Title: A β-catenin:MSI2 axis regulates the expression of *LEF1* and subsequent human haematopoietic stem/progenitor cell proliferation.

Short title: β-catenin:MSI2 regulation of *LEF1* in HSPC

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MW performed experiments, analysed data, co-wrote the manuscript and managed laboratory. DN provided experimental advice and AB provided clinical samples. BT and SN provided experimental guidance and reagents. RGM performed experiments, analysed data, co-wrote the manuscript and directed project.

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Abstract

Wnt/β-catenin signaling is important for normal hematopoietic stem/progenitor cell (HSPC) biology and heavily implicated in many hematological malignancies. The central mediator, β-catenin, is an attractive therapeutic target in myeloid neoplasms however, the design of targeted agents has been hampered by a limited understanding of its molecular interactions in hematopoietic cells. To address this shortfall, we recently published the first β-catenin interactome (Morgan *et al*, 2019) in haematological cells and identified a plethora of novel partners which included the significant enrichment of RNA binding proteins (Wagstaff et al, 2022), including Musashi-2 (MSI2). MSI2 has been extensively implicated in normal HSC activity and also promotes aggressive myeloid leukemias where its expression confers inferior survival. Interaction between MSI2 and Wnt signalling has not previously been reported in a hematopoietic context. In this study we identify an interaction between MSI2 and β-catenin in both myeloid cell lines and primary AML patient samples where there is a significant degree of correlation between the proteins. We demonstrate that MSI2 can impact Wnt signalling output (TCF/LEF activity) through modulating LEF-1 expression and localization, and that MSI2 can further bind *LEF1* transcript, an interaction augmented by β-catenin. Finally, we show that MSI2 mediated expansion of HSPCs could be partly driven through *LEF1* regulation. To our knowledge this is the first study to demonstrate an interaction between MSI2 and β-catenin in haematopoietic cells, and shows the potential for β-catenin to cooperate with RBPs to govern HSPC proliferation. Finally, these data indicate β-catenin may also serve critical post-transcriptional functions (in addition to transcriptional roles) in haematopoietic cells, which could extend to regulating expression of Wnt signalling components.

Dear Editor,

Wnt/β-catenin signalling is an evolutionary conserved pathway which regulates multiple stem cell systems, including hematopoietic stem/progenitor cells (HSPC).[1] The activity of this pathway is tightly regulated to ensure normal hematopoietic development, but its dysregulation has been associated with numerous hematological malignancies including acute myeloid leukemia (AML).[1] Despite this knowledge, there are few agents which specifically and efficaciously target β-catenin in leukemia, hampered by a poor understanding of its molecular interactions in a haematopoietic context. To address this shortfall, we recently characterized β-catenin's interaction network in haematological cells for the first time by mass spectrometry and discovered a plethora of novel protein partners,[2] including the significant enrichment of RNA binding proteins (RBP).[1] RBPs are a diverse family of proteins that bind and regulate the stability, splicing, transport and translation of target RNAs, and are heavily implicated in normal hematopoiesis and leukemia.[3]

Within the network of β-catenin interacting RBPs with low probability of being contaminants (as deduced by <10% presence in the CRAPome database: https://reprint-apms.org), we identified the significant enrichment of Mushashi-2 (MSI2) in the cytoplasmic fraction of K562 cells and detected it in the cytoplasmic fraction of HEL cells (Figure 1A). We also detected this interaction in the cytoplasmic and nuclear fractions of colorectal SW620 cells suggesting this interaction may occur beyond haematopoietic tissue (Supplemental Figure S1A). The highly conserved Musashi family of RBPs were originally discovered in *Drosophila* where they were shown to control cell fate in neural stem cells.[4] They include two tandem RNA recognition motifs (RRM1 and 2) which bind to GU-rich and/or structured RNA to repress or promote protein translation by binding to 5' and/or 3'UTRs of their target

RNAs. Given that MSI2 is an important regulator of post-transcriptional gene expression,[4] and like β-catenin,[1] has been previously shown to control HSC activity[5] and the progression of myeloid leukaemias,[6] we deemed this interaction worthy of further investigation. To identify suitable models for onward study we assessed MSI2 expression across a panel of 13 myeloid cell lines and identified 8 lines (62%) with co-expression of both β-catenin and MSI2 (Figure 1B), which significantly correlated (Figure 1C). To validate the interaction between β-catenin and MSI2 we performed the reciprocal MSI2 co-immunoprecipitation (Co-IP) from K562 and HEL cells and observed consistent MSI2:β-catenin protein interaction under both basal (DMSO) and Wnt signalling activated (CHIR99021; GSK3β inhibitor) conditions (Figure 1D). We further validated the interaction in two other myeloid cell lines, KG1 and NB4, also under basal and Wnt signalling stimulated conditions, (Supplemental Figure S1B). Since MSI2 is an RBP,[4] and β-catenin has also been shown to bind RNA,[7] we assessed whether the MSI2:β-catenin interaction was an aretefact of co-accupancy on common RNAs. However, after repeating MSI2 co-IPs +/-RNase A, and confirming complete digestion of RNA,[8] the MSI2:β-catenin protein interaction was unaffected suggesting these proteins are complexed irrespective of RNA presence (Figure 1D).

To ascertain any potential clinical relevance of the β-catenin:MSI2 association, we screened 20 AML patients for protein expression of MSI2 and β-catenin (patient clinical details provided in Supplemental Table S1). Like myeloid cell lines, MSI2 was frequently co-expressed with β-catenin to similar frequency as observed in myeloid cell lines (12/20 samples [60%]; Figure 1E), with a significant degree of correlation between the two proteins (Figure 1F). To examine if the MSI2:β-catenin interaction could be detected in patient cells, we performed an MSI2 Co-IP in a primary AML sample containing abundant levels of both proteins and where ample cellular material existed (patient 10), and confirmed MSI2 interaction with β-catenin (Figure 1G). Taken together these data indicate that β-catenin and MSI2 interact in AML cells, and could represent a clinically relevant association.

We next aimed to assess the functional impact of this interaction given crosstalk between MSI2 and Wnt signalling has been reported in other tissues.[9] We lentivirally transduced two high MSI2 expressing cell lines (K562 and NB4 cells) with two unique MSI2 shRNAs (Figure 2A) and assessed Wnt signalling output (TCF/LEF activity) using the β-catenin activated reporter (BAR), as previously described (Supplemental Figure 1C).[8] As observed in Figures 2B and 2C, cells harbouring MSI2 shRNA had significantly diminished TCF/LEF activity. To better understand the basis for this reduced TCF/LEF activity in MSI2-deficient cells, we performed nuclear/cytoplasmic fractionation followed by immunoblotting and examined the expression/localisation of the Wnt signalling transcription factor LEF-1, which has critical roles in normal HSC biology and AML.[2, 10, 11] We observed limited impact of MSI2 knockdown on β-catenin level or nuclear localisation in response to Wnt signalling activation but did note marked reduction of LEF-1 expression and nuclear localisation across both cell lines under both basal and Wnt signalling active states (Figures 2D). We further observed that ectopic MSI2 expression increased LEF-1 expression and TCF/LEF activity in K562 cells (Supplemental Figure S1D, E and F) in Wnt signalling active conditions. In summary, these data suggest that MSI2 can impact Wnt signalling output in leukaemia cells through the modulation of LEF-1 level and localisation.

To understand the basis for reduced LEF-1 expression upon MSI2 knockdown we examined mRNA levels using qRT-PCR and observed significantly reduced *LEF1* (but not that of *TCF7L2,* another critical Wnt effector) expression in MSI2-deficient K562 and NB4 cells (Figure 2E). Given the role of MSI2 as an RBP and regulator of post-transcriptional gene expression, we next assessed whether *LEF1* could be an mRNA partner. After first confirming efficient MSI2 RNA immunoprecipitation (RIP) in K562 cells (Figure 2F), and enrichment of known mRNA partners *MYB* and *MYC*,[12] we observed a significantly increased fold-enrichment of *LEF1* (and *TCF7L2*) mRNA relative to IgG RIP in K562 cells (Figure 2G). Enrichment of *LEF1* and *TCF7L2* was also observed in MSI2 RIP from NB4 cells (Supplemental Figure S1G). We next enquired how the MSI2:β-catenin protein interaction might affect MSI2 binding to *LEF1* mRNA. Using β-catenin deficient cells we first confirmed no impact on total MSI2 expression (Figure 2H) before performing MSI2 RIP from each shRNA line. We observed significant attenuation of MSI2 binding to *LEF1* mRNA in the presence of β-catenin knockdown in Figure 2I (despite no overall change in total *LEF1* levels; Figure 2J). No consistent impact of β-catenin depletion on MSI2 binding to *TCF7L2* was observed (Supplemental Figures S1H and I), nor did β-catenin knockdown consistently affect *MYB* or *MYC* binding by MSI2 (without overall total mRNA levels changing in response to β-catenin loss; Supplemental Figures S1J-M). These data indicate β-catenin might facilitate MSI2 binding to specific RNAs, including those directly encoding Wnt signalling components.

Finally, to understand any potential physiological relevance for the MSI2:*LEF1* axis we determined the impact on human HSPC activity since both proteins have well documented roles in this setting.[5, 6, 10, 11, 13] Previously, MSI2 has been shown to promote HSC expansion and we hypothesized that some of this activity could be mediated through *LEF1*. Human cord blood derived CD34$^+$ HSPC were isolated and lentivirally transduced with ectopic MSI2 expression (Figure 2K) alongside either non-targeting, or LEF-1 shRNAs (Figure 2L). From day 4 onwards (3 days post-lentiviral transduction) we observed a modest but consistent increase in the expansion rates of HSPCs expressing ectopic MSI2 versus control cultures, which was significantly higher (32%±2 increase) at day 8 of liquid culture (Figure 2M). A significantly higher percentage of CD34$^+$ population were also observed in MSI2 overexpressing cultures versus controls at this timepoint (Supplemental Figure S1N). Increases in the expansion rates and CD34 positivity of MSI2 overexpressing cultures were abrogated in the presence of LEF-1 shRNA (Figure 2M). Primary HSPC cultures harbouring LEF-1 shRNA exhibited consistently reduced growth rates which were significantly lower at days 8, 11 and 13 (shRNA#2 only) of liquid culture (Figure 2M). These data are consistent with our previous study showing LEF-1 can impact the proliferation of myeloid leukaemia cells,[2] and implies that part of MSI2's capacity to expand human HSPCs could be mediated through LEF-1.

In summary, this study has identified a novel MSI2:β-catenin protein interaction and desmonstrated that a β-catenin:MSI2 axis can bind and regulate *LEF1* expression. Recent work has shown that MSI2 can bind to Internal Ribosome Entry Sites (IRES) located in the 5'UTRs of oncogenic mRNAs including *MYC*, *JUN* and *VEGFA* promoting protein expression by increasing translation.[14] Interestingly, LEF-1 also includes a potential IRES in its 5'UTR,[15] which coupled to our data suggest that MSI2 (together β-catenin) might bind the 5'UTR IRES of *LEF1* to enhance its translation. This study further highlights novel roles for β-catenin in the RBP/RNA biology of haematopoietic cells which could be an important axis regulating human

HSPC activity. Finally, β-catenin could regulate post-transcriptional mechanisms (in addition to transcriptional) by facilitating RBP interaction with specific RNAs, including those encoding Wnt signalling components.

Acknowledgements

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Figure legends

Figure 1 - β-Catenin interacts with MSI2 in myeloid cells. A) Scatter plots showing MSI2 detection in β-catenin interactomes performed in cytosolic fractions of K562 and HEL cells. Vertical dashed red line indicates the threshold for 2-fold change in protein binding at $\log_2$ (=1) relative to IgG Co-IP. Horizontal red line represents threshold for significant interactions at p=0.05 on $\log_{10}$ scale (=1.3). Highlighted red dots indicate statistically significant interactions, green labels

highlight position of MSI2, and blue labels highlight position of β-catenin bait. Data are screened to remove any peptides with high probability of being contaminants as deduced by over 10% presence in the CRAPome database (https://reprint-apms.org/). **B)** Immunoblot of myeloid leukemia cell lines showing the relative level of β-catenin and MSI2 protein, with GAPDH used to assess protein loading. **C)** Summary scatter plot showing the correlation (Spearman Rank R=0.6, P<0.03) between relative β-catenin and MSI2 protein expression in 13 myeloid cell lines (normalised to GAPDH expression within each cell line). **D)** Immunoblots showing the level of β-catenin protein present in MSI2 Co-IPs derived from K562 and HEL whole cell lysates +/- 20µg/mL RNaseA +/-5µM CHIR99021 overnight. ID= immunodepleted lysate. All immunoblots shown are representative of 3 independent biological replicates. **E)** Immunoblot screen of 20 primary AML patient samples showing the relative level of β-catenin and MSI2 protein (light and dark exposures). * denotes samples co-overexpressing both β-catenin and MSI2 relative 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 containing no protein as deduced from negative GAPDH detection. **F)** Summary scatter plot showing the correlation (Spearman Rank R=0.79, P<0.0001) between relative β-catenin and MSI2 protein expression in 20 primary AML patient samples (normalised to GAPDH expression in each sample). **G)** Immunoblot showing the level of β-catenin protein present in an MSI2 Co-IP performed from primary AML patient sample #10 of sample screen.

Figure 2 – MSI2 knockdown impairs Wnt signalling output and reduced HSC expansion through LEF-1. A) Immunoblots showing MSI2 level in K562 and NB4 cells harbouring MSI2 shRNA or non-targeting shRNA controls. GAPDH indicates protein loading. **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 K562 and NB4 cells +/- MSI2 shRNA following treatment with 5μM CHIR99021 overnight. The fuBAR (dashed), non-targeting control shRNA (grey filled), and two MSI2 shRNAs (blue or red) histograms are shown. **C**) Summary graphs showing the median fluorescence intensity (MFI) generated from the BAR/fuBAR in K562 and NB4 cells +/- MSI2 shRNA with +/- 5μM CHIR99021. All data represents mean□±□1 s.d (*n*□=□3). Statistical significance is denoted by ***P*<0.01, and *****P*<0.0001 as deduced from a student's t-test. Data shown are representative of 3 independent biological replicates. **D)** Immunoblots showing total β-catenin, LEF-1 and MSI2 subcellular localization in K562 and NB4 cells lentivirally transduced with two different MSI2 shRNAs +/- 5μM CHIR99021. Lamin A/C and α-tubulin indicate the purity/loading of the nuclear (N) and cytosol (C) fractions respectively. **E**) Summary graph showing the fold change in *LEF1* and *TCF7L2* mRNA expression as assessed by qRT-PCR in K562 and NB4 cells expressing MSI2 shRNA. Fold change is relative to matched respective controls (black dashed line) and overall expression was normalized to the housekeeping gene β-actin (*ACTB*). **F**) Immunoblot showing MSI2 level in MSI2 RIP performed from K562 cells. ID= immunodepleted lysate. **G**) Summary graph showing the fold enrichment of *TCF7L2*, *MYC*, *MYB*, and *LEF1* mRNA in MSI2 RIPs performed from K562 cells. Fold enrichment is relative to matched IgG RIPs (dashed

black line). **H**) Immunoblots showing MSI2 and β-catenin level in K562 cell lines harbouring β-catenin shRNAs versus non-targeting shRNA control. GAPDH indicates protein loading. **I**) Summary graphs showing the fold enrichment of *LEF1* mRNA (versus IgG RIP) in MSI2 RIPs generated from K562 cells +/- β-catenin shRNAs. **J**) Summary graph showing the fold change in *LEF1* mRNA expression in input K562 cells harbouring β-catenin shRNA versus non-targeting shRNA control (dashed black line) used for MSI2 RIP. **K)** Immunoblot showing the level of MSI2 in day 4 primary cord blood derived HSPC cultures following 3 days post lentiviral transduction with empty vector (EV) or human MSI2. GAPDH indicates protein loading. **L**) Representative day 4 flow cytometry plots showing percentage GFP^+ events in primary HSPC cultures lentivirally transduced with ectopic MSI2 +/- LEF-1 or non-targeting (NT) shRNA versus matched untransduced cells. **M**) Line graph showing fold-expansion of HSPC cultures following lentiviral transduction with ectopic MSI2 +/- LEF1 shRNAs. All data represents mean ± 1 s.d ($n = 3$). All data represents mean ± 1 s.d ($n = 3$). Statistical significance is denoted by *P<0.05, **P<0.01, and ****P<0.0001 as deduced from a student's t-test. Data shown are representative of 3 independent biological replicates.

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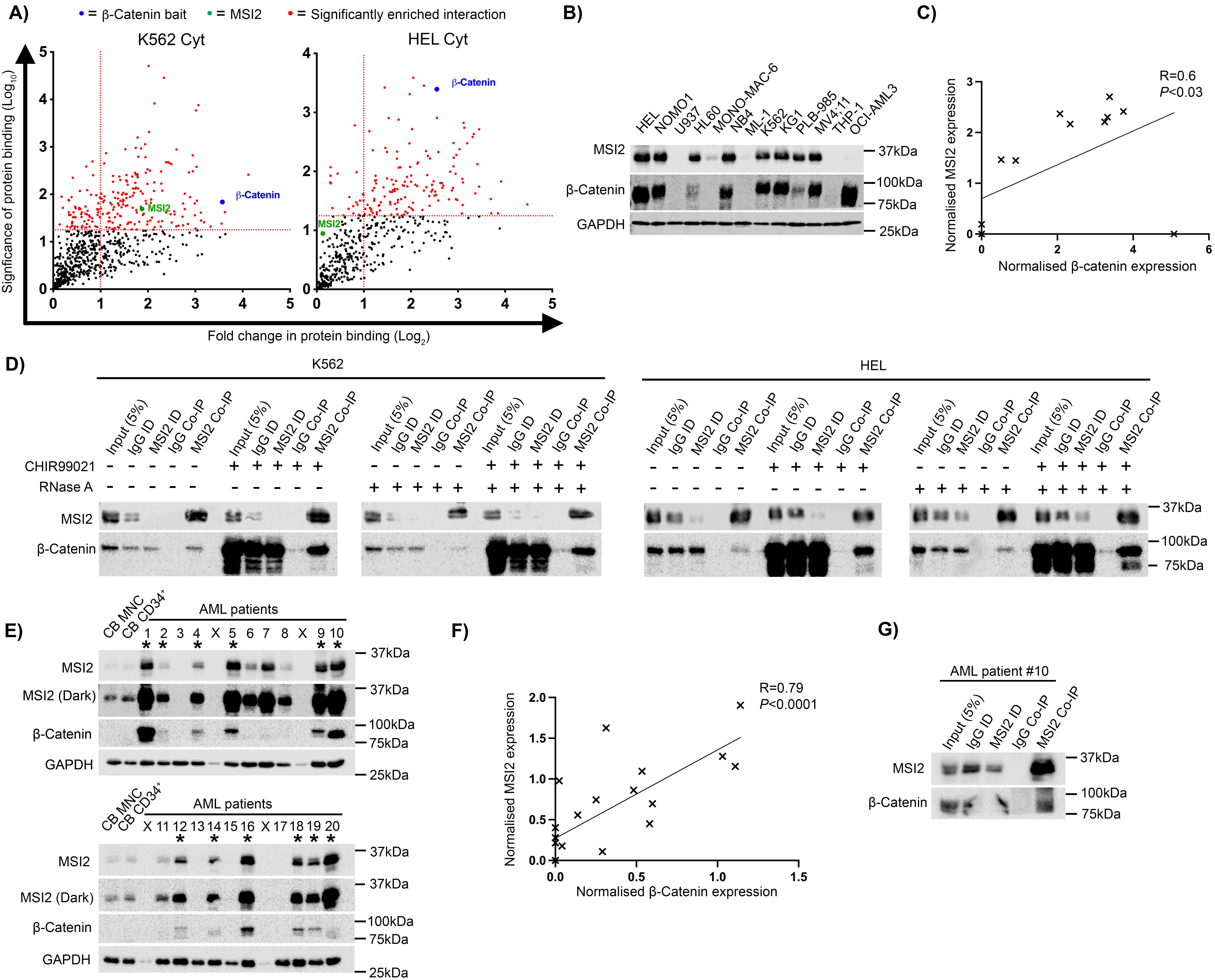
Increased Wnt Signalling
HSPC proliferation/maintenance
LEF1 mRNA
MSI2
β-catenin
LEF1 mRNA
MSI2
β-catenin


A)
● = β-Catenin bait
● = MSI2
● = Significantly enriched interaction
K562 Cyt
HEL Cyt
Signficance of protein binding (Log$_{10}$)
Fold change in protein binding (Log$_2$)
MSI2
β-Catenin
B)
HEL
NOMO1
U937
HL60
MONO-MAC-6
NB4
ML-1
K562
KG1
PLB-985
MV4;11
THP-1
OCI-AML3
MSI2
β-Catenin
GAPDH
37kDa
100kDa
75kDa
25kDa
C)
R=0.6
P<0.03
Normalised MSI2 expression
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K562
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Input (5%)
IgG ID
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MSI2 Co-IP
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RNase A
MSI2
β-Catenin
37kDa
100kDa
75kDa
E)
CB MNC
CB CD34$^+$
AML patients
1 2 3 4 X 5 6 7 8 X 9 10
MSI2
MSI2 (Dark)
β-Catenin
GAPDH
37kDa
100kDa
75kDa
25kDa
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F)
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Normalised MSI2 expression
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IgG ID
MSI2 ID
IgG Co-IP
MSI2 Co-IP
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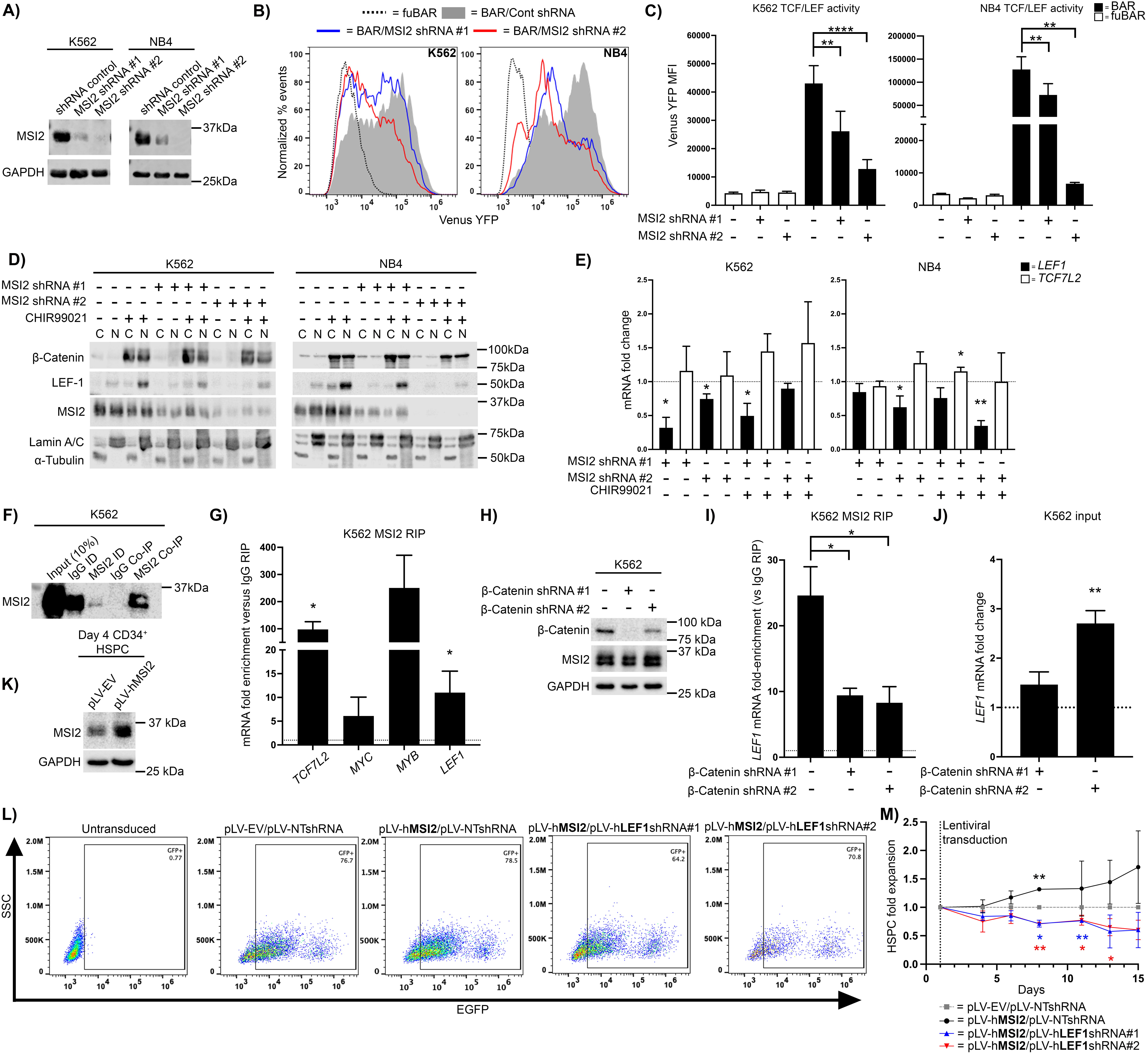
β-Catenin
37kDa
100kDa
75kDa


A)
K562
NB4
shRNA control
MSI2 shRNA #1
MSI2 shRNA #2
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GAPDH
37kDa
25kDa
B)
= fuBAR
= BAR/Cont shRNA
= BAR/MSI2 shRNA #1
= BAR/MSI2 shRNA #2
K562
NB4
Normalized % events
Venus YFP
C)
K562 TCF/LEF activity
NB4 TCF/LEF activity
■ = BAR
□ = fuBAR
Venus YFP MFI
MSI2 shRNA #1
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D)
K562
NB4
MSI2 shRNA #1
MSI2 shRNA #2
CHIR99021
C N C N C N C N C N C N
β-Catenin
LEF-1
MSI2
Lamin A/C
α-Tubulin
100kDa
75kDa
50kDa
37kDa
75kDa
50kDa
E)
K562
NB4
■ = LEF1
□ = TCF7L2
mRNA fold change
MSI2 shRNA #1
MSI2 shRNA #2
CHIR99021
F)
K562
Input (10%)
IgG ID
MSI2 ID
IgG Co-IP
MSI2 Co-IP
MSI2
37kDa
G)
K562 MSI2 RIP
mRNA fold enrichment versus IgG RIP
TCF7L2
MYC
MYB
LEF1
H)
K562
β-Catenin shRNA #1
β-Catenin shRNA #2
β-Catenin
MSI2
GAPDH
100 kDa
75 kDa
37 kDa
25 kDa
I)
K562 MSI2 RIP
LEF1 mRNA fold-enrichment (vs IgG RIP)
β-Catenin shRNA #1
β-Catenin shRNA #2
J)
K562 input
LEF1 mRNA fold change
β-Catenin shRNA #1
β-Catenin shRNA #2
K)
Day 4 CD34⁺ HSPC
pLV-EV
pLV-hMSI2
MSI2
GAPDH
37 kDa
25 kDa
L)
Untransduced
pLV-EV/pLV-NTshRNA
pLV-hMSI2/pLV-NTshRNA
pLV-hMSI2/pLV-hLEF1shRNA#1
pLV-hMSI2/pLV-hLEF1shRNA#2
GFP+ 0.77
GFP+ 76.7
GFP+ 78.5
GFP+ 64.2
GFP+ 70.8
SSC
EGFP
M)
Lentiviral transduction
HSPC fold expansion
Days
= pLV-EV/pLV-NTshRNA
= pLV-hMSI2/pLV-NTshRNA
= pLV-hMSI2/pLV-hLEF1shRNA#1
= pLV-hMSI2/pLV-hLEF1shRNA#2