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KRas^{G12D}-evoked leukemogenesis does not require β -catenin

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The targeting of self-renewal pathways commonly activated in leukemia serves as a potential strategy for multiple subtypes of this disease regardless of genetic, clonal, or cellular heterogeneity. Elevation of β -catenin above physiological conditions enhances the self-renewal of normal hematopoietic stem cells (HSCs), and this attribute appears to be commonly utilized by leukemia cells.¹ Dependency on elevated β -catenin activity in leukemia stem cells (LSCs) demonstrated in several different types of leukemia strongly suggest an essential and universal role for β -catenin in LSC function in leukemia.^{2–6} Since normal adult HSCs do not require its basal activity,⁷ β -catenin has emerged as a potential LSC-specific therapeutic target.

Mutations in the Ras pathway are some of the most common in all human malignancies and occur across the spectrum of human blood neoplasms.⁸ These mutations usually in *KRAS*, *NRAS*, or *NFI* lead to stabilization of GTP-bound active state of small Ras GTPases leading to over-activation of downstream Ras effector pathways.⁸ Endogenous levels of gain-of-function Ras proteins in mice lead to myeloproliferative neoplasms (MPN) and/or T-ALL.^{9–11} While this pathway has been intensely studied, direct pharmacological inhibition of mutant Ras proteins has proven to be extremely challenging.

To determine if β -catenin is required for activated-Ras pathway-evoked leukemia, we first utilized mice that express from the endogenous promoter a conditionally active gain-of-function allele of KRas (*loxP*-stop cassette-*loxP* [*LSL*]-*KRas*^{G12D}), that develop a Juvenile Myelomonocytic Leukemia (JMML)/Chronic Myelomonocytic Leukemia (CMML)-like MPN upon Cre-mediated excision of the stop cassette.^{9,10} *LSL-KRas*^{G12D} mice were crossed with mice carrying conditional loss-of-function alleles of β -catenin and to interferon-inducible transgenic-*Mx1Cre* mice, allowing for recombination upon administration of pIpC. However, we found as previously reported,⁷ that pIpC administered to *Mx1Cre*; β -catenin^{loxP/loxP} mice results in early non-hematopoietic lethality (data not shown). Consistent with previous results, we found high efficiency spontaneous excision of

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CONFLICT OF INTEREST

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the stop-cassette in the absence of Cre induction and found that β -catenin could also be excised concurrently in the $\text{Mx1Cre}^+\text{LSL-KRas}^{\text{G12D}}$ setting (Figure 1a).^{10,11} We thus utilized mice of the following genotypes, $\text{Mx1Cre}^+\beta\text{Cat}^{\text{loxP/loxP}}$ ($\beta\text{Cat}^{\text{loxP/loxP}}$), $\text{Mx1Cre}^+\text{LSL-KRas}^{\text{G12D}}$ ($\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}$), $\text{Mx1Cre}^+\text{LSL-KRas}^{\text{G12D}}\beta\text{-catenin}^{+/loxP}$ ($\beta\text{cat}^{+/+}\text{KRas}^{\text{G12D}}$), and $\text{Mx1Cre}^+\text{LSL-KRas}^{\text{G12D}}\beta\text{-catenin}^{\text{loxP/loxP}}$ ($\beta\text{Cat}^{-/-}\text{KRas}^{\text{G12D}}$) and assessed them without pIpC administration.

We confirmed Cre-mediated (in the absence of pIpC administration) excision within the β -catenin locus by qRT-PCR as early as 4 weeks of age in the peripheral blood of $\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}$ and $\beta\text{Cat}^{-/-}\text{KRas}^{\text{G12D}}$ mice (data not shown) and in the bone marrow (BM) of 13-17 weeks old mice (Figure 1a). We found no statistical differences in the survival of all mice expressing oncogenic $\text{KRas}^{\text{G12D}}$, regardless of β -catenin status (Figure 1b). Further examination of mice euthanized at 13-17 weeks revealed that all $\beta\text{Cat}^{-/-}\text{KRas}^{\text{G12D}}$ and $\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}$ mice demonstrated leukocytosis, and splenomegaly with myelomonocytic expansion indistinguishable from $\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}$ mice (Figure S1 and Table S1).

Transplanted $\text{KRas}^{\text{G12D}}$ -expressing BM cells give rise to an aggressive TALL.¹¹ To determine the requirement for β -catenin in $\text{KRas}^{\text{G12D}}$ -induced T-ALL, we transplanted donor BM cells with helper cells into lethally-irradiated congenic recipient mice, and found that all $\text{KRas}^{\text{G12D}}$ -expressing cells, regardless of β -catenin status, exhibited increased chimerism (>80%) when compared to mice transplanted with control ($\beta\text{Cat}^{\text{loxP/loxP}}$) BM cells (~60%) (Figure 1c). All mice transplanted with $\text{KRas}^{\text{G12D}}$ -expressing BM cells, even those with loss of β -catenin, were moribund within 3.5 months of transplant, while none of the recipients transplanted with control cells died during this observation period (Figure 1d and Figure S2a and S2b). Consistent with previous findings,¹¹ we found that all recipient mice transplanted with $\text{KRas}^{\text{G12D}}$ -expressing cells developed both a mild MPN (Table S1 and data not shown), and a more aggressive T-ALL disease, characterized by thymus enlargement filled with abnormal CD8^+ single positive (SP) and $\text{CD4}^+\text{CD8}^+$ double positive (DP) cells (Table S1 and Figure S2c).

To further assess the role of β -catenin in $\text{KRas}^{\text{G12D}}$ -induced T-ALL, we performed a secondary limiting-dilution transplant using thymocytes from primary recipients for injection into sublethally-irradiated recipients. Despite a slight difference in the frequency of leukemia-initiating cells (LICs) (Table S2a), the loss of β -catenin did not alter the survival nor disease phenotype of mice transplanted with $\text{KRas}^{\text{G12D}}$ -expressing thymocytes (Figure 1e and Figure S3).

We and others demonstrated that β -catenin is required for MLL-rearranged-driven AML.^{4,5} As Ras pathway mutations are common in AML and can co-occur with MLL-rearrangements,^{4,5} we sought to determine if β -catenin would still be required for leukemogenesis in a $\text{KRas}^{\text{G12D}}$ -expressing MLL-rearranged setting. We transduced the HSPC-enriched $\text{Lin}^-\text{Sca}^-\text{1}^+\text{c-Kit}^+$ (LSK) cell fraction with MSCV-MLL-AF9-ires-GFP retrovirus from the following mice: $\text{MxCre}^+\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}$, $\text{MxCre}^+\beta\text{Cat}^{-/-}\text{KRas}^{\text{G12D}}$, $\text{MxCre}^-\beta\text{Cat}^{\text{loxP/loxP}}$, and $\text{MxCre}^+\beta\text{Cat}^{\text{loxP/loxP}}$; and transplanted these cells into sub-lethally irradiated C57BL/6 recipients. We found that mice transplanted with $\text{KRas}^{\text{G12D}}\text{MLL-AF9}$

cells, regardless of β -catenin status, developed a lethal AML, characterized by leukocytosis and splenomegaly with myeloid infiltration (Figure 2a, Figure S4 and Table S1). Mice transplanted with β Cat^{+/+}MLL-AF9 and β Cat^{-/-}MLL-AF9 cells exhibited a significantly longer latency (Figure 2a). In support of the requirement of β -catenin for MLL-AF9 AML, we found that β Cat^{-/-}MLL-AF9 cells tended to have a lower level of chimerism and white blood cells (wbc) in the peripheral blood than β Cat^{+/+}MLL-AF9 (Figure 2b and Figure S4b). All disease parameters assessed, including survival, peripheral blood chimerism and wbc counts, were indistinguishable between β Cat^{+/+}KRas^{G12D}MLL-AF9 and β Cat^{-/-}KRas^{G12D}MLLAF9 recipient mice.

We further explored if the loss of β -catenin and/or the gain of oncogenic KRas affected the frequency of leukemia-initiating cells (LICs) in this AML model by performing a secondary limiting-dilution transplantation using BM cells from primary AML recipients. Remarkably, only the loss of β -catenin in MLL-AF9 leukemia led to a decrease in the frequency of LICs, which translated into a significant difference in survival between β Cat^{loxp/loxp}MLL-AF9 and β Cat^{-/-}MLL AF9 recipients (Figure 2c and Table S2b). Interestingly, the loss of β -catenin (β Cat^{-/-}MLL-AF9 compared to β Cat^{+/+}MLL-AF9) appeared to be compensated for by KRas^{G12D} expression, as demonstrated by the comparable frequencies of LICs, survival and similar disease parameters between β Cat^{+/+}MLL-AF9 and β Cat^{-/-}KRas^{G12D}MLL-AF9 (Figure 2c and Table S2b).

In an attempt to decipher the underlying molecular mechanisms for this compensation, we performed gene-expression analysis using RNA from LSC-enriched Lin⁻Kit^{hi}GFP^{hi} BM cells of secondary AML transplant recipients and found that gene expression levels which were altered with the loss of β -catenin in MLL-AF9 were in part rescued with the co-expression of KRas^{G12D} in AML (Figure 2d). In particular, CD99 and DPPIV piqued our interest since they displayed changes in surface expression due to loss of β -catenin in MLL-AF9 AML and are brought to normal levels upon KRas^{G12D} expression (Figure S5b).

We found that β -catenin is dispensable for leukemogenesis evoked by expression of KRas^{G12D}. Furthermore, KRas^{G12D} expression appears to rescue the effects of β -catenin loss in an MLL-AF9 AML model. We sought to determine if self-renewal pathways activated by β -catenin are generally required in leukemia, and found that in contrast to BCR-ABL-driven CML,^{2,6} MLL-rearrangement-driven AML,^{4,5} and Pten-loss driven T-ALL,³ KRas^{G12D} can function independently or in parallel to β -catenin-dependent pathways to generate leukemia. These data suggest alternative mechanisms of leukemogenesis and leukemia maintenance independent of β -catenin, and are in line with data demonstrating the lack of major effects due to β -catenin knockdown in leukemia generation by some primary human AML samples.¹² In keeping with our previous findings, we found differential dependence on beta-catenin in MLL-AF9 leukemia.^{4,13} It is important to note that AMLs derived from granulocyte monocyte progenitor cells show a much more absolute dependence on β -catenin than do LSK derived AML cells, further supporting the findings that the cell of origin influences pathway dependencies in the fully developed leukemia (A.K. unpublished data).^{4,13}

Our analysis has also uncovered potential mechanisms of bypassing the need for β -catenin. Of note, CD99 levels diminish upon loss of β -catenin in our AML model, but are rescued upon induction of KRas^{G12D} (Figure 2d and Figure S5b). Significantly, CD99 expression is high in human LSC.¹⁴ DPPIV/CD26 levels, on the other hand, increase upon β -catenin loss in our AML model, and its levels remain decreased upon KRas^{G12D} induction in the absence of β -catenin (Figure 2d and Figure S5b). Interestingly, DPPIV/CD26 was previously demonstrated to impede HSC function, and our data suggest it may act similarly in leukemia cells.¹⁵

In this study we demonstrated that β -catenin is not universally required for leukemia development. We have specifically shown that activated KRas can bypass the need for this molecule in leukemogenesis and propose a potential mechanism of resistance to β -catenin inhibition in cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

β-catenin is dispensable for KRas^{G12D}-induced MPN and T-ALL (a-b) Primary mice were analyzed for evidence of MPN at 13-17 weeks of age. (a) Quantitative real-time (qRT) PCR was carried out on genomic DNA harvested from BM cells to assess for Cre-mediated excision of floxed β-catenin allele(s) from mice of the indicated number (n) and genotypes. (b) Kaplan–Meyer (KM) survival curve is shown from mice of the indicated genotypes with the number of mice analyzed. Log-rank test was used to calculate p values: βCat^{loxpl/loxpl} (black) vs βCat^{+/+}KRas^{G12D} (blue), p<0.0001; βCat^{+/+}KRas^{G12D} (blue) vs βCat^{-/-}KRas^{G12D}

(red), $p=0.0718$. Abbreviations: * = $p<0.05$. **(c-e)** Analysis of $Kras^{G12D}$ -induced T-ALL in transplanted mice. **(c-d)** Bone marrow transplantation (BMT) was performed using 1×10^6 bone marrow (BM) cells from donor mice (CD45.2), together with an equal number of helper cells (CD45.1) into lethally-irradiated congenic (CD45.1) recipients. **(c)** Chimerism of donor cells was monitored at 28, 70 and 90 days after transplantation by assessing the percentage of CD45.2 donor cells in the peripheral blood of recipients. For each of the time points: $\beta Cat^{+/+}Kras^{G12D}$, $n=10, 8$ and 7 , respectively; $\beta Cat^{+/-}Kras^{G12D}$, $n=10, 7$ and 6 , respectively; $\beta Cat^{-/-}Kras^{G12D}$, $n=9, 5$ and 5 , respectively; $\beta Cat^{loxp/loxp}$, $n=8$ at all time points. Average percentage chimerism for $\beta Cat^{+/+}Kras^{G12D}$ and $\beta Cat^{-/-}Kras^{G12D}$ at each time point was not significantly different and p values are as follows: $0.9141, 0.1056$ and 0.5021 . At all time points, $\beta Cat^{+/+}Kras^{G12D}$, $\beta Cat^{+/-}Kras^{G12D}$ or $\beta Cat^{-/-}Kras^{G12D}$ compared to $\beta Cat^{loxp/loxp}$, $p<0.05$. Green arrows indicate when pIpC was administered. **(d)** KM curve assessing survival of recipient mice from (c). $\beta Cat^{+/+}Kras^{G12D}$, $\beta Cat^{+/-}Kras^{G12D}$ or $\beta Cat^{-/-}Kras^{G12D}$ vs $\beta Cat^{loxp/loxp}$, $p<0.05$; $\beta Cat^{+/+}Kras^{G12D}$ vs $\beta Cat^{-/-}Kras^{G12D}$, $p=0.3421$. **(e)** KM curve assessing survival of recipient mice transplanted with either 1×10^6 (solid line), 1×10^5 (dotted line) or 1×10^4 (fine dotted line) thymocytes from mice with T-ALL [mice as in (c-d)] is shown. Recipients transplanted with $\beta Cat^{+/+}Kras^{G12D}$ and $\beta Cat^{-/-}Kras^{G12D}$ thymocytes are shown in blue and red, respectively. Survival between $\beta Cat^{+/+}Kras^{G12D}$ and $\beta Cat^{-/-}Kras^{G12D}$ at all cell numbers transplanted were not significantly different and the p values are as follows: 1×10^6 , $p=0.857$; 1×10^5 , $p=0.411$; 1×10^4 , $p=0.317$.

Figure 2.

β -catenin is dispensable for $\text{KRas}^{\text{G12D}}$ -MLL-AF9 acute myeloid leukemia (AML).

(a-b) BMT was performed using LSK-derived BM cells transduced with MSCV MLL-AF9-ires-GFP , followed by injection of cells into sub-lethally irradiated C57/BL6 recipients. (a) KM curve assessing survival of recipient mice is shown. Green arrows indicate pIpC administration. Statistically significant differences ($p < 0.05$) in survival between $\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}\text{MLL-AF9}$ and $\beta\text{Cat}^{\text{loxp/loxp}}\text{MLL-AF9}$ as well as $\beta\text{Cat}^{-/-}\text{KRas}^{\text{G12D}}\text{MLL-AF9}$ and $\beta\text{Cat}^{-/-}\text{MLL-AF9}$ were found; $\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}\text{MLL-AF9}$ vs

$\beta\text{Cat}^{-/-}\text{KRas}^{\text{G12D}}\text{MLL-AF9}$ ($p=0.4142$); $\beta\text{Cat}^{\text{loxp/loxp}}\text{MLL-AF9}$ vs $\beta\text{Cat}^{-/-}\text{MLL-AF9}$ ($p=0.1185$). **(b)** Number of circulating WBC is shown. $\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}\text{MLL-AF9}$, $n=3$; $\beta\text{Cat}^{-/-}\text{KRas}^{\text{G12D}}\text{MLL-AF9}$, $n=2$; $\beta\text{Cat}^{\text{loxp/loxp}}\text{MLL-AF9}$, $n=3$; $\beta\text{Cat}^{-/-}\text{MLL-AF9}$, $n=3$. Abbreviations: N.S. = not significant ($p>0.05$); * = $p<0.05$. **(c-d)** BMT was performed by injection of BM cells from primary AML recipient mice, as in (a), into sub-lethally irradiated C57/BL6 recipients. **(c)** KM curves assessing survival of recipient mice transplanted with three different cell concentrations (1×10^4 , 1×10^3 and 1×10^2) are shown. Green arrows indicate pIpC administration. 1×10^4 cells BMT: Statistically significant differences ($p<0.05$) in survival were found when comparing each of the other groups ($\beta\text{Cat}^{+/+}\text{KRas}^{\text{G12D}}\text{MLL-AF9}$, $\beta\text{Cat}^{-/-}\text{KRas}^{\text{G12D}}\text{MLL-AF9}$ and $\beta\text{Cat}^{\text{loxp/loxp}}\text{MLL-AF9}$) to $\beta\text{Cat}^{-/-}\text{MLL-AF9}$; All other analyses failed to reach statistically significant differences in survival ($p>0.05$). 1×10^3 and 1×10^2 cells BMT: All analyses showed the lack of statistical difference in survival ($p>0.05$). **(d)** Gene expression analysis was carried out using RNA from $\text{Lin}^{-}\text{kit}^{\text{hi}}\text{GFP}^{+}$ BM cells obtained from secondary BMT recipients transplanted with 1×10^4 primary AML cells ($n=3$ per group). Heat maps show gene expression levels that are altered with the loss of β -catenin in MLL-AF9 AML cells which are rescued with the co-expression of $\text{KRas}^{\text{G12D}}$; genes that are decreased (left panel) and increased (right panel), respectively, with the loss of β -catenin in MLL-AF9 AML are shown.