β-Arrestin2 mediates the initiation and progression of myeloid leukemia

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β-Arrestins were initially discovered as negative regulators of G protein-coupled receptor signaling. Although β-arrestins have more recently been implicated as scaffold proteins that interact with various mitogenic and developmental signals, the genetic role of β-arrestins in driving oncogenesis is not known. Here we have investigated the role of β-arrestin in hematologic malignancies and have found that although both β-arrestin1 and -2 are expressed in the hematopoietic system, loss of β-arrestin2 preferentially leads to a severe impairment in the establishment and propagation of the chronic and blast crisis phases of chronic myelogenous leukemia (CML). These defects are linked to a reduced frequency, as well as defective self-renewal capacity of the cancer stem-cell population, in mouse models and in human CML patient samples. At a molecular level, the loss of β-arrestin2 leads to a significant inhibition of β-catenin stabilization, and ectopic activation of Wnt signaling reverses the defects observed in the β-arrestin2 mutant cells. These data cumulatively show that β-arrestin2 is essential for CML disease propagation and indicate that β-arrestins and the Wnt/β-catenin pathway lie in a signaling hierarchy in the context of CML cancer stem cell maintenance.

chronic myeloid leukemia | leukemia stem cell | hematopoiesis

Chronic myelogenous leukemia (CML) is initiated by a translocation resulting in the BCR-ABL fusion oncogene, which leads to the dysregulated activation of the tyrosine kinase ABL. Although CML is routinely treated with ABL inhibitors, significant issues remain because these approaches are unable to completely eradicate disease (1–3). Emerging evidence suggests that although differentiated cells within the tumor are addicted to ABL and can be eliminated by kinase inhibition, CML cancer stem cells are in fact no longer dependent on the presence of BCR-ABL and continue to renew despite effective kinase inhibition (4–6). These findings underscore the importance of identifying new players and pathways that drive CML cancer stem cell growth and renewal to allow for the development of novel approaches that will promote long-term, cancer-free survival.

β-Arrestins comprise a small family of multifunctional scaffold proteins that regulate G protein-coupled receptor (GPCR) signaling. Although β-arrestins were initially discovered as desensitizers of GPCR signaling (7, 8), they have recently been shown to be signaling molecules in their own right (9, 10). Among the many processes that β-arrestins regulate are cell proliferation and differentiation, primarily through the activation of mitogenic pathways, including the MAPK and AKT signaling cascades (10, 11). Additionally, β-arrestins have been implicated as important mediators of developmental signals, such as those involved in the Wnt and Hedgehog pathways (12–15). Because these pathways have been shown to be required for the initiation and propagation of hematopoietic malignancies (16–19), we tested if β-arrestins regulate normal and malignant growth in the hematopoietic system.

Results

To test whether loss of β-arrestins influences normal hematopoietic development, we analyzed bone marrow from wild-type, β-arrestin1 $(\beta\text{-arr1})^{-/-}$, and β-arrestin2 $(\beta\text{-arr2})^{-/-}$ mice. These mice appear normal but have been shown to display deficiencies in response to specific stimuli along with impaired chemotaxis and proliferation of T and B lymphocytes (20, 21). Thus, we examined the hematopoietic system in both mutants. We first determined the relative frequency and absolute number of the hematopoietic stem cell (HSC)-enriched cells (c-Kit^+Lin^- Sca-1^+, KLS) (Fig. 1 *A* and *B* and Fig. S1) and the distribution of mature blood cells (Fig. S2), all of which were similar to wild-type in both mutants, suggesting that the establishment of the hematopoietic compartment is not dependent on β-arr. Interestingly, the absence of β-arr2 preferentially led to significant defects in the function of HSCs, including a dramatic reduction in the frequency of colonies in serial replating assays (Fig. 1 *C* and *D*), and an eightfold reduction in their ability to self-renew in the long term and repopulate lethally irradiated recipient mice (Fig. 1 *E* and *F*). Equivalent numbers of donor cells from wild-type, $\beta\text{-arr1}^{-/-}$, or $\beta\text{-arr2}^{-/-}$ mice could be found in the bone marrow 8 h after transplantation, indicating that the reduced repopulation ability is unlikely to be because of changes in homing to the bone marrow (Fig. S3). These data collectively suggest that long-term HSC self-renewal is dependent on β-arr2–mediated signaling, primarily under conditions that trigger a high degree of proliferation but not in homeostasis.

Because signals governing stem cell self-renewal can be aberrantly reused in cancer development, we examined whether β-arr2 influences the incidence and progression of CML, a disease driven by a translocation of the BCR and ABL genes. The onset, progression, and physiologic outcomes of BCR-ABL–mediated CML in human patients can be recapitulated in mice in vivo by retrovirally transducing the p210 form of BCR-ABL into the HSC-enriched population (22). To test if β-arr is required for CML development, KLS cells isolated from wild-type, $\beta\text{-arr1}^{-/-}$, and $\beta\text{-arr2}^{-/-}$ were infected with viral BCR-ABL and serially replated in vitro (Fig. 2 *A* and *B*). Significantly fewer colonies formed in both primary and secondary plating in the β-arr2–deficient HSCs, suggesting that loss of β-arr2 can disrupt

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Conflict of interest statement: R.J.L., T.R., M.F., and J.J.K. are named inventors on published PCT application WO/2011/133211 (publication date 10/27/2011) relating to β-arrestin2 as a therapeutic target in myeolgenous leukemia.

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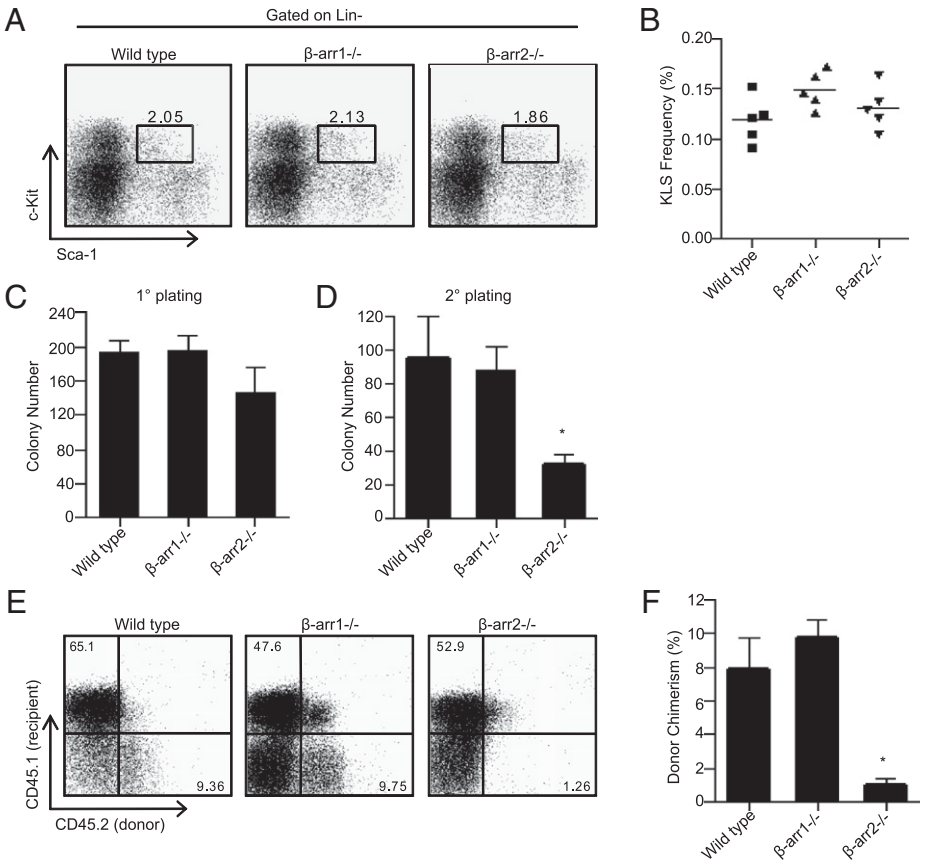

Fig. 1. Analysis of HSC-enriched cells in β-arr2–null mice. (*A*) Representative analysis of KLS frequency in wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ bone marrow. (*B*) Average frequency of KLS cells in wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ mice ($n = 5$). (*C*) Colony formation by KLS cells from wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ mice plated in quadruplicate (1,000 cells/well) in methylcellulose media. The graph shows an average of three independent experiments. (*D*) Colonies from wild-type, β-arr1$^{-/-}$ and β-arr2$^{-/-}$ mice were serially replated in quadruplicate (10,000 cells/well) in methylcellulose media. The graph shows an average of three independent experiments (*$P < 0.05$). (*E*) Representative FACS analysis of the donor-derived chimerism: 500 KLSF cells from wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ mice were transplanted together with competitor bone marrow cells into lethally irradiated congenic recipients. Donor-derived chimerism was analyzed at 14 wk (six mice in each cohort). (*F*) Plots show the average donor-derived chimerism after long-term reconstitution of the wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ KLSF cells (*$P < 0.05$).

CML cancer stem cell renewal in vitro. To test if β-arrs are required for the propagation of CML in vivo, HSCs from wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ mice were infected with BCR-ABL and transplanted into primary wild-type recipients. Mice transplanted with either wild-type and β-arr1$^{-/-}$ BCR-ABL cells displayed symptoms of CML disease onset, which included weight loss and splenomegaly (Fig. S4*A*); 92% of these mice died within 2 mo of transplantation (12 of 13 mice) (Fig. 2*C*). In contrast, only 7% of the mice transplanted with BCR-ABL–infected β-arr2$^{-/-}$ HSCs succumbed to disease (1 of 14 mice) (Fig. 2*C*). These data indicate that β-arr2 signaling is essential for normal CML establishment in vitro and in vivo.

The genetic models harboring germ-line deletion of β-arr2 demonstrated that chronic loss of β-arr2 inhibits the initiation of the disease; however, it was unclear whether acute removal of β-arr2 could inhibit fully established disease. We thus designed a β-arr2 retroviral shRNA to down-regulate β-arr2 in CML stem cells. To ensure knockdown of β-arr2, we measured β-arr2 mRNA levels in c-Kit^{+}Lin^{-} cells infected with β-arr2 shRNA and observed a clear reduction in β-arr2 mRNA levels (Fig. S5). Subsequently, sorted CML stem cells (BCR-ABL^{+} KLS cells) were retrovirally transduced with either a control shRNA or β-arr2 shRNA, resorted, and then plated (Fig. 2*D*). Loss of β-arr2 significantly reduced the number of colonies formed by CML stem cells in serial replating assays, suggesting that targeting β-arr2 is not only required for the initiation of CML but also the sustained growth and maintenance of the disease (Fig. 2 *E* and *F*).

To define a possible mechanism by which β-arr2 may influence leukemogenesis, we focused on the Wnt/β-catenin pathway. This was of particular interest because β-arr2 has been shown to be required for the activation of β-catenin in mouse embryonic fibroblasts (23) and to regulate Wnt5a-driven endocytosis of Frizzled 4 (Fzd4) through its interaction with phosphorylated disheveled-2 (12). However, whether this connection is physiologically relevant is unknown. We first examined whether the absence of β-arr2 affected the activity of β-catenin. Wild-type or β-arr2–deficient KLS cells were transformed with BCR-ABL and the levels of activated β-catenin were measured. As shown in Fig. 3 *A* and *B*, the loss of β-arr2 led to a significant reduction in activated β-catenin levels. To test if β-catenin was in fact functionally downstream of β-arr2, we tested the capacity of activated β-catenin to complement the CML growth defect in the BCR-ABL transduced β-arr2$^{-/-}$ cells. Introduction of constitutively activated β-catenin (18) rescued the defect in colony formation in the β-arr2$^{-/-}$ BCR-ABL–infected cells (Fig. 3*C*). Taken together, these data suggest that β-arr2 plays a critical role in BCR-ABL–induced CML through its downstream regulation of β-catenin signaling, and indicates that these proteins may lie in a hierarchy in context of leukemia development.

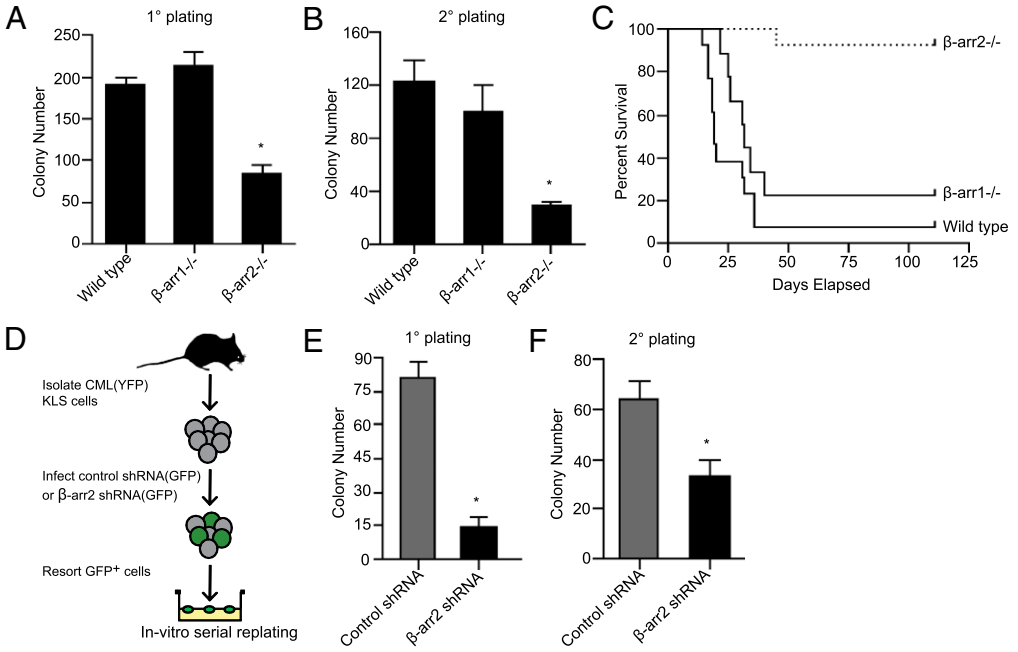

Fig. 2. Loss of β-arr2 impairs CML development. (*A*) KLS cells from wild-type, β-arr1$^{-/-}$, or β-arr2$^{-/-}$ bone marrow were infected with BCR-ABL retrovirus and plated in quadruplicate (1,000 cells/well) in methylcellulose media. The graph shown is an average of three independent experiments (*$P < 0.05$). (*B*) Secondary plating of wild-type, β-arr1$^{-/-}$, or β-arr2$^{-/-}$ bone marrow KLS cells infected with BCR-ABL in quadruplicate (10,000 cells/well) in methylcellulose media. The graph shown is an average of two independent experiments (*$P < 0.05$). (*C*) Survival curve of mice transplanted with KLS cells infected with BCR-ABL retrovirus (wild-type, $n = 13$; β-arr1$^{-/-}$, $n = 13$; or β-arr2$^{-/-}$, $n = 14$) (*D*) Experimental scheme for the transduction of CML cells with control shRNA-IRES-GFP or β-arr2 shRNA-IRES-GFP for 48 h and their subsequent plating in methylcellulose media. (*E*) Colonies from BCR-ABL^{+} KLS cells infected with control shRNA or β-arr2 shRNA plated in quadruplicate in methylcellulose (*$P < 0.05$, $n = 2$). (*F*) Secondary plating of CML BCR-ABL^{+} KLS cells with control shRNA or β-arr2 shRNA in quadruplicate (10,000 cells/well) in methylcellulose media (*$P < 0.05$, $n = 2$).

The chronic phase of myeloid leukemia can progress to the more aggressive blast-crisis phase (bcCML). bcCML is characterized by the severe arrest of myeloid cell differentiation and hyperproliferation of immature cells (24, 25) and a greater inability to respond to kinase inhibition. Human bcCML can be closely mimicked in mice by expression of NUP98-HOXA9 (26–28), which is a translocation in humans associated with bcCML as well as de novo acute myeloid leukemia (29–31), together with BCR-ABL in KLS cells. Thus, the leukemia generated by the combined expression of BCR-ABL and NUP98-HOXA9 in the KLS population is referred to here as bcCML. Given the impairment of CML incidence in chronic phase in the absence of β-arr2, we investigated whether β-arr2 remains important in the onset and maintenance of CML in the blast-crisis phase. We first tested the in vitro colony forming capacity of β-arr2$^{-/-}$ cells infected with both BCR-ABL and NUP98-HOXA9 and found a significant reduction in primary and secondary colony formation (Fig. 4 *A* and *B*). To test if β-arrestins are required for establishment of bcCML in vivo, cells from wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ mice were infected with BCR-ABL and NUP98-HOXA9 and transplanted into primary and secondary recipients (Fig. 4*C* and Fig. S6). Over a period of 3 wk, 100% of the wild-type and β-arr1$^{-/-}$ transplanted animals succumbed to bcCML. In contrast, all of the mice receiving BCR-ABL and NUP98-HOXA9 transduced β-arr2$^{-/-}$ cells were still alive after 3 mo (Fig. 4*C*), suggesting that β-arr2 signaling is required for establishment of the more aggressive blast crisis phase of CML as well.

We next assessed if β-arr2 deficiency could impair the growth and propagation of established bcCML. To this end we isolated the Lin^{-} stem cell-enriched population from established bcCML samples, infected them with either control or β-arr2 shRNA, and tested their ability to form colonies in vitro. Significantly fewer colonies formed in the bcCML samples infected with β-arr2 shRNA than with the control shRNA (Fig. 4 *D* and *E*). We further tested if the acute depletion of β-arr2 in bcCML stem cells via shRNA-mediated knockdown leads to a defect in disease incidence in vivo. One-hundred percent of the recipient mice that received vector-transduced cells and 80% of the recipient mice that received the β-arr2–depleted cells succumbed to disease (Fig. 4*F* and Fig. S4). The loss of β-arr2 clearly affected the self-renewal and propagative ability of bcCML cancer stem cells; in the secondary transplants, 90% of mice receiving β-arr2–depleted bcCML cells survived (Fig. 4*G*), whereas none of the control mice survived. Analysis of the spleens from the control shRNA-treated bcCML mice demonstrated significant expansion in the frequency of undifferentiated cells (Lin^{-}), consistent with bcCML (Fig. 4 *H* and *I*). In contrast, the β-arr2–deficient bcCML samples displayed a notable increase in the percentage of mature myeloid cells (Fig. S7) and a significant increase of differentiated cell types (Fig. 4 *H* and *I*, and Figs. S8 and S9). The in vivo serial transplantation studies indicate that depletion of β-arr2 promotes the differentiation of bcCML cells, thereby exhausting the cancer stem cell population and consequently impairing disease development and propagation in vivo. These results cumulatively implicate β-arr2 as a unique molecular target in leukemia that has the capacity to regulate CML in both its chronic and highly aggressive, therapy-resistant blast-crisis phase.

To define if β-arrestin could potentially serve as a therapeutic target, we examined whether inhibition of β-arr2 also impairs growth in human leukemia cells. Delivery of β-arr2 lentiviral shRNA into an established human bcCML cell line (K562) reduced β-arr2 mRNA levels 44-fold (Fig. 4*J*) and significantly impaired colony formation (Fig. 4*K*). To functionally test the consequence of β-arr2 depletion in primary cells we transduced β-arr2 shRNA into isolated primary CD34^{+} cells from two independent human bcCML patient samples. Depletion of β-arr2

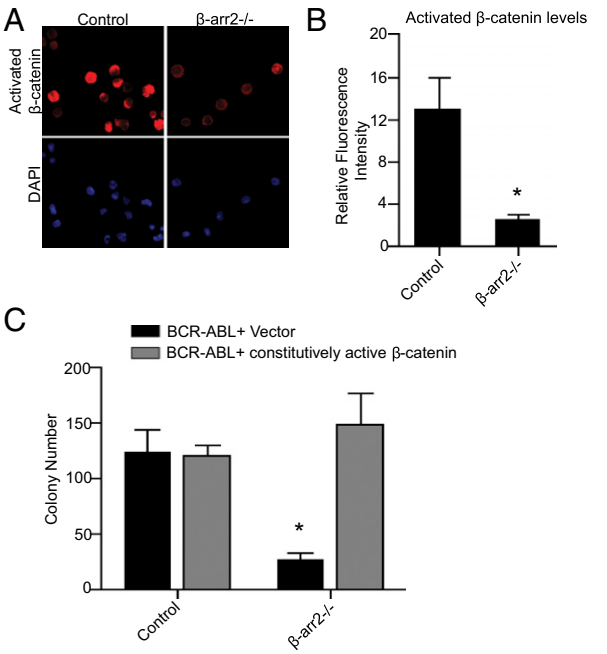

Fig. 3. β-arr2 loss impairs Wnt/β-catenin signaling in BCR-ABL^{+} leukemia. (*A*) Expression of activated β-catenin in wild-type or β-arr2$^{-/-}$ KLS cells infected with BCR-ABL retrovirus. Red represents nonphosphorylated β-catenin and blue represents DAPI. (Original magnification, 20×.) (*B*) Quantification using Metamorph software of the fluorescence intensities per cell for activated β-catenin in control BCR-ABL infected KLS cells to β-arr2$^{-/-}$ KLS cells ($n = 2$, $^{*}P < 0.05$). (*C*) Bone marrow KLS cells from wild-type or β-arr2$^{-/-}$ mice were infected with control vector or constitutively active mutant β-catenin together with BCR-ABL and were plated in quadruplicate in methycellulose media ($^{*}P < 0.01$, $n = 2$).

(Fig. 4*L*) reduced the number of colonies formed in both patient samples (Fig. 4 *M* and *N*), further demonstrating that β-arr2 regulates the expansion of bcCML cells. Importantly, this suggests that inhibition of β-arr2–mediated signaling could provide an alternative target in patients who have become resistant to kinase inhibitors.

Discussion

Targeting CML with kinase inhibitors has been successful in keeping the disease at bay. However the inability to eradicate CML cancer stem cells with this approach necessitates lifelong drug dependence and increased risk of relapse and resistance. Thus finding the signals that drive CML cancer stem cell growth and renewal and can also be targeted to allow for successful elimination of disease is critical. Furthermore, because CML can mutate from a slow-growing chronic phase to a highly drug-resistant blast-crisis phase, there is a clear need to identify BCR-ABL–independent pathways and targets that underlie this progression as well.

To study the potential role of β-arrs in hematopoiesis and oncogenesis, we made use of the β-arr1 and β-arr2 knockout mice. We found that deletion of either β-arr1 or β-arr2 did not influence normal hematopoiesis under homeostatic conditions. However, β-arr2 knockout mice display a defect in HSC self-renewal in vivo when serially transplanted. Recently, Yue et al. reported that β-arr1 depletion in Zebrafish embyros resulted in down-regulation of hematopoietic progenitor markers; in contrast, most of the hematopoietic genes were not down-regulated when β-arr2 was depleted (32). Consistent with this finding, we observed that β-arr1$^{-/-}$ mice displayed reduced self-renewal in vitro upon secondary plating (Fig. 1*D*). However, in both normal and malignant contexts, the loss of β-arr2 in the murine models had a significantly more dramatic impact. It is possible that the differences in the dependence of hematopoiesis on β-arr2 in mouse and zebrafish models are because of differences in the developmental origin or spatial location of HSCs between these organisms.

Several in vitro studies have highlighted a critical role for β-arrs in certain transformed cell lines (33, 34). However, whether genetic loss of β-arrs affects the progression of cancers in general, and of leukemia in particular, was unknown. Our data suggest that deletion of the multifunctional adaptor protein β-arr2 leads to the virtual inability of BCR-ABL and other cooperating oncogenes to induce CML and the more aggressive bcCML in vivo. Although none of the mice transplanted with BCR-ABL infected β-arr2–null cells developed CML, we were able to use β-arr2–null bcCML cells in serial transplant assays to show that defects in disease formation are primarily because of deficiencies in long-term self-renewal.

To determine whether independent pathways implicated in the maintenance of CML could act in a hierarchy we examined the relationship between β-arr2 and β-catenin. The evolutionarily conserved Wnt/β-catenin developmental pathway has been found to be highly active in, and required for, the propagation of myeloid leukemias (16, 35–38). Furthermore, β-arr2 has been implicated in canonical Wnt/β-catenin signaling in cultured cell lines (12, 23). Whether this connection may be important for a primary physiologically relevant disease model prompted us to test if loss of β-arr2 mediates Wnt/β-catenin signaling in leukemic stem cells. Loss of β-arr2 effectively reduced the activation of β-catenin and Wnt-related targets in both CML and bcCML (Fig. 3, and Figs. S10 and S11) and the effects of β-arr2 deficiency could be rescued by the ectopic overexpression of activated β-catenin. These findings better delineate the mechanism of action of β-arr2 in myeloid leukemia and may allow in the long-term identification of new intervention points at which the pathway could be targeted. Further insight into the mechanism by which β-arr2 mediates β-catenin function may come from studies focused on identifying whether β-arr2 also affects Fzd or disheveled function in vivo. Additionally, whether β-arr2 influences other developmental signals, such as the Hedgehog/Smoothened and TGF-β pathways, in CML and bcCML will also be important avenues of future research. Both pathways are known mediators of hematopoietic malignancies and may be influenced by β-arr signaling (12–14, 39).

Collectively, these results identify β-arr2 as a potential target for therapeutics in patients with chronic and bcCML. The fact that β-arr2–null mice appear to develop normally without overt defects suggests that there may be a therapeutic window for targeting β-arr2, although only further preclinical work will enable proper assessment of this possibility. Our data also suggests that the requirement for β-arr2 in the potentiation of CML and bcCML rests, at least in part, in its ability to positively regulate Wnt/β-catenin signaling. The mediation of β-catenin signaling by β-arr2 raises the possibility that other solid cancers driven by aberrant Wnt signaling may also be dependent upon β-arr2 for disease onset and progression.

Experimental Procedures

Mice. The wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ mice used were on the C57BL/6J background (40, 41) and B6-CD45.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) mice were used as transplant recipients. All mice were 8–10 wk of age. All mice were bred and maintained on acidified, antibiotic water in the animal care facilities at Duke University Medical Center and University of California, San Diego. All animal experiments were performed according to protocols approved by the Duke University and University of California, San Diego Institutional Animal Care and Use Committee.

HSC Isolation and Analysis. Isolation of HSCs from bone marrow and their transplantation for in vivo analysis of function were performed as previously described (42). For analysis of lineage markers (Lin), bone marrow cells from wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ mice were incubated with antibodies

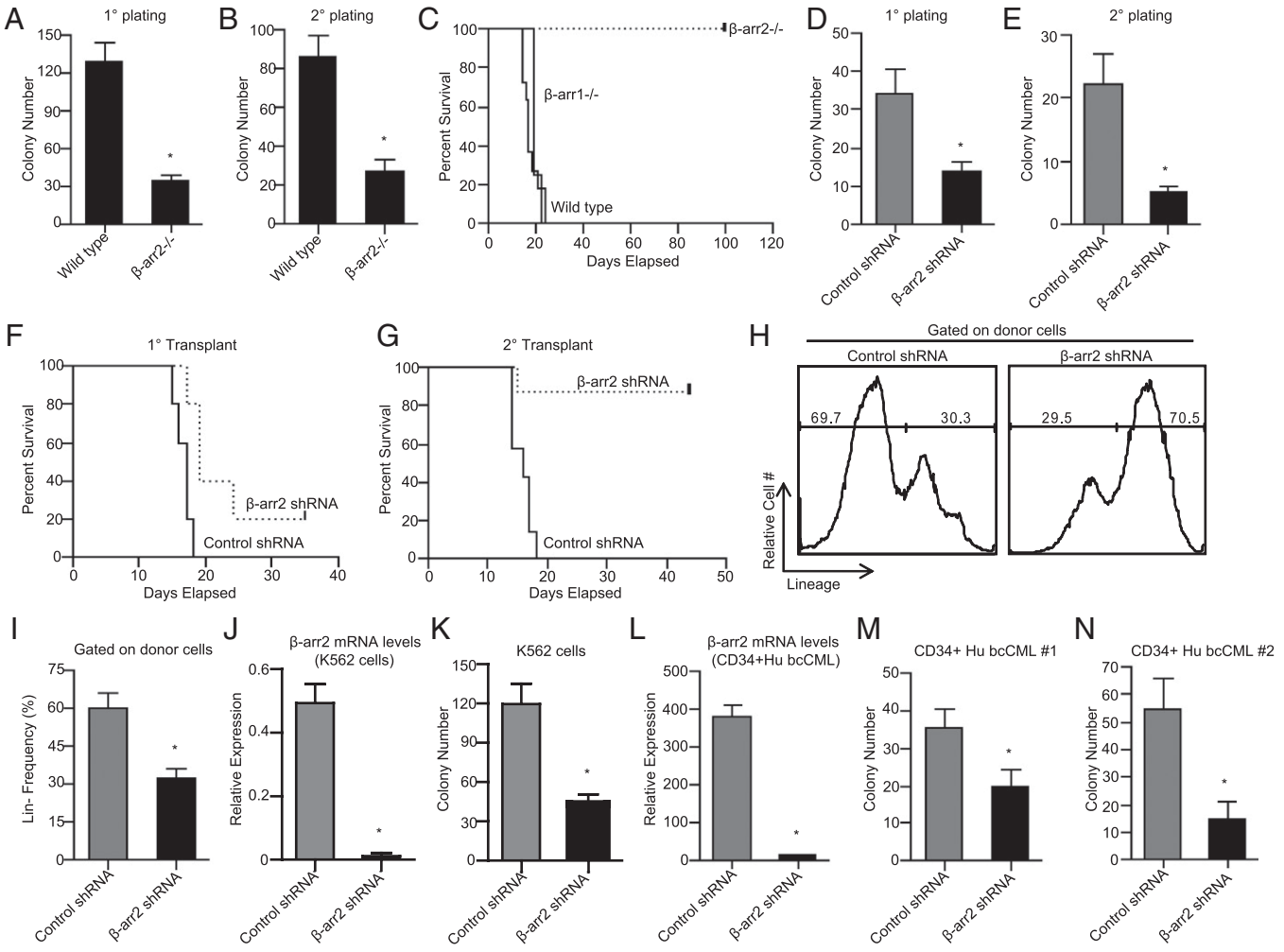

Fig. 4. Loss of β-arr2 impairs the propagation of highly aggressive bcCML. (*A*) Colony-forming ability of wild-type and β-arr2$^{-/-}$ KLS cells transduced with BCR-ABL and NUP98-HOXA9 retroviruses and plated in methylcellulose media. The graph shown is an average of two independent experiments (*$P < 0.05$). (*B*) Secondary plating of the wild-type and β-arr2$^{-/-}$ cells infected with BCR-ABL and NUP98-HOXA9 (*$P < 0.05$, $n = 2$). (*C*) Survival curve of recipient mice transplanted with 10,000 wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ KLS cells infected with BCR-ABL and NUP98-HOXA9 viruses ($n = 8$ each). (*D*) Colonies from Lin$^-$ bcCML cells expressing control or β-arr2 shRNA plated in quadruplicate in methylcellulose (*$P < 0.05$, $n = 2$). (*E*) Secondary plating of bcCML cells expressing control or β-arr2 shRNA in quadruplicate (10,000 cells/well) in methylcellulose media (*$P < 0.05$, $n = 2$). (*F*) Survival curve of mice transplanted with established bcCML cells infected with control or β-arr2 shRNA ($n = 5$ mice each, *$P < 0.01$). (*G*) Survival curve of mice secondarily transplanted with Lin$^-$ cells from primary shRNA expressing leukemias (control shRNA, $n = 17$; β-arr2 shRNA, $n = 15$, *$P < 0.0001$). (*H*) Representative FACS analysis of Lin$^+$ cells in control or β-arr2 shRNA-expressing bcCML. (*I*) Graph showing the average frequency of Lin$^-$ cells in wild-type and β-arr2$^{-/-}$ bcCML splenocytes (*$P < 0.05$, $n = 3$). (*J*) β-arr2 mRNA levels in K562 (human bcCML cells) infected with lentiviral control or β-arr2 shRNA. Gene-expression levels were normalized to human actin (*$P < 0.001$, $n = 2$). (*K*) K562 cells were infected with control shRNA or β-arr2 shRNA and plated in quadruplicate (1,000 cells/well) in methylcellulose media. Colonies were counted after 10–14 d (*$P < 0.01$, $n = 3$). (*L*) β-arr2 mRNA levels in CD34$^+$ human bcCML cells infected with lentiviral control or β-arr2 shRNA. Gene-expression levels were normalized to human actin (*$P < 0.001$, $n = 2$). (*M* and *N*) Colony-forming ability of CD34$^+$ cells infected with lentiviral control or β-arr2 shRNA from two independent primary human bcCML patients (*$P < 0.05$).

to murine Ter119, Mac-1, Gr-1, B220, CD4, CD8, and CD3 (eBiosciences) and analyzed by FACS. For the transplantation assays, bone marrow cells from wild-type, β-arr1$^{-/-}$, or β-arr2$^{-/-}$ mice were stained, and 500 wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ c-Kit$^+$Lin$^{-/lo}$Sca-1$^+$Flk2$^-$ (KLSF) cells were injected along with 200,000 competing bone marrow cells into lethally irradiated CD45.1 recipients. Recipient mice were killed and analyzed for donor chimerism at 14 wk.

Generation and Analysis of CML and bcCML. KLS cells from wild-type, β-arr1$^{-/-}$, and β-arr2$^{-/-}$ mice were isolated and cultured overnight in X-vivo with 10% (vol/vol) FBS, 100 ng/mL stem cell factor, and 20 ng/mL thrombopoietin in a 96-well U-bottom plate (40,000 per well). Subsequently, for the CML experiments cells were infected with MSCV-BCR-ABL-IRES-GFP. Cells were harvested 48 h later and transplanted retro-orbitally with 200,000 whole bone marrow cells into lethally irradiated allelically mismatched recipients. To generate bcCML, KLS cells were infected with MSCV-BCR-ABL-IRES-GFP and MSCV-NUP98-HOXA9-IRES-YFP. Where indicated, MSCV-BCR-ABL-IRES-YFP and MSCV-NUP98-HOXA9-IRES-tNGFR viruses were used. Cells were harvested 48 h later and transplanted retro-orbitally into allelically mismatched recipients. After transplantation, recipient mice were evaluated daily for signs of morbidity, weight loss, failure to groom, and splenomegaly. Premorbid animals were killed and relevant tissues were harvested and analyzed by flow cytometry and histopathology. For flow cytometric analysis of CML and bcCML stem cells, leukemic cells were stained with antibodies for KLS and Lin$^-$, respectively and data analyzed on FACS-Vantage (BD) and Flowjo software (Tree Star).

Methylcellulose Colony Formation Assays. For the CML colony formation assays, BCR-ABL$^+$ KLS cells were sorted and plated with complete methylcellulose medium (M3434; Stem Cell Technologies). For the bcCML colony formation assays Lin$^-$ BCR-ABL$^+$ NUP98-HOXA9$^+$ cells were plated with complete methylcellulose medium. Colonies for both leukemia types were

counted 8–10 d after plating. Cells were harvested and counted, and 10,000 cells were replated into 24-well plates for the serial replating analysis.

Immunofluorescence Staining. KLS cells were sorted by FACS and infected with BCR-ABL-IRES-GFP retrovirus and resorted for infected cells after 72 h. Cells were cytospun, air-dried, fixed in 4% (wt/vol) paraformaldehyde, and then stained with mouse antinonphosphorylated β-catenin (Upstate; Clone 8E4) and AlexaFluor 594-conjugated anti-mouse IgG (Molecular Probes). Cells were counterstained with DAPI (Molecular Probes). Slides were viewed on an Axio Imager (Zeiss) at 20× magnification. Fluorescence intensity analysis was quantified using Metamorph software (Molecular Devices).

Real-Time RT-PCR Analysis. RNA was isolated using RNAqueous-Micro (Ambion) and converted to cDNA using SuperScript II (Invitrogen). Quantitative real-time PCR was performed using an iCycler (BioRad) by mixing equal amounts of cDNAs, iQ SYBR Green Supermix (BioRad) and gene-specific primers. Primer sequences are available upon request. Human AXIN2 (Hs00610344_m1) and ARRB2 (Hs01034135_m1) gene levels were analyzed with TaqMan Gene Expression Assays (Applied Biosystems). All real-time data were normalized to Gapdh or actin.

Human bcCML Experiments. Studies on primary human bcCML samples were carried out with approval from the Duke University Institutional Review Board. Mononuclear cells were isolated from peripheral blood samples using density-gradient centrifugation. RNA was isolated using RNAqueous-Micro (Ambion) and was converted to cDNA using SuperScript II (Invitrogen). Quantitative real-time PCR was performed with primers specific to human β-arr2 or human actin. Primer sequences are available on request. For methylcellulose colony formation assays, $CD34^+$ cells were FACS-sorted from primary human bcCML patients, infected with control or β-arr2 lentiviral shRNA, and plated into a 24-well plate (50,000 cells per well) with complete methylcellulose medium (Stem Cell Technologies). Lentiviral shRNA construct to human β-arr2 was cloned in FG12 lentiviral vector. Colony numbers were counted 10–14 d after plating.

Statistical Analysis. Statistical analyses were performed using one-way ANOVAs or Student *t* test (normal distribution). $P < 0.05$ was considered statistically significant.

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