Title: Dual targeting a LIN28B:β-catenin axis in acute myeloid leukaemia

Short title: Targeting a LIN28B:β-catenin axis in AML

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Highlights

- **β-Catenin interacts with the *let7* regulating RNA-binding protein (RBP) LIN28B in myeloid and lymphoid cell lines, and primary fetal hematopoietic stem cells (HSC).**
- **LIN28B regulates LEF-1 expression and subsequent Wnt/β-catenin signalling output, through a novel post-transcriptional mechanism in leukaemia cells.**
- **Dual targeting of β-catenin and LIN28B through either genetic or pharmacological means enhances the killing of leukaemia cell lines.**

Summary

Wnt/β-catenin signalling is dysregulated across several haematological malignancies, including acute myeloid leukaemia (AML), where is lacks effective targeting strategies. Previously, we discovered that β-catenin interacts with several RNA-binding proteins (RBP), and binds mRNA indirectly, indicating contributions to the post-transcriptional landscape of leukaemia cells. Here, we found the most frequent RBP-binding motif amongst β-catenin-bound mRNAs was the GGAG motif targeted by the oncofoetal expressed miRNA-regulating RBP LIN28B. We detected the β-catenin:LIN28B interaction in lymphoid and myeloid cell lines, and primary human CD34$^+$ fetal liver-derived haematopoietic stem cells. LIN28B positively regulated Wnt signalling capacity by regulating *LEF1* expression through a post-transcriptional mechanism requiring the *let7* miRNA axis. Further miRNA sequencing of β-catenin- and LIN28B-depleted myeloid cells revealed both potential cooperative and antagonistic function in miRNA regulation. Finally, dual-targeting both β-catenin and LIN28B through either genetic or pharmacological means preferentially killed AML cells. These data reveal a potential novel synthetically lethal relationship in AML which could be exploited in the rare AML subsets where LIN28B expression becomes reactivated.

Introduction

Canonical Wnt signalling is a transduction cascade critical for normal embryonic, fetal and adult development, and heavily dysregulated in human cancers.[1] The pathway regulates several key adult tissue stem cell systems including the gut, breast, hair follicle, liver and blood.[2] In particular, Wnt/β-catenin signalling plays a vital role in regulating the self-renewal,[3,4] and differentiation,[5-8] capacity of haematopoietic stem/progenitor cells (HSPC) in a dose-dependent fashion,[9,10] through both fetal and adult phases of haematopoiesis.[11-15] The importance of Wnt/β-catenin signalling to normal haematopoietic development means its activity is frequently hijacked in the development of haematological malignancies including acute myeloid leukaemia (AML). Indeed, disruption to the level, localisation and activity of the central mediator β-catenin has been observed across numerous AML subtypes,[15] and is known to contribute towards the emergence, maintenance of leukaemia stem cells (LSC), particularly those driven from *mixed-lineage leukaemia* (MLL) gene rearrangements.[16-19]

Despite extensive knowledge of aberrant Wnt signalling activity over three decades both in AML and human cancer beyond, effective β-catenin targeting strategies remain largely absent from the clinic. Previous approaches to drug Wnt/β-catenin signalling have shown promise with a number of agents reaching Phase I or II clinical trials predominantly in solid cancers,[20] as exemplified by WNT974 (PORCN inhibitor preventing Wnt ligand palmitoylation and secretion).[21,22] However, very few of these have been deployed in a haematological setting which might partly be explained by β-catenin's divergent molecular interactions in this context. For example, the well-characterised *APC*, *AXIN* and *CTNNB1* mutations which lead to Wnt signalling hyperactivation and initiate solid tumours such as colorectal cancer, are seldom reported in AML. Furthermore, β-catenin's well characterised alternative function at the cell membrane in cell-cell homotypic adhesion via cadherin complexes is likely not a prominent feature of haematopoietic tissue which is fluid in nature. Despite its extensive characterisation in epithelial tissues, β-catenin's molecular interactions/functions within haematological cells are much less well-defined and recent reports in AML suggest novel partnerships such as that with the transcription factor IRF7.[23] Our previous exploration of the β-catenin interactome in myeloid cells revealed the substantial enrichment of RNA binding proteins (RBP)[15,24] indicating a potential novel role for β-catenin in the post-transcriptional landscape of gene regulation in leukaemia cells.[25] Since this time we have characterised several β-catenin:RBP interactions including WT1,[26] TOE1,[27] and MSI2[28], and revealed β-catenin associates with an mRNA network of Wnt signalling and metabolic transcripts in leukaemia cells.[28] This study also suggested that β-catenin's interaction with RNA is likely indirect and probably through its various RBP partners. One putative interacting partner identified from our previous β-catenin interactome study was the oncofetal expressed, and miRNA regulating, RBP; LIN28B.[24]

Like OCT4 and SOX2, LIN28B is a critical pluripotency factor maintaining primitive stem cells,[29] through both its role as a suppressor of *let7* miRNA biogenesis,[30] and a direct binder of partner mRNAs.[31] In a normal haematopoietic setting, its expression is confined entirely to fetal HSPCs,[32] where it maintains stemness and suppresses differentiation through *let7*-Cbx2,[33] *let7*-Hmga2,[34] and IGF2BP3 axes.[35] Despite previous reports of β-catenin and LIN28/let7 crosstalk in several tissues including breast,[36] intestine,[37] retina,[38] and lung,[39-41] there are no studies examining the relationship of these two proteins in a haematopoietic context. This study aimed to examine the relationship between β-catenin and LIN28B in AML and evaluate any dual-targeting potential in human leukaemia.

Results

β-Catenin and LIN28B interact in myeloid leukaemia cell lines and fetal HSPC

Our previous exploration of the β-catenin interactome in haematopoietic cells revealed a dense RBP network,[24] and subsequent β-catenin RIP-seq of myeloid cells identified β-catenin enrichment with mRNAs encoding regulators of Wnt signalling pathway and metabolism.[28] These studies also suggested β-catenin has little direct RNA binding capacity and so RNA association is likely mediated through its many canonical RBP partners.[15,24] To shortlist the RBP partners most important for β-catenin's mRNA interactions, we analysed the most frequently occurring RBP binding motifs amongst the 190 commonly and significantly enriched transcripts derived from β-catenin RIP-seq in K562 and HEL cells. From this analysis we identified the GGAG motif to be the most frequently enriched binding motif (p=0.019) which is bound by the *let7* miRNA regulating and oncofetal expressed RBP; LIN28 (Figure 1A).[42] LIN28B was identified as a putative β-catenin interacting protein in myeloid cells from our original interactome study,[24] suggesting β-catenin influence in post-transcription could be mediated through a LIN28B interaction. To evaluate the merit of targeting this interaction in AML we explored *LIN28B* RNA expression across human leukaemias using the Leukaemia MILE Study dataset via BloodSpot[43] which indicated a small number of ALL, CML, and AML cases exhibit *LIN28B* reactivation (Figure 1B).

To interrogate this interaction further we examined the LIN28B and β-catenin expression across a panel of 18 myeloid cell lines. We observed variable expression of both the long and short LIN28B isoforms in approximately 50% of myeloid cell lines (Figure 1C), and co-expression of both LIN28B and β-catenin was detected in numerous cell lines which didn't particularly align with any specific geno- or phenotypic features. Notably, the alternative LIN28 paralogue, LIN28A, was not detectable in any of the myeloid cell lines examined but was detected in the T47D breast cancer cell line, confirming antibody functionality (Supplementary Figure S1A). Based on this screen, we opted to validate the β-catenin:LIN28B interaction in the two cell lines used from the original TMT mass spectrometric analysis of the original β-catenin interactome study.[24] As observed in Figure 1D, LIN28B was efficiently Co-IP'd from both K562 and HEL cells under both basal and Wnt stimulated conditions (CHIR99021 treated) and exhibited β-catenin interaction in both contexts. We also confirmed the β-catenin:LIN28B interaction in lymphoid SEM cells suggesting this interaction extends beyond a myeloid context (Supplementary Figure S1B). Given the association of both LIN28B[44] and β-catenin[25] with RNA, we wanted to rule out non-specific interaction of both proteins due to RNA co-occupancy. Complete digestion of RNA in input lysates prior to co-IP through RNase A treatment, had no impact on the β-catenin:LIN28B interaction (Figure 1E), suggesting the two molecules may be complexed together regardless of RNA presence. However, AlphaFold modelling suggests against a direct interaction between the two proteins suggesting other common intermediate proteins or structures could mediate the association (Supplementary Figure S1C).

We next wanted to test whether this interaction could be detected in primary AML samples and screened LIN28B expression across primary AML patient samples we've previously examined for β-catenin expression.[26,28] We were unable to detect LIN28B in our 30 patient cohort (Supplementary Figure S1D) in keeping with low LIN28B expression in adult haematopoietic tissue.[45] Instead, given LIN28B's well characterised role as a regulator of fetal HSC emergence and development,[32-35] a setting also requiring active Wnt/β-catenin signalling,[11-13] we repeated the LIN28B Co-IP from human fetal liver (FL)-derived CD34$^+$ HSPC. After confirming enrichment of FL-CD34$^+$ HSPC to >85% purity (Figure 1F), and abundant levels of both proteins in input lysates, we confirmed β-catenin enrichment through LIN28B Co-IP (Figure 1G). Taken together, these findings identify LIN28B as a novel interacting partner of β-catenin in both myeloid and lymphoid cell lines, and primary human FL-CD34$^+$ HSPCs.

LIN28B modulates Wnt/β-catenin signalling output through LEF1

Given previous reports of LIN28B and Wnt signalling crosstalk in solid tissues,[36,37] we next wanted to assess the degree and direction of crosstalk in leukaemia cells. To examine this, we employed the β-catenin activated reporter (BAR) system adopted previously,[26,28,46] to assess TCF/LEF activity upon LIN28B modulation. Upon confirmation of efficient LIN28B knockdown using shRNA (Figure 2A), we observed a ~2-fold decrease in TCF/LEF activity in K562 cells upon Wnt signalling activation (Figure 2B and C, $p < 0.05$). Reciprocally, ectopic LIN28B promoted a ~2-fold increase in TCF/LEF activity under active Wnt signalling conditions (Figures D and E, $p < 0.05$). Collectively, these results indicate that LIN28B is a positive regulator of Wnt/β-catenin signalling in myeloid cells.

To investigate the underlying mechanism of LIN28B regulation of Wnt signalling output we first examined β-catenin expression and localization following LIN28B ablation. We observed no significant changes to overall β-catenin level (Figure 2F) or subcellular localization (Figure 2G). We next checked the level of the downstream Wnt transcription factors LEF1 and TCF4 (*TCF7L2*) and observed a consistent reduction in both *LEF1* mRNA and protein levels in K562 cells following LIN28B depletion (Figure 2F, G, and H). Reciprocally, ectopic expression of LIN28B in K562 cells led to substantially increased LEF-1 protein levels (Figure 2D). Finally, we wanted to determine whether LIN28B regulation of LEF-1 occurred through transcriptional mechanisms since LIN28B has been linked with transcriptional regulation previously.[47] To test this, we designed *LEF1* primers spanning intronic regions of *pre-LEF1*, and assessed abundance following LIN28B depletion. As a control, we first validated these intronic primers in K562 cells treated with CHIR99021 to induce Wnt signalling activation since *LEF1* is a known Wnt signalling target gene,[48] which confirmed a 4-fold increase compared to DMSO-treated cells (Supplementary Figure S2A). Using these validated primers, we next measured *LEF1* levels in LIN28B-depleted K562 cells and observed no change versus control NT-shRNA K562 cells (Supplementary Figure S2B), suggesting regulation through post-transcriptional means. Taken together, these findings indicate that LIN28B can positively influence Wnt/β-catenin signalling activity through post-transcriptional modulation of LEF-1.

LIN28B regulation of LEF1 is partly mediated through let-7 miRNA

Next, we aimed to investigate the mechanism through which LIN28B regulates LEF-1 expression in myeloid cells. Initially we hypothesised this could be through direct transcript binding since LIN28B is a well-known mRNA binding protein,[44] and such interactions influence target expression/stability as evidenced with TP53,[49] and several splicing factors (*FUS/TLS*, *hnRNP F*, *TDP-43* and *TIA-1*).[42] Adopting both LIN28B RIP and CLIP approaches from K562 cells, we first confirmed efficient enrichment of *LIN28B* mRNA as a positive control, since LIN28B is known to bind and autoregulate its own transcript (Supplementary Figure S3A-C).[42] We also confirmed the significant enrichment of *LEF1* mRNA (Supplementary Figure S3A-C), however subsequent assessment of *LEF1* mRNA stability in LIN28B-depleted cells failed to show any marked differences from control cells (Supplementary Figure S3B). We next considered whether regulation of *LEF1* could occur through *let-7*-dependent mechanisms given the well-characterised role of LIN28B in the repression of *let-7* miRNA maturation.[30] To first evaluate if LIN28B impacted *let-7* expression in myeloid cells we performed miRNA-seq in K562 cells with LIN28B shRNA (Supplementary Figure S4A). We also examined miRNA expression in β-catenin depleted cells (Supplementary Figure S4A) given its previous link regulating *let-7* expression in breast cancer cells,[36] and to reveal any potential overlapping function with LIN28B. Clustering analysis revealed clear and distinct miRNA expression profiles across control, LIN28B and β-catenin shRNA samples (Supplementary Figure S4B; E-MTAB-16028; ArrayExpress). Comparison of miRNA expression levels between both LIN28B-depleted and β-catenin–depleted cells relative to control (NT shRNA), revealed significantly upregulated and downregulated miRNAs in each condition (Supplementary Figures S4C-H; see also Supplemental miRNA-seq data sheet). As expected, numerous *let-7* miRNAs, including *let-7d*, *let-7i* and *let-7f-1*, were significantly upregulated in response to LIN28B ablation (Supplementary Figures S4C). In contrast, we observed no significantly differential *let-7* miRNA expression with β-catenin depletion in K562 cells (Supplementary Figure S4D), suggesting β-catenin is not a major regulator of mature *let-7* miRNA expression in a myeloid setting. However, several other miRNA species were differentially regulated in response to β-catenin modulation (Supplementary Figures S4E-H and Supplemental Table S1), including several overlapping with LIN28B depletion (Supplementary Figure S4C and Supplemental Table S2), suggesting novel miRNA regulating roles which could be mediated in cooperation with LIN28B.

Given that multiple *let-7* miRNAs were induced following LIN28B suppression we opted to globally inhibit their expression, rather than interrogating individual *let-7* family members, as a means of evaluating *LEF1* regulation. For this we deployed C1632, a small molecule inhibitor (SMI) which blocks LIN28:*let-7* interaction resulting in *let-7* induction.[50] Following overnight treatment with two doses of C1632 we observed substantially reduced levels of LEF-1 protein (Figure 2I) and *LEF1* exonic mRNA (Figure 2J), in both K562 and HEL cells which was significantly depleted at 100µM, however intronic *LEF-1* remained unchanged (Figure 2K) confirming post-transcriptional, rather than transcriptional regulation, by C1632. Examination of TCF/LEF activity in C1632-treated cells, also revealed dose dependent depletion of Wnt signalling capacity in response to C1632 treatment in both K562 and HEL cells (Figure 2L). In summary, these findings indicate that LIN28B may regulate LEF-1 expression through a *let-7* miRNA dependent mechanism.

Dual β-catenin and LIN28B targeting decreases AML cell death viability

Public expression databases suggest LIN28B reactivation can occur in discrete human leukaemia subtypes including normal/complex karyotype and *MLL*-rearranged AML (Figure 1B), settings where aberrant β-catenin level, localisation or activity is also reported.[16-19,51,52] Therefore, dual-targeting of the LIN28B:β-catenin axis could be clinically viable given the molecular cooperation indicated from previous experiments. To test this, we initially deployed two doses of C1632 (10 and 100µM) against THP1 and HEL cells expressing either control or β-catenin shRNA (Figure 3A) across 24, 48 and 72 hours and assessed cell viability. As seen in Figures 3B and C, 100µM C1632 significantly reduced the viability of both β-catenin depleted AML cell lines relative to NT-shRNA controls at 72hrs treatment with both shRNAs, also impacting HEL cells from 48hrs onwards. However, no increased C1632 sensitivity was observed in other β-catenin depleted myeloid cell lines (K562 and KG-1 cells; Supplementary Figure S5), implying subgroup specificity for where the β-catenin:LIN28B axis promotes survival.

Reciprocally, we also aimed to assess the impact of targeting β-catenin through SMIs in a LIN28B-depleted setting. We reasoned that compounds targeting β-catenin stability, rather than TCF/LEF or CBP/p300 interaction, were more applicable in this scenario given its interaction and subsequent miRNA regulation with LIN28B is likely post-transcriptional. We first needed to identify an efficient β-catenin degrader in leukaemia cells, since many SMIs were optimised in solid tissues where mechanisms of Wnt/β-catenin disruption differ from leukaemia cells.[15,25] To our surprise, many of the proposed β-catenin degraders were ineffective at reducing β-catenin stability across a 24hrs exposure in myeloid cells including LGK974, MSAB, KYA1797K, XAV-939, and BC2059 (Tegatrabetan), and the β-catenin targeted PROTAC xStAx-VHLL (Supplementary Figure S6). These data suggest β-catenin stability is governed by completely distinct mechanisms in leukaemia cells. The only agent mildly effective at reducing β-catenin stability from 5µM was pyrvinium pamoate (PP; Figure 4A) which is an FDA-approved Casein Kinase-1α (destruction complex component) agonist and anthelmintic known to also have anti-cancer activity, especially against B-ALL[53] and MLL-rearranged AML.[54,55] Therefore, we treated LIN28B shRNA expressing HEL and THP1 cells (Figure 4B) with 5µM PP and assessed cell viability 24-72hrs. As observed in Figure 4C, PP significantly increased the death of LIN28B-depleted HEL cells versus NT-shRNA control counterparts. THP1 cells were exquisitely sensitive to PP with NT-shRNA control cells exhibiting ≥ 90% cell death with a 5µM dose after just 24 hours making any cooperative cell killing challenging to observe (Supplementary Figure S7A). Therefore, we dropped the PP concentration to 0.5µM and assessed cell viability 24-72hrs but failed to observe differential PP sensitivity between THP1 cells with LIN28B shRNA versus NT-shRNA controls (Supplementary Figure S7B). However, it should be noted that upon immunoblotting of β-catenin levels, 0.5µM PP was insufficient to reduce β-catenin stability until 72hrs in THP1 cells (Supplementary Figure S7C) which was much quicker in HEL cells. Finally, to evaluate a proof-ofconcept approach to targeting LIN28:β-catenin in AML cells we deployed a dual targeting strategy using both SMIs. As shown in Figure 4D, dual treatment of HEL cells with C1632 and PP led to more cell death in combination than using either drug individually. Taken together, these data support the notion that dual β-catenin:LIN28B targeting could be an effective strategy in discrete subgroups of AML where LIN28B is reactivated and β-catenin can be efficiently degraded.

Discussion

This study has demonstrated β-catenin and LIN28B interact for the first time in any context. AlphaFold modelling suggests this interaction is indirect, with our data suggesting an RNA-independent association that may be part of a wider protein complex. Indeed, this is now the third RBP interaction with β-catenin that we've demonstrated has regulatory capacity over LEF-1 expression,[27,28] and points to β-catenin constituting a wider post-transcriptional regulatory network that governs expression of this Wnt transcription factor. We've not been able to characterise the functional significance of the β-catenin:LIN28B interaction but our data suggests no capacity for either to regulate the stability/localisation of the other. Instead we speculate this interaction could impact miRNA expression since our miRNA-seq experiment identified co-regulated targets from LIN28B and β-catenin depletion, and cooperation between β-catenin and LIN28A in miRNA regulation has been reported in the expansion of breast cancer stem cells.[36] This study also demonstrated that LIN28B is able to regulate expression of LEF-1, likely through a post-transcriptional or translational mechanism. Crosstalk between Wnt/β-catenin signalling and LIN28 has been observed frequently in both normal development,[38,41,56-58] and cancer,[37,59] however this is the first report in a haematopoietic context and the first to implicate LEF-1 regulation. Our experiments using the LIN28:*let7* inhibitor C1632 suggest this regulation is primarily post-transcriptional in nature and potentially via miRNA regulation of *LEF1*. One previous study reports *let7* regulation of LEF-1 expression (via *let-7i-3p*),[60] however there are numerous miRNAs reported to regulate *LEF1* expression including miR-26b,[61,62] miR-34a,[63-67] miR-181a,[68] miR-218,[69] miR-223,[64] miR-381,[70] miR-557,[71] and miR-621,[72] suggesting further mechanistic characterisation in relation to LIN28B will be important in a haematopoietic setting. Interestingly, miR-181a and miR-34a were amongst the most significantly increased miRNAs following LIN28B and β-catenin depletion, respectively, in our study, indicating a potential novel route for *LEF1* regulation in haematopoietic cells.

LIN28B itself has an enigmatic and complex role in normal and malignant haematopoiesis. Expression is reportedly restricted to fetal haematopoiesis,[32] where it regulates fetal HSC development through *let-7*–Hmga2,[34] *let-7*-Cbx2,[33] and IGF2BP3 axes.[35] Active Wnt/β-catenin signalling also contributes to these early stages of haematopoiesis,[11-13] and our finding that β-catenin interacts with LIN28B in purified human fetal liver CD34$^+$ HSPCs raises the intriguing possibility of crosstalk/cooperation between these proteins in early haematopoietic development. Upon transition to adult haematopoiesis, LIN28B expression is rapidly downregulated (permitting mature *let-7* expression) with expression only reactivated in a small number of leukaemias. There is evidence for LIN28B serving both tumour suppressive and oncogenic functions in malignant haematopoiesis. *In vivo* models of MLL-derived myeloid leukaemogenesis (MLL-AF9 and MLL-ENL) have demonstrated that LIN28B expressing fetal progenitors have reduced transformation capacity, and only the reduction of LIN28B expression in these cell provides a leukaemia-permissive environment.[45,73] In myeloid neoplasms, LIN28B reactivation appears to have a pro-leukaemic role contributing to differentiation arrest,[74] growth/survival,[75] and promotion of a stem-like transcriptional programme.[76] In CML, LIN28B expression is enhanced in blast phase versus chronic phase, and promotes proliferation through a miR-181d-PDCD4 axis (miR-181d also upregulated in our β-catenin shRNA miRNA-seq) which involves a LIN28B DNA-binding function.[77] A novel fetal-like subgroup of juvenile myelomonocytic leukaemia (JMML) cases also exhibit LIN28B reactivation where its expression predicts inferior survival.[78,79] Collectively this implies whilst pre-existing LIN28B expression appears to inhibit leukaemogenesis, in rare number of AML cases where LIN28B becomes reactivated it can serve oncogenic roles likely through its ability to confer stem-like properties.

Although this study has not elucidated the functional role of the β-catenin:LIN28B interaction, we have shown that dual targeting of both molecules may have therapeutic merit in subsets of myeloid leukaemia where they are both expressed. Notably, one of the cell lines susceptible to the β-catenin shRNA/C1632 combination was THP-1 cells which is an MLL-rearranged myeloid cell line, and expression databases suggest LIN28B reactivation occurs in a small subset of MLL leukaemias of both myeloid and lymphoid origin (Figure 1B). LIN28B inhibitors remain in their infancy as the importance of this RBP in human cancer continues to emerge. Although C1632 has shown activity against several solid cancers,[80-84] it's not a particularly efficient compound requiring high doses such as those used in this study (100µM) to be effective. However, this evidence shows in principle that LIN28B targeting could be a worthwhile approach in the treatment of specific AML cohorts if efficacious inhibitors could be developed. Several new LIN28 inhibitors are currently in development,[85-88] such as ln268,[89] and if successful it's likely they'll exhibit a favourable toxicity profile in AML given LIN28B is not expressed in normal adult haematopoietic tissues limiting on-target toxicity.

Even if LIN28B inhibition can be refined, our study highlights that β-catenin drug targeting in leukaemia remains a formidable challenge. Many of the compounds proposed to reduce β-catenin level in solid tissues, including LGK974 (PORCN inhibitor), MSAB (β-catenin degrader), KYA1797K (Axin agonist), XAV-939 (Tankyrase inhibitor), BC2059 (Transducin β-Like 1 blocker), and xStAx-VHL (β-catenin PROTAC) were largely ineffective in leukaemia cells, pointing to alternative modes of β-catenin stabilisation/regulation in myeloid leukaemia yet to be defined. The only compound that showed any capacity for β-catenin degradation was PP which is an FDA-approved quinoline-derived cyanine dye and antihelminth drug that boosts the activity of Casein Kinase-1α, a critical destruction complex component responsible for providing the serine/threonine phosphorylation necessary for β-catenin's subsequent ubiquitination and proteasomal degradation. In recent years, PP has emerged as an anti-tumour compound with activity against leukaemias,[53-55] however PP is not a specific Wnt/β-catenin inhibitor, also acting through mitochondrial disruption, PI3K/AKT inhibition, and UPR activation.[90] Therefore, there remains an urgent and unmet need for specific and efficient β-catenin degraders in myeloid leukaemia.

In summary, we've identified an interaction between β-catenin and LIN28B, and shown LIN28B can impact Wnt signalling activity in haematopoietic cells through the post-transcriptional regulation of LEF-1 expression. This study has demonstrated that dual targeting of β-catenin and LIN28B could represent an novel and effective strategy in specific AML subgroups where both proteins are actively contributing to disease biology.

Author contributions

OS performed experiments, analysed data and co-wrote the manuscript. MW managed laboratory, performed experiments and provided experimental guidance. RL assisted with FL-CD34 HSC experiments whilst AG, DP, HP and KH generated contributing data, and AB provided primary AML samples. LC and SN provided reagents and experimental advice, while AR provided primary FL-CD34 HSCs and conceptual guidance. BT and RGM performed experiments, analysed data, co-wrote the manuscript and conceived/directed the study.

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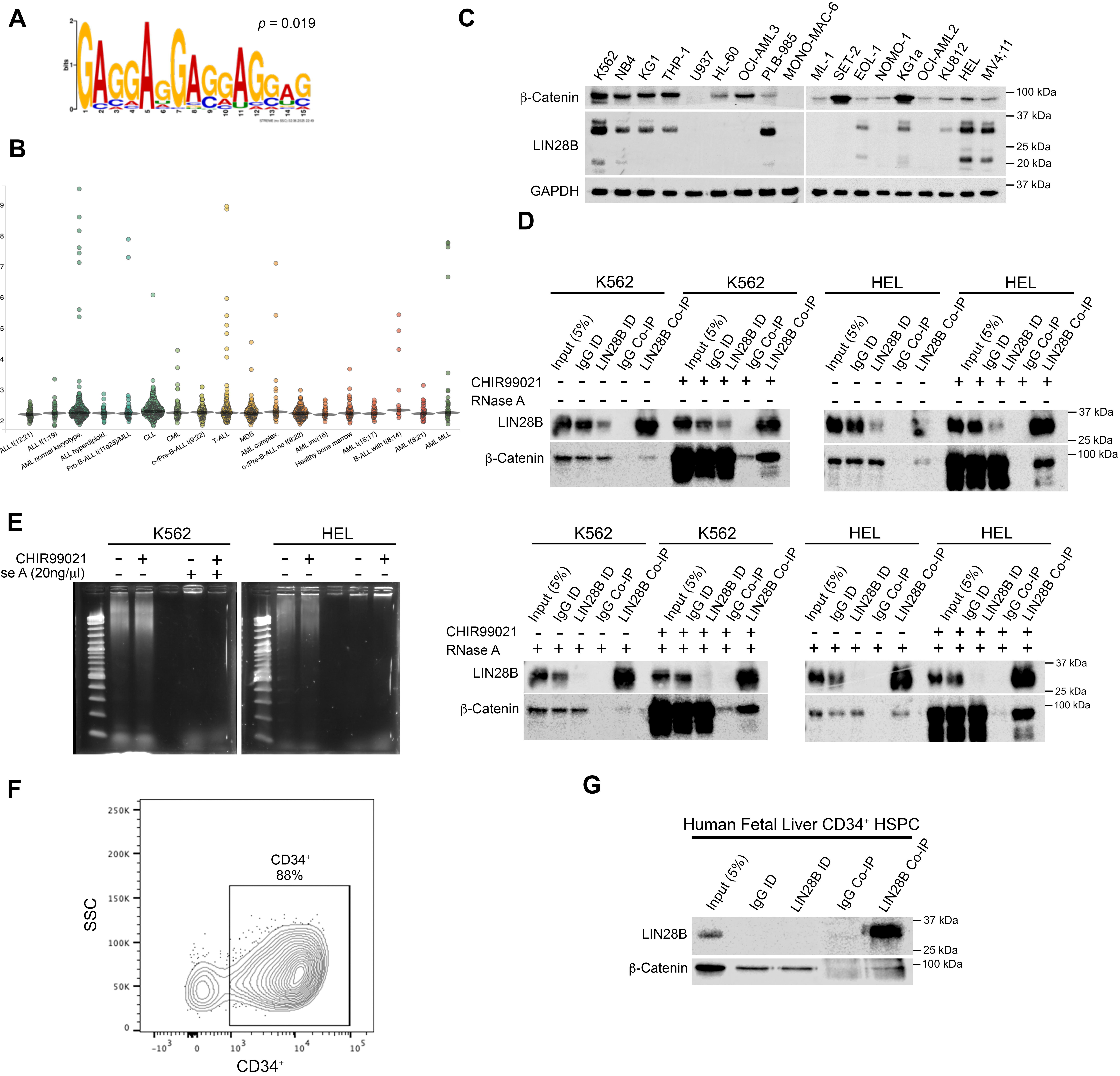
Figure legends

Figure 1. (A) RBP binding motif analysis from transcripts commonly and significantly enriched from β-catenin-RIP-seq performed in K562 and HEL cells.[28] **(B)** RNA expression profiles from the Leukeamia MILE study on BloodSpot showing *LIN28B* mRNA expression across healthy bone marrow and numerous subtypes of human myeloid and lymphoid leukaemias. **(C)** Immunoblots showing β-catenin and LIN28B (long and short isoforms) expression across 18 myeloid leukaemia cell lines. GAPDH was used as a loading control. Immunoblots show β-catenin level in LIN28B Co-IPs from K562 and HEL whole cell lysates following overnight ± 5µM CHIR99021 treatment. **(D)** Immunoblots showing β-catenin levels in LIN28B Co-IPs from K562 and HEL whole-cell lysates following overnight treatment ± 5µM CHIR99021, without RNase. **(E)** Same as in **(D)**, performed with RNase A treatment (20µg/mL). **(F)** Flow-cytometric contour plot demonstrating the purity of $CD34^+$ HSCs isolated from human fetal liver. **(G)** Detection of β-catenin protein in LIN28B Co-IPs from human fetal liver $CD34^+$ HSCs. ID: immunodepleted lysate.

Figure 2. (A) Immunoblots showing LIN28B expression in K562 cells harbouring the BAR (β-catenin–activated reporter) or fuBAR (found unresponsive BAR; negative control) system with ± LIN28B shRNA. **(B)** Representative flow-cytometric histograms showing Venus Yellow Fluorescent Protein (YFP) intensity, reflecting TCF-dependent activity, in K562 cells with ± LIN28B shRNA following overnight treatment ± 5µM CHIR99021. The dashed histogram represents the fuBAR control, while the grey-filled histogram corresponds to non-targeting control shRNA. Blue and red histograms represent two LIN28B shRNAs. **(C)** Summary bar graph showing YFP MFI from the BAR/fuBAR system in K562 cells with ± LIN28B shRNA following overnight treatment ± 5µM CHIR99021. **D)** Immunoblot showing LIN28B and LEF-1 protein expression in K562 cells transduced with full-length human LIN28B cDNA (pLV-hLIN28B) versus an empty-vector (EV) control with GAPDH used as a loading control. **(E)** Summary bar graph showing YFP MFI from the BAR/fuBAR system in K562 cells ± pLV-hLIN28B following overnight treatment ± 5µM CHIR99021. **(F)** Immunoblots showing expression of LIN28B, β-catenin, LEF-1, and TCF4 in K562 cells with ± LIN28B shRNA. GAPDH was used as a loading control. **(G)** Immunoblots showing β-catenin, LEF-1, and TCF4 protein expression in cytoplasmic (C) and nuclear (N) fractions of K562 cells with ± LIN28B shRNA following ± 5µM CHIR99021. Lamin A/C and α-tubulin were used as fraction purity and loading controls. **(H)** Scatter plot showing fold changes in *LIN28B*, *LEF1*, *TCF7*, and *TCF7L2* mRNA levels in K562 cells with ± LIN28B shRNA, normalized to *GAPDH*. **(I)** Immunoblots showing the levels of LEF-1 protein in K562 and HEL cells following overnight treatment with 10µM or 100µM C1632. DMSO was used as a vehicle control. GAPDH served as a loading control. Bar graphs summarizing the fold change in *LEF1* mRNA levels in K562 and HEL cells after overnight treatment ± C1632 (10µM, 100µM) using primers to **(J)** exonic or **K**) intronic regions, normalized to *GAPDH*. **(L)** Summary bar graphs showing MFI generated from BAR/fuBAR system in K562 and HEL cells following overnight treatment ± C1632 (10µM, 100µM) and ± 5µM CHIR99021. Data are presented as mean ± SD (standard deviation) from three independent biological replicates. Statistical significance was assessed using Student's t-test (*$p < 0.05$; **$p < 0.01$; **$p < 0.005$; ns: $p > 0.05$).

Figure 3. (A) Immunoblots showing the expression levels of β-catenin protein in HEL and THP1 cells with ± β-catenin shRNA. GAPDH served as a loading control. Bar graphs display the percentage of live cells in **(B)** HEL and **(C)** THP-1 cells with ± β-catenin shRNA following treatment with ± C1632 (10µM, 100µM) for 24, 48 and 72 hours. Data are presented as mean ± SD from three independent biological replicates. Statistical significance was assessed using Student's t-test (*$p < 0.05$; **$p < 0.01$; ns: $p > 0.05$).

Figure 4. (A) Immunoblots showing the expression level of β-catenin protein in HEL cells following overnight treatment with different concentrations of PP. **(B)** Immunoblots showing LIN28B protein expression level in HEL and THP-1 cells with ± LIN28B shRNA. GAPDH served as a loading control. **(C)** Bar graphs display the percentage of live cells in HEL cells with ± LIN28B shRNA following treatment with ± 5µM PP for 24, 48 and 72 hours. **(D)** Bar graphs display the percentage of live cells in HEL cells after treatment with DMSO, 100µM C1632, 5µM PP and 100µM C1632 & 5µM PP for 24, 48 and 72 hours. Data are presented as mean ± SD from three independent biological replicates. Statistical significance was assessed using Student's t-test (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$; ns: non-significant).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bits
B
Log2 LIN28B mRNA Expression
ALL t(12;21)
ALL t(1;19)
AML normal karyotype.
ALL hyperdiploid.
Pro-B-ALL t(11q23)/MLL
CLL
CML
c-/Pre-B-ALL t(9;22)
T-ALL
MDS
AML complex.
c-/Pre-B-ALL no t(9;22)
AML inv(16)
Healthy bone marrow
AML t(15;17)
B-ALL with t(8;14)
AML t(8;21)
AML MLL
C
K562
NB4
KG1
THP-1
U937
HL-60
OCI-AML3
PLB-985
MONO-MAC-6
ML-1
SET-2
EOL-1
NOMO-1
KG1a
OCI-AML2
KU812
HEL
MV4;11
β-Catenin
LIN28B
GAPDH
100 kDa
37 kDa
25 kDa
20 kDa
37 kDa
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K562
K562
HEL
HEL
Input (5%)
IgG ID
LIN28B ID
IgG Co-IP
LIN28B Co-IP
CHIR99021
RNase A
LIN28B
β-Catenin
37 kDa
25 kDa
100 kDa
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HEL
CHIR99021
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88%
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CD34+
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LIN28B Co-IP
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β-Catenin
37 kDa
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= BAR/LIN28B shRNA #2
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LIN28B shRNA#2
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β-Catenin
TCF-4
LEF-1
Lamin A/C
α-Tubulin
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LIN28B-shRNA#2
Fold Change (relative to NT-shRNA)
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-2
-4
-8
-16
-32
LIN28B
LEF1
TCF7L2
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DMSO
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LEF1
Fold Change (relative to DMSO)
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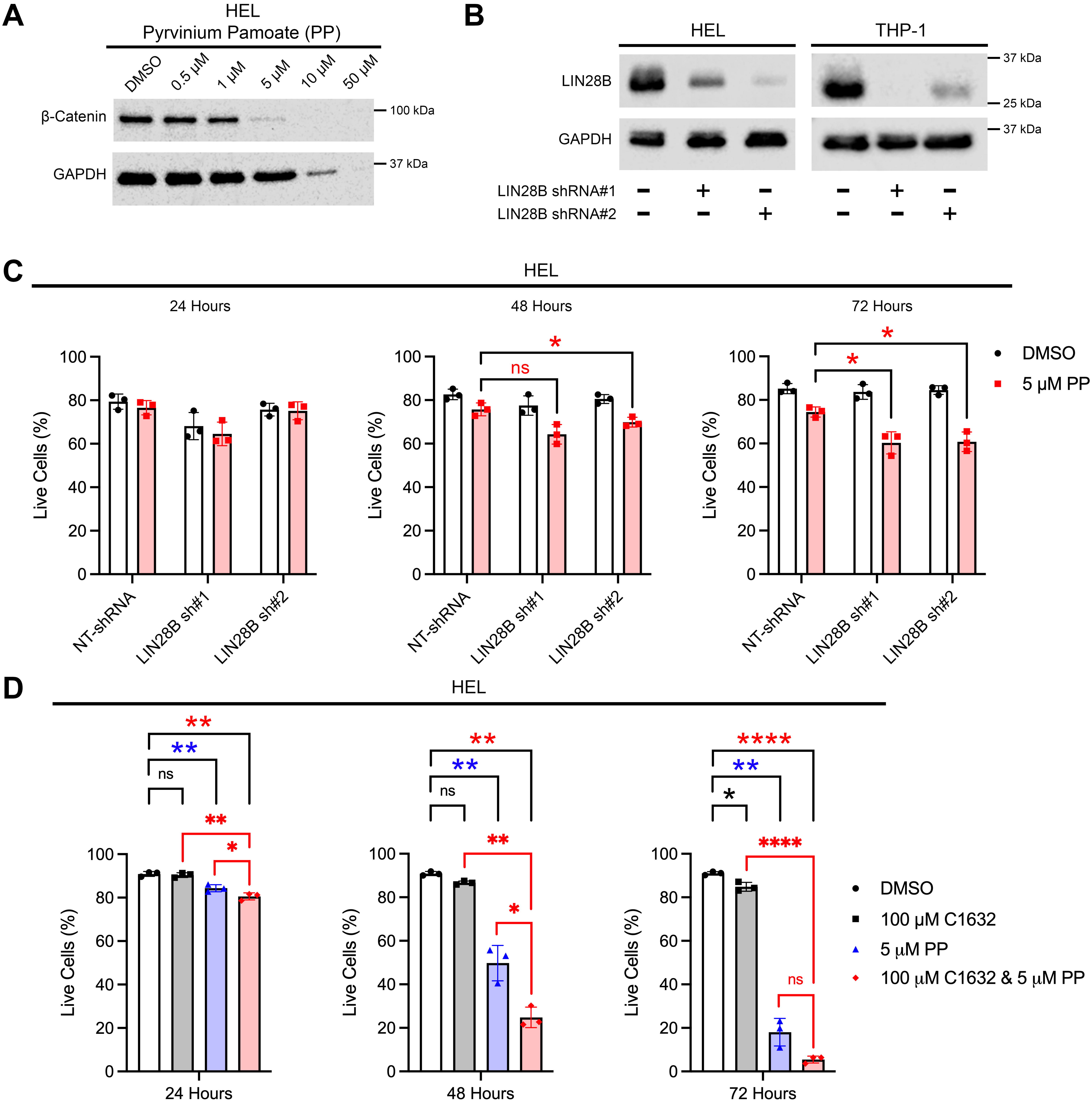
100 μM
ns
*
ns
*
K
K562
LEF1 Intronic Primers
HEL
LEF1 Intronic Primers
Fold Change (relative to DMSO)
1.0
0.5
0.0
-0.5
-1.0
DMSO
10 μM
100 μM
L
K562
CHIR99021
HEL
CHIR99021
Venus YFP MFI
60000
40000
20000
0
BAR
fuBAR
ns
**
*
**
DMSO
10 μM C1632
100 μM C1632

A
HEL
THP-1
β-Catenin
GAPDH
100 kDa
37 kDa
β-Catenin shRNA#1 − + −
β-Catenin shRNA#2 − − +
B
HEL
24 Hours
48 Hours
72 Hours
Live Cells (%)
0
20
40
60
80
100
NT-shRNA
β-Cat sh#1
β-Cat sh#2
**
*
ns
*
● DMSO
■ 10 µM C1632
▲ 100 µM C1632
C
THP-1
24 Hours
48 Hours
72 Hours
Live Cells (%)
**
**
● DMSO
■ 10 µM C1632
▲ 100 µM C1632


A
HEL
Pyrvinium Pamoate (PP)
DMSO
0.5 µM
1 µM
5 µM
10 µM
50 µM
β-Catenin
GAPDH
100 kDa
37 kDa
B
HEL
THP-1
LIN28B
GAPDH
37 kDa
25 kDa
37 kDa
LIN28B shRNA#1 − + − − + −
LIN28B shRNA#2 − − + − − +
C
HEL
24 Hours
48 Hours
72 Hours
Live Cells (%)
0
20
40
60
80
100
NT-shRNA
LIN28B sh#1
LIN28B sh#2
ns
*
DMSO
5 µM PP
D
HEL
Live Cells (%)
0
20
40
60
80
100
24 Hours
48 Hours
72 Hours
ns
**
*
****
DMSO
100 µM C1632
5 µM PP
100 µM C1632 & 5 µM PP