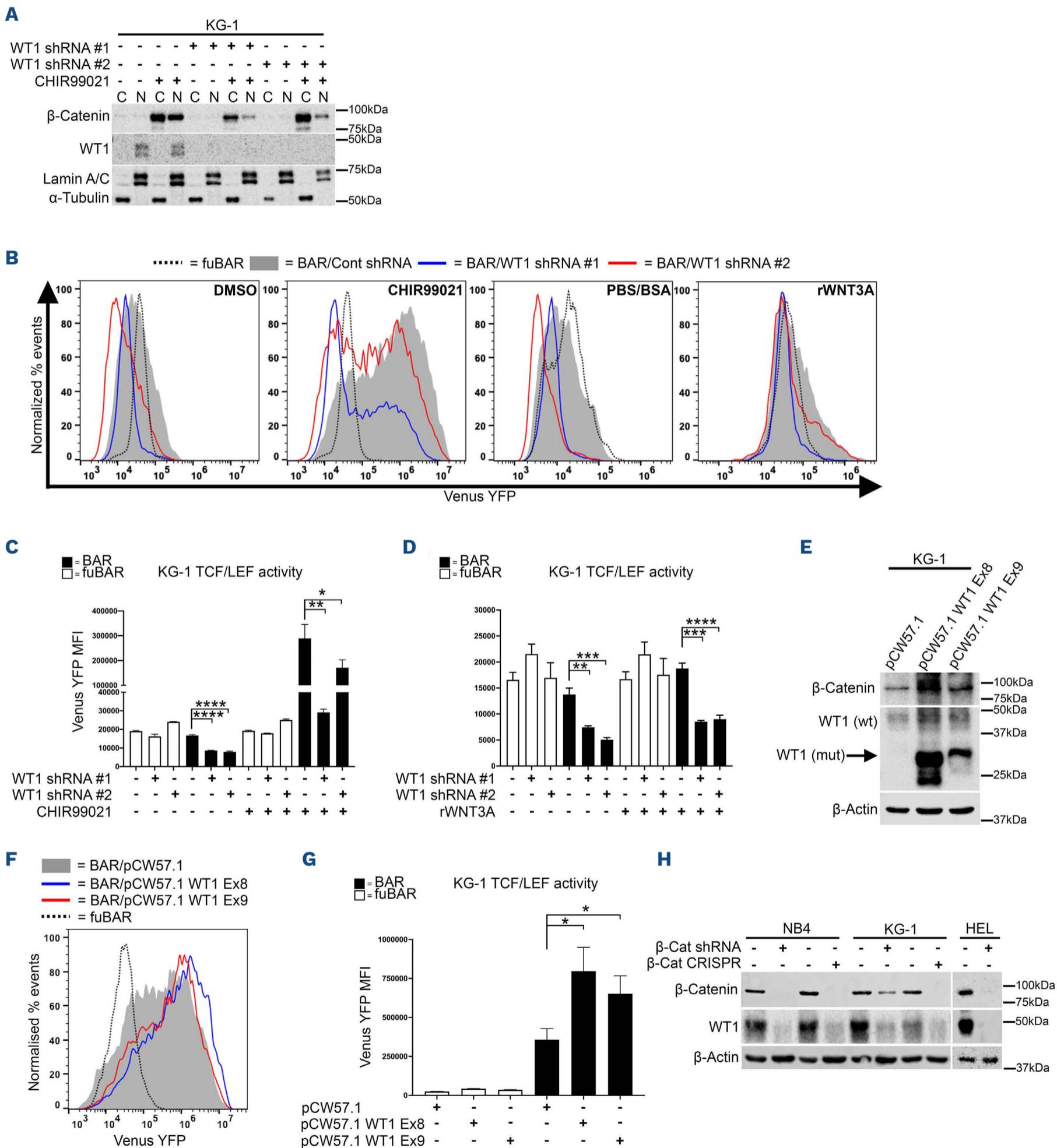


Figure 2. Co-localization and clinical relevance of the β -catenin:WT1 interaction. (A) Representative confocal laser scanning microscopy Z-sections showing β -catenin and WT1 subcellular localization in KG-1, K562 and HL60 cells \pm 5 μ M CHIR99021. Phase (gray), WT1 (red), β -catenin (green), DAPI (blue) and merged WT1/ β -catenin images are shown. Data shown are representative of 20 individual cells derived from three independent experiments, white scale bar indicates 5 μ m. (B) Correlation between β -catenin and WT1 signal in KG-1, K562 and HL60 cells \pm 5 μ M CHIR99021, as determined by the Pearson correlation coefficient (-1 = inverse correlation, 0 = no correlation, +1 = positive correlation). All data represent mean \pm 1 standard deviation as determined from a minimum of three independent experiments each containing a minimum of 20 cells per field. Statistical significance is denoted by * P <0.05 and ** P <0.01 as deduced from a Student t-test. (C) Immunoblot screen of 30 primary samples from patients with acute myeloid leukemia (AML) showing the relative level of β -catenin and WT1 protein; *denotes samples overexpressing both WT1 and β -catenin relative to levels in cord blood-derived mononuclear cells (CB MNC) and a CD34⁺ enriched fraction (CB CD34⁺) pooled from five independent cord blood samples. X = void sample as deduced from β -actin value used to assess protein loading. (D) Immunoblot showing total β -catenin, active β -catenin and WT1 localization in selected AML patients' samples co-overexpressing both β -catenin and WT1 (from the initial screen). Lamin A/C and GAPDH indicate the purity/loading of the nuclear [N] and cytosol [C] fractions. (E) Immunoblot showing the level of β -catenin protein present in WT1 Co-IP from primary AML sample from patient #4 of the sample screen. AML: acute myeloid leukemia; PCC: Pearson correlation coefficient; ID: immunodepleted lysate; N: nuclear fraction; C: cytosolic fraction; Co-IP: co-immunoprecipitation.

response to β -catenin loss. Like β -catenin, WT1 protein is ubiquitinated and regulated by the proteasome,¹⁴ therefore we hypothesized that the resultant WT1 loss upon β -catenin reduction might be a result of β -catenin protecting WT1 from proteasome-mediated degradation. However, treatment of both NB4 and KG-1 cells harboring β -catenin knockdown with the proteasome inhibitor MG132 failed to

restore WT1 protein level, and instead reduced stability further (*Online Supplementary Figure S1E*). The loss of WT1 following proteasome inhibition has been reported previously in the context of the treatment of myeloid cells with bortezomib which targeted WT1 transcripts.¹⁵ Finally, to assess the impact of β -catenin on WT1 signaling activity we used quantitative reverse transcriptase polymerase chain



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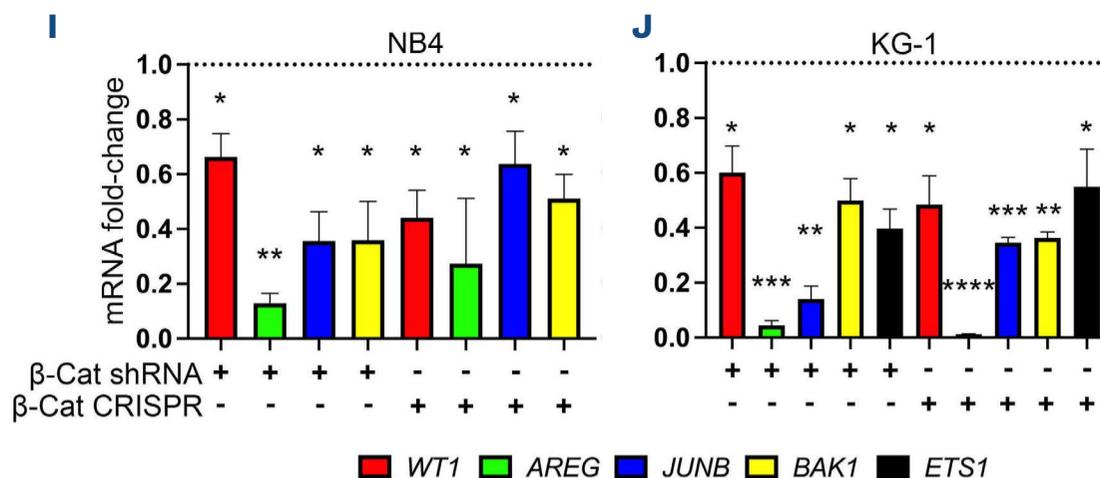


Figure 3. Crosstalk between Wnt and WT1 signaling in acute myeloid leukemia cells. (A) Immunoblot showing total β -catenin and WT1 subcellular localization in KG-1 cells lentivirally transduced with two different WT1 shRNA \pm 5 μ M CHIR99021. Lamin A/C and α -tubulin indicate the purity/loading of the nuclear [N] and cytosol [C] fractions, respectively. (B) Representative flow cytometric histograms showing the intensity of the TCF-dependent expression of Venus Yellow Fluorescent Protein (YFP) from the β -catenin activated reporter (BAR) or negative control ‘found unresponsive’ BAR (fuBAR; containing mutated promoter binding sites) in KG-1 cells \pm WT1 shRNA \pm 5 μ M CHIR99021 or \pm 1 μ g/mL rWNT3A. The fuBAR (dashed), non-targeting control shRNA (gray filled), and two WT1 shRNA (blue or red) histograms are shown. Summary graphs showing the median fluorescence intensity (MFI) generated from the BAR/fuBAR in KG-1 cells \pm WT1 shRNA and (C) \pm 5 μ M CHIR99021 or (D) \pm 1 μ g/mL rWNT3A. (E) Immunoblot showing total β -catenin and WT1 protein in doxycycline-treated KG-1 cells expressing empty pCW57.1 vector, or pCW57.1 containing inducible exon 8 and exon 9 truncating mutations of WT1. The arrow indicates the position of mutant WT1 protein and β -actin was used to assess protein loading. (F) Representative flow cytometric histograms showing the MFI of fuBAR/BAR in doxycycline-treated KG-1 cells expressing empty pCW57.1 vector, or pCW57.1 containing inducible exon 8 or exon 9 WT1 mutations following treatment with 5 μ M CHIR99021. The fuBAR (dashed), empty pCW57.1 (gray filled), pCW57.1 exon 8 mutant WT1 (blue) and pCW57.1 exon 9 mutant WT1 (red) histograms are shown. (G) Summary graph showing the MFI generated by BAR/fuBAR from doxycycline-treated KG-1 cells expressing empty pCW57.1 vector, or pCW57.1 containing inducible exon 8 or exon 9 WT1 mutations following treatment with 5 μ M CHIR99021. (H) Immunoblot showing total β -catenin and WT1 protein in KG-1, NB4 and HEL cells following lentiviral transduction with either β -catenin shRNA or CRISPR/Cas9 targeting β -catenin alongside matched respective controls. β -Actin was used to assess protein loading. (I, J) Summary graphs showing the fold change in WT1 target gene mRNA expression as assessed by quantitative reverse transcriptase polymerase chain reaction analysis in (I) NB4 and (J) KG-1 cells expressing either β -catenin shRNA or CRISPR/Cas9. Fold change is relative to matched respective controls (dashed line) and overall expression was normalized to the housekeeping gene β -actin (*ACTB*). All data represent mean \pm 1 standard deviation (n=3). Statistical significance is denoted by * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001 as deduced from a Student *t*-test. Data shown are representative of three independent biological replicates. DMSO: dimethylsulfoxide; TCF/LEF: T-cell factor/lymphoid enhancer factor; PBS/BSA: phosphate-buffered saline/bovine serum albumin.

reaction analysis to examine the mRNA expression of a panel of previously identified WT1 target genes, including *WT1*, *AREG*, *JUNB*, *BAK1* and *ETS1*,⁶ which were previously validated in KG-1 cells (*Online Supplementary Figure S1F*). In both NB4 and KG-1 cells we observed a significant reduction in all WT1 target genes assessed (except for *ETS1* in NB4 cells; *data not shown*), including *WT1* mRNA itself, upon either shRNA or CRISPR/Cas9-mediated β -catenin knockdown (Figure 3I, J). This suggests β -catenin-mediated regulation of WT1 expression is at least in part transcriptionally driven. WT1 or its targets have not previously been identified as direct Wnt target genes (https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes); however, very few such studies have been performed in a hematopoietic context, and our original study did not detect the β -catenin:WT1 interaction in colorectal cancer cells meaning this association could be highly context dependent.³ To our knowledge, this is the first report of β -catenin-mediated regulation of WT1 expression/activity in AML. Like β -catenin,¹ WT1 is overexpressed in AML where it confers inferior prognosis,^{4,5} and also similarly to β -cate-

nin,² WT1 cooperates with common genetic aberrations in AML, such as t(8;21) *RUNX1:RUNX1T1*¹⁶ and t(9;11) *MLL:AF9*¹⁴ to promote leukemogenesis. The results of this study, and previous studies suggesting functional overlap, raise the intriguing possibility of cooperation between these two frequently dysregulated proteins in AML. Such findings could be important for informing novel therapeutic strategies for targeting these oncoproteins in myeloid malignancies.

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Disclosures

No conflicts of interest to disclosure.

Contributions

MW performed experiments, analyzed data and co-wrote the manuscript. OT assisted with experiments and managed laboratory work and GC acquired flow cytometry data. HG performed and offered guidance in qRT-PCR experiments. LH and EM provided reagents and guidance for recombinant protein experiments while AB provided primary AML samples. KJ directed and performed mass spectrometry analyses. AT and RLD supplied reagents, expression constructs and

experimental guidance, and SGR supplied WT1 reagents and directed experiments. RGM performed experiments, analyzed data, co-wrote the manuscript and provided project direction.

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Data-sharing statement:

All data are available in the Online Supplementary Files and further data or methodology is available upon reasonable request.

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