Title: Crosstalk between β-catenin and WT1 signalling activity in acute myeloid leukemia

Short title: β-Catenin and WT1 crosstalk in AML

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Author contributions:

MP performed experiments, analysed data and co-wrote the manuscript. OT assisted with experiments and managed laboratory 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 whilst 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, analysed data, co-wrote the manuscript and provided project direction.

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Article summary

- β-Catenin is frequently dysregulated in acute myeloid leukemia (AML) and protein interactions govern its stability, localisation and activity, but these are poorly defined in AML.
- This study shows for the first time that β-catenin and Wilms tumour protein (WT1) interact and influence each other's expression level and signalling activity in AML cells, which could inform future therapeutic strategies.

Dear Editor,

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 that target specific molecular aberrations, reduce toxicity, and induce long lasting remissions. One such molecular target of considerable interest given it's frequent dysregulation in AML is Wnt/β-catenin signaling.[1]

β-Catenin is the central mediator of Wnt signaling and frequently overexpressed in AML where it is associated with poor prognosis.[2] Wnt/β-catenin is also known to drive the emergence and maintence of leukemia stem cells in AML.[3] Protein interactions are critical to the stability, localisation and activity of β-catenin, and we recently performed the first protemeomic analyses of the β-catenin interactome in myeloid cells.[4] This study identified Wilms tumour protein (WT1) as a putative novel interaction partner in myeloid cells.[4] WT1 is also overexpressed and mutated in AML where it confers inferior survival,[5,6] yet the interplay between these two signalling proteins has not been examined previously within a hematopoietic context.

In order to idenitfy 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 and B). To validate the interaction between β-catenin and WT1 we perfomed the reciprocal WT1 co-immunoprecipitation (Co-IP) in β-catenin/WT1 co-expressing cell lines (KG1, K562, HEL) and confirmed protein interaction under both basal (DMSO) and activated Wnt signalling (CHIR99021;

GSK3β inhibitor) conditions (Figure 1C). WT1 is an RNA-binding protein (RBP),[7] and β-catenin has also been shown to bind RNA,[8] so to confirm this interaction was not indirect via RNA binding we repeated the same Co-IPs with RNase pre-treated cell lysates (+/- CHIR99021). As shown in Figure 1D, the β-catenin:WT1 interaction remained in both K562 and HEL cells under both basal (DMSO) and stimulated Wnt signalling (CHIR99021), despite the complete digestion of RNA in cell lysates (Figure 1E). 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 association (Supplemental Figure S1A and B). This suggested that perhaps the interaction is mediated through some of the many common partners, such as WTX,[9,10] or a cellular structure like DNA, given their well documented roles in transcription. To examine the subcellular location of the β-catenin:WT1 interaction we performed co-localisation studies using confocal laser scanning microscopy (CLSM). Using HEL, KG-1, K562 and NB4 cell lines, we observed WT1 is mainly a nuclear protein with little colocalisation with β-catenin (mainly cytosolic) during basal Wnt signalling. However increased nuclear colocalisation is observed for both proteins under stimulated Wnt signalling when β-catenin tranlocates to the nucleus (Figure 1F).

To evaluate the clinical relevance of this protein interaction we examined β-catenin and WT1 expression across a panel of primary AML patient samples by immunoblotting (clinical details in Supplemental Table S1). We observed that approximately one third (9/30) of this cohort co-overexpressed both proteins relative to normal $CD34^+$ cord blood (CB)-derived HSPC (Figure 1G) – a similar frequency of coexpression to that observed in myeloid cell lines (Figure 1A). We detected β-catenin expression in HSPC in keeping with its self-renewal role in this context,[11] but WT1 was undetectable as expected given only 1.2% of the $CD34^+$ HSPC pool are estimated to express this protein.[12] Overall, we observed WT1 overexpression in around 47% (14/30) of our AML patient blast screen, consistent with previous estimates of high WT1 overexpression in this disease.[6,13] From this screen we used AML patient sample (#4) which expressed high levels of both proteins (and where ample viable cellular material was available) to perform a WT1 Co-IP and confirmed the β-catenin:WT1 interaction by immunoblot (Figure 1H). Finally, we performed a single tandem mass tag (TMT) labelled mass spectrometric (MS) analysis of the β-catenin interactome

(using β-catenin Co-IP versus IgG Co-IP) in the highest β-catenin expressing AML patient sample (#1) and revealed a ~70% enrichment of WT1 in the nucleus (Supplemental Table S2 and Supplementary MS data). 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 signalling have been heavily implicated in AML we wanted to assess the potential signalling interplay between these two proteins in AML cells given crosstalk has been identified in other contexts.[14] 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 localisation capacity (Figure 2A). This corresponded with a significant reduction in both basal and induced Wnt signalling output using the β-catenin activated reporter (BAR) system (Figure 2B and C). This supports previous studies demonstrating cooperation between WT1 and Wnt signalling in other systems.[15,16]

WT1 mutations are frequent in AML, presenting in ~10% of cases but the impact on Wnt/β-signalling has not previously been investigated.[6] Using doxycycline (DOX)-inducible mutant WT1 expression constructs (kind gift of Constanze Bonifer)[5] we examined the impact of WT1 mutations on β-catenin expression and TCF activity using frequently reported WT1 mutations. These variants include frameshift mutations to exons 8 and 9, which truncate the protein at different Zn^{2+}-finger domains.[6] The presence of these mutations has recently been found to significantly increase the growth and clonogenicity of AML cells as well as decreasing apoptosis.[5] Using KG1 cells we confirmed the expression of the truncated WT1 mutant proteins following DOX exposure, with the exon 8 mutant expressed more abundantly than the exon 9 mutant (Figure 2D). The presence of both mutations resulted in increased expression of the endogenous wild type WT1 as reported previously,[17] and also a concomitant increase in total β-catenin expression (Figure 2D). Examination of Wnt signaling output using the BAR reporter showed that both WT1 mutants significantly augmented TCF activity (Figure 2E and F). These are the first studies to examine Wnt signalling in the context of WT1 mutations in AML and suggest the presence of WT1 mutations in AML cells can augment Wnt signalling activation.

To complement these studies, we also performed the reciprocal experiments to examine the effect of β-catenin knockdown on WT1 expression and signalling activity. Using both shRNA and CRISPR/Cas9 we successfully reduced β-catenin in AML cell lines able to tolerate its loss; both KG1 and NB4 (Figure 2G). 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 2G) in response to β-catenin loss. Like β-catenin, WT1 protein is ubiquitinated and regulated by the proteasome,[18] therefore we hypothesised 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 KG1 cells harbouring a β-catenin knockdown with the proteasome inhibitor MG132 failed to restore WT1 protein level, and instead reduced stability further (Supplemental Figure S1C). The loss of WT1 following proteasome inhibition has been reported previously following the treatment of myeloid cells with bortezomib which targeted WT1 transcript.[19,20] Finally, to assess the impact of β-catenin on WT1 signalling activity we examined the mRNA expression of a panel of previously identified WT1 target genes using qRT-PCR including *WT1*, *AREG*, *JUNB*, *BAK1 and ETS-1*[7] which were previously validated in KG1 cells (Supplemental Figure S1D). In both NB4 and KG1 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. 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.[4] To our knowledge, this is the first report of β-catenin mediated regulation of WT1 expression/activity in AML. Like β-catenin,[1,2] WT1 is overexpressed in AML where it confers inferior prognosis,[5,6] and also similar to β-catenin,[3,21] WT1 cooperates with common genetic aberrations in AML such as t(8;21) *RUNX1::RUNX1T1*[22] and t(9;11) *ML::AF9*[18] 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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Figure legends

Figure 1. Association between β-Catenin and WT1 in myeloid cell lines and primary AML samples. (**A**) Immunoblot of myeloid leukemia cell lines showing the relative level of β-catenin (~92kDa) and WT1 (~50kDa) protein, with β-actin 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 expression in myeloid cell lines (normalised to β-actin expression within the cell line). (**C**) Immunoblots showing the level of β-catenin protein present in WT1 Co-IPs derived from KG1, K562 and HEL cells under basal (DMSO) and induced Wnt signalling (5μM CHIR99021) conditions. ID= immunodepleted lysate. (**D**) Immunoblots showing the level of β-catenin protein present in WT1 Co-IPs derived from K562 and HEL cells +/- 5μM CHIR99021 and +/- 20μg/mL RNaseA. (**E**) Agarose gel electrophoresis showing the stability of total RNA in K562 or HEL cell lysates +/- 5μM CHIR99021 treated overnight +/- 20μg/mL RNaseA prior to WT1 Co-IP analysis. (**F**) Representative CLSM Z-sections showing β-catenin and WT1 subcellular localisation in HEL, KG1, K562 and NB4 cells +/- 5μM 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 3 independent experiments, white scale bar indicates 5μm. (**G**) Immunoblot screen of 30 primary AML patient samples 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 $CD34^+$ enriched fraction (CB $CD34^+$) pooled from five independent cord blood samples. X = void sample as deduced from β-actin which assessed protein loading. (**H**) Immunoblot showing the level of β-catenin protein present in WT1 Co-IP performed from primary AML patient sample #4 from initial sample screen.

Figure 2. Crosstalk between Wnt and WT1 signalling in AML cells. (**A**) Immunoblot showing total β-catenin and WT1 subcellular-localization in KG1 cells lentivirally transduced with two different WT1 shRNAs +/- 5μM 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 intensity of the TCF-dependent expression of Venus Yellow Fluorescent Protein (YFP) from the β-

catenin activated reporter (BAR) reporter, or negative control 'found unresponsive' BAR (fuBAR) (containing mutated promoter binding sites) in KG1 cells +/- WT1 shRNA +/- 5μM CHIR99021. The fuBAR (dashed), non-targeting control shRNA (grey filled), and two WT1 shRNAs (blue or red) histograms are shown. (**C**) Summary graph showing the median fluorescence intensity (MFI) generated from the BAR/fuBAR in KG1 cells +/- WT1 shRNA and +/- 5μM CHIR99021. (**D**) Immunoblot showing total β-catenin and WT1 protein in doxycycline treated KG1 cells expressing empty pCW57.1 vector, or pCW57.1 containing inducible exon 8 and exon 9 truncating mutations of WT1. Arrow indicates position of mutant WT1 protein and β-actin was used to assess protein loading. (**E**) Representative flow cytometric histograms showing the MFI of fuBAR/BAR in doxycycline treated KG1 cells expressing empty pCW57.1 vector, or pCW57.1 containing inducible exon 8 or exon 9 WT1 mutations following treatment with 5μM CHIR99021. The fuBAR (dashed), empty pCW57.1 (grey filled), pCW57.1 exon 8 mutant WT1 (blue) and pCW57.1 exon 9 mutant WT1 (red) histograms are shown. (**F**) Summary graph showing the MFI generated by BAR/fuBAR from doxycycline treated KG1 cells expressing empty pCW57.1 vector, or pCW57.1 containing inducible exon 8 or exon 9 WT1 mutations following treatment with 5μM CHIR99021. (**G**) Immunoblot showing total β-catenin and WT1 protein in KG1, 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. Summary graph showing the fold change in WT1 target gene mRNA expression as assessed by qRT-PCR in (**H**) NB4 and (**I**) KG1 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 represents mean ± 1 s.d ($n = 3$). Statistical significance is denoted by *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$.

Figure 1

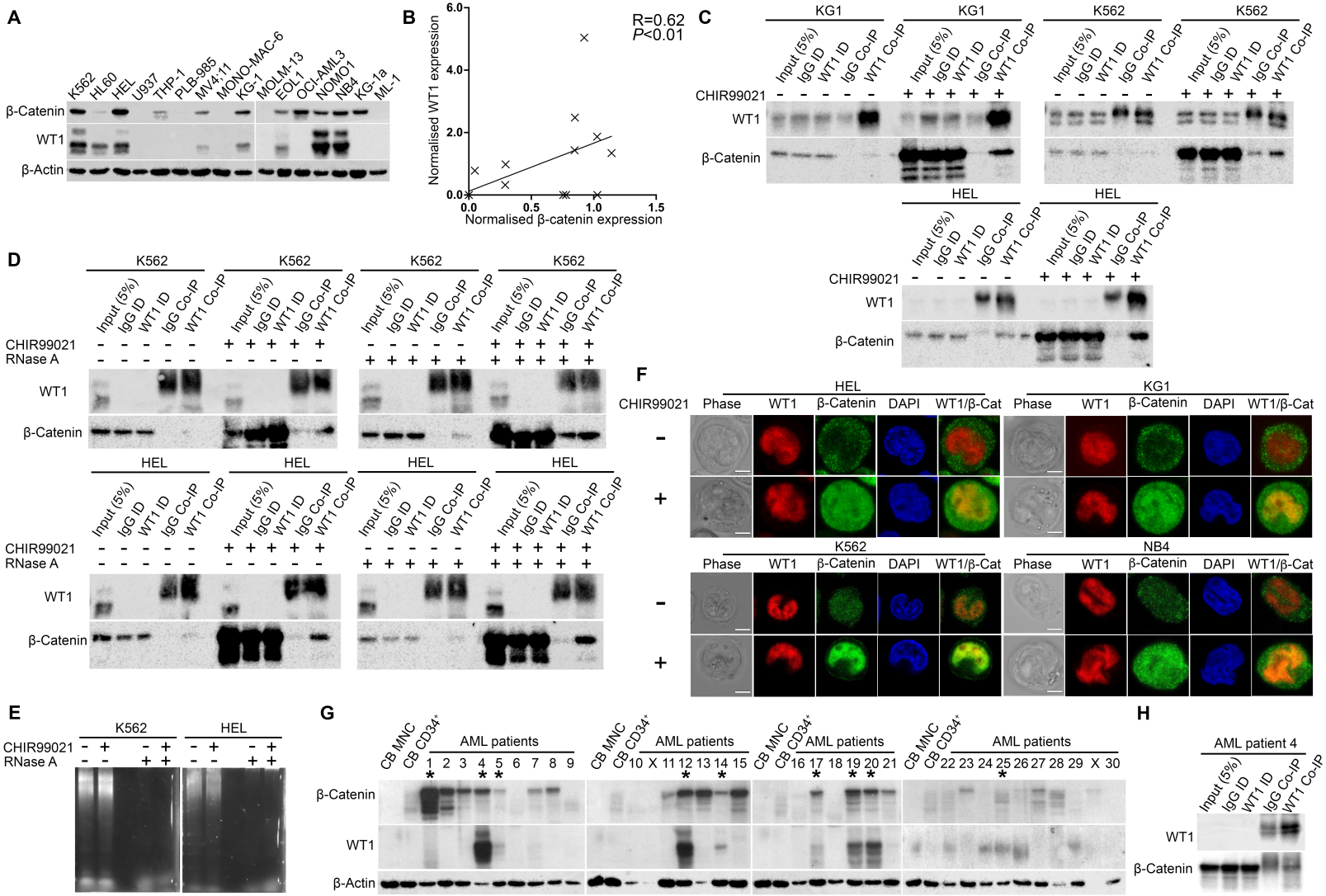
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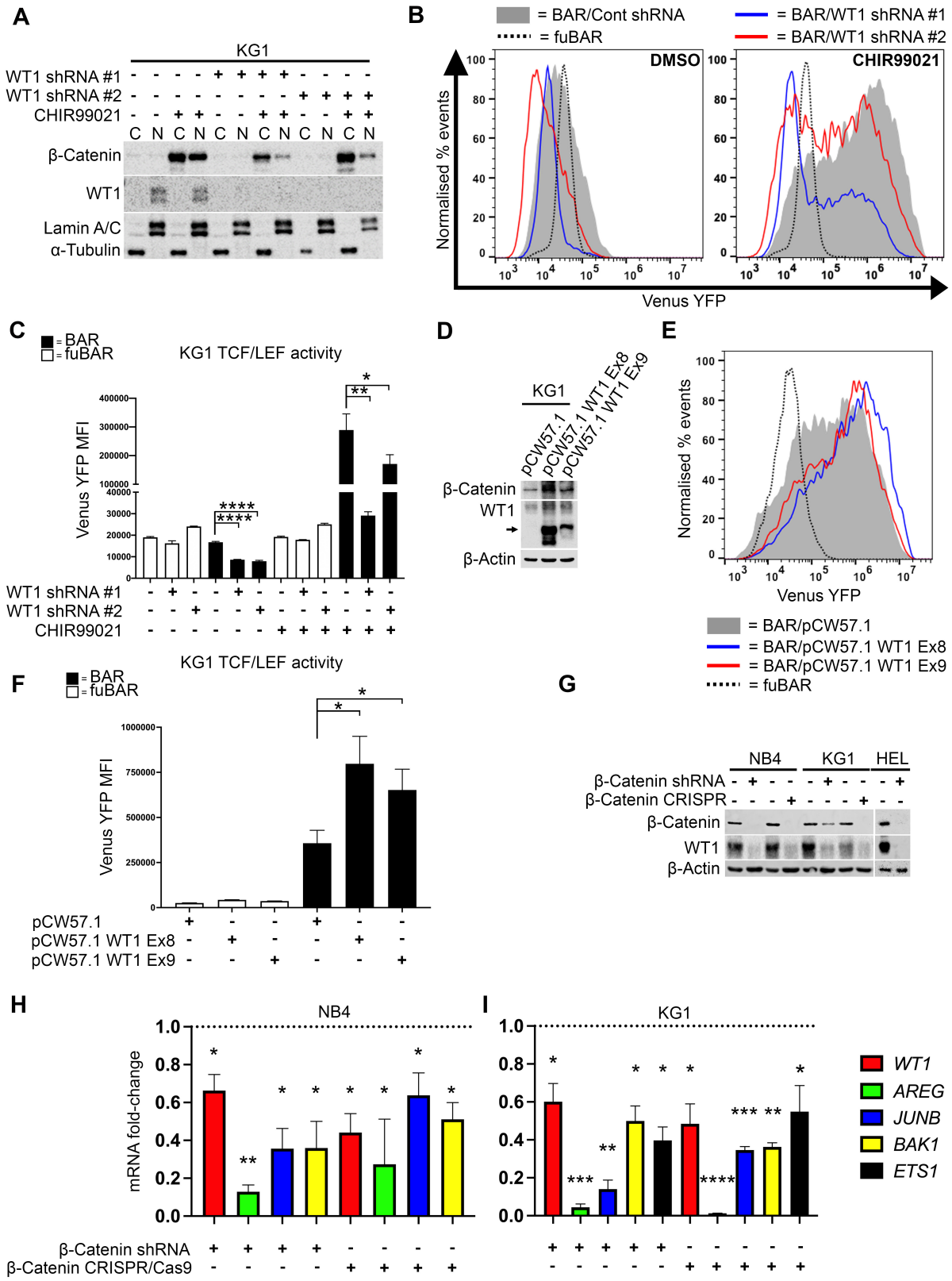
Figure 2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