β-Arrestin1 promotes the progression of chronic myeloid leukaemia by regulating BCR/ABL H4 acetylation

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Background: β-Arrestins are scaffold proteins that interact with various cellular signals. Although β-arrestin2 mediates the initiation and progression of myeloid leukaemia, the critical role of β-arrestin1 in the chronic myeloid leukaemia (CML) is still unknown. The aim of this study is to investigate the essential function of β-arrestin1 in CML.

Methods: The expressions of β-arrestin1 and BCR/ABL in CML patients, animal models and K562 cells were measured by RT–PCR, immunofluorescence and western blotting. The effect of β-arrestin1 on CML animal models and K562 cells by colony formation, MTT and survival analysis were assessed. BCR/ABL H4 acetylation was analysed through the use of Chromatin-immunoprecipitation (ChIP) -on-chip and confirmed by ChIP respectively. Co-immunoprecipitation and confocal were examined for the binding of β-arrestin1 with enhancer of zeste homologue 2 (EZH2).

Results: The higher expression of β-arrestin1 is positively correlated with clinical phases of CML patients. Depletion of β-arrestin1 decelerates progression of K562 and primary cells, and increases survival of CML mice. Importantly, silenced β-arrestin1 results in the decrease of BCR/ABL H4 acetylation level in K562 cells. Further data illustrate that nuclear β-arrestin1 binds to EZH2 to mediate BCR/ABL acetylation and thus regulates cell progression in K562 cells and the survival of CML mice.

Conclusions: Our findings reveal a novel function of β-arrestin1 binding to EZH2 to promote CML progression by regulating BCR/ABL H4 acetylation.

Chronic myeloid leukaemia (CML), a clonal disorder of hemato-poietic stem cells, constitutes about 15% of adult leukaemia. Its clinical phases typically progresses over time from an early chronic phase (CP) through an accelerated phase (AP) and terminal blast crisis phase (BC). The outcome of CML is positively correlated with the advanced clinical stages. The molecular hallmark of CML, BCR/ABL fusion oncogene, resulting from the translocation between chromosomes 9 and 22, has a critical role in the pathogenesis of CML. In fact, BCR/ABL levels were increased during disease progression (Neviani et al, 2005).

β-Arrestins, including four superfamily members, are multi-functional adaptor and scaffold proteins (Lefkowitz and Whalen, 2004). Only β-arrestin1 and β-arrestin2 are ubiquitously expressed. The traditional function of β-arrestin proteins is to mediate the

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desensitization and internalisation of G protein-coupled receptors (GPCRs) (Luttrell and Lefkowitz, 2002; Katanaev *et al*, 2005) and other seven transmembrane-spanning receptors in an agonist-dependent manner (Lefkowitz and Shenoy, 2005). Emerging evidence has demonstrated that *β*-arrestin1 mediates cellular function in some solid tumours. The best known examples are prostate cancer (Lakshmikanthan *et al*, 2009), lung cancer (Raghuwanshi *et al*, 2008), breast cancer (Ge *et al*, 2004) ovarian cancer (Rosano *et al*, 2009) and others. Recently, although *β*-arrestin2 was reported to participate in the initiation and progression of myeloid leukaemia (Fereshteh *et al*, 2012), the critical role of *β*-arrestin1 in human haematological malignancies remains unresolved. More interestingly, *β*-arrestin1 has the ability of nuclear translocation and histone H4 acetylation modification with the delta-opioid receptors activated (Kang *et al*, 2005). The acetylation status of histone H4 terminal tails is one component of chromatin remodelling and chromatin-based processes including gene expression (Brower-Toland *et al*, 2005). Several studies underscored histone H4 hyperacetylation associated with BCR/ABL tyrosine kinase *in vitro* and *in vivo* and proceeding, at least in part. Histone H4 acetylation status, in particular at the BCR/ABL promoter, influences the rearranged gene transcription, supporting a role for epigenetic chromatin modifications in the expression of the causative gene for CML (Brusa *et al*, 2006). Therefore, we are curious whether *β*-arrestin1 could regulate the acetylation of BCR/ABL in CML progression.

Covalent modifications of core histone tails including acetylation, methylation, phosphorylation, ADP-rybosilation and ubiquitination, determine the chromatin structure and create binding surfaces for protein recognition, such as the bromodomain for acetylated lysines and the chromodomain for methylated lysines, therefore dictate gene transcription rate. In particular, the acetylation at specific lysine residues of H3 and H4 histone NH2-terminal tails weakens electrostatic interactions that tether octamer tails to the DNA phosphate backbone and induces reversible chromatin decondensation required for DNA transcription, replication and recombination (Strahl and Allis, 2000). Covalent modification could be regulated by other factors. Among the pivotal regulators, enhancer of zeste homologue 2 (EZH2), a Polycomb Group (PcG) protein homologous to Drosophila enhancer of zeste, is involved in gene silencing (Kleer *et al*, 2003). EZH2 catalyses the addition of methyl groups to histone H3 at Lys 27 (H3K27) forming H3K27me3, the activated form of H3K27, in target gene promoters, leading to epigenetic silencing (Wei *et al*, 2011). EZH2 is reported to be over-expressed, amplified and with an altered localisation in lymphoma or acute myeloid leukaemia (AML) (Roman-Gomez *et al*, 2007; Strathdee *et al*, 2007). However, whether EZH2 is involved in CML is largely unknown.

Herein, we revealed that enhanced *β*-arrestin1 expression with the advanced clinical stages of CML patients, and depletion of *β*-arrestin1 reduced the CML progression *in vitro* and *in vivo*, by regulating BCR/ABL H4 acetylation through binding to EZH2.

MATERIALS AND METHODS

Patients and samples. The use of human samples was approved by the Research Ethics Committee of Chongqing Medical University. All the patients involved in this study were admitted patients from haematological ward, and the nonmalignant haematological adult in-patient patients as the healthy control, from the First Affiliated Hospital of Chongqing Medical University who were diagnosed with clinically defined chronic myeloid leukaemia. All the information of patients is listed in Table 1.

Table 1. The clinical demographics of CML patients						
Patient no.	Age (Y)	Gender (M/F)	PB WBC count ($\times 10^9 l^{-1}$)	BM blast cell count (%)	BCR/ABL fusion gene	Clinical stages
1	28	F	40.3	6.5	+	CP
2	43	F	36	5	+	CP
3	38	M	28.8	5.6	+	CP
4	31	F	70.4	7.8	+	CP
5	47	F	60.7	3.4	+	CP
6	43	M	56.7	6.8	+	CP
7	32	F	76.7	8.3	+	CP
8	50	M	39.4	5.5	+	CP
9	40	F	49.7	6.7	+	CP
10	35	F	60.3	7.1	+	CP
11	23	M	67.1	9	+	CP
12	46	M	41.2	5.9	+	CP
13	48	F	80.5	15.2	+	AP
14	34	M	101.5	16.9	+	AP
15	36	F	89.8	14.8	+	AP
16	50	F	66.4	14.5	+	AP
17	51	F	153.3	16.3	+	AP
18	32	M	98	15.1	+	AP
19	39	M	60.6	12.5	+	AP
20	36	F	94.4	13	+	AP
21	47	F	160.2	71	+	BC
22	48	M	150.3	60.9	+	BC
23	28	M	108.9	61.4	+	BC
24	47	F	405.3	81.2	+	BC
25	37	F	190.6	78.6	+	BC
26	50	M	98.4	57.7	+	BC

Abbreviations: AP = accelerated phase; BC = blast crisis phase; BM = bone marrow; CP = chronic phase; F = female; M = male; no. = number; PBC = peripheral blood cells; WBC = white blood cells; Y = years.

Bone marrow samples were obtained from the subjects. All patients completed and signed the informed consent form.

Antibodies, virus particles and reagents. Primary antibodies used were: mouse monoclonal anti-*β*-arrestin1, anti-*β*-arrestin2, anti-*β*-actin (Santa Cruz, Santa Cruz, CA, USA), anti-EZH2 (Cell Signaling, Danvers, MA, USA), rabbit monoclonal anti-*β*-arrestin1 (Abcam, Cambridge, MA, USA), anti-H3, anti-H3K27me3 (Cell Signaling), rabbit polyclonal anti-BCR (Santa Cruz) and as secondary antibodies for immunoprecipitation (IP) assays, goat anti-mouse or anti-rabbit conjugated with DyLight (Abcam). Antibodies against histone H4, acetyl-histone H4, acetyl-histone H4 (specific against acetylated Lys 12, Lys 16) were obtained from Upstate Biotechnology (Lake Placid, NY, USA). 3-deazaneplanocin A (DZNep), the inhibitor for EZH2, was purchased from Sigma (St Louis, MO, USA). All the *β*-arrestins related lentivirus package and control plasmid cDNAs were kindly gifted by Professor Gang Pei (Shi *et al*, 2007).

Cell culture and mice. The primary CML cells were isolated mononuclear cells from the newly diagnosed CML patients' bone marrow and then cultured in 10% FBS IMEM. CML K562 and HEK293T cells (American Type Culture Collection, Manassas,

MD, USA) were cultured and maintained in RPMI 1640 medium and DMEM (Gibco-BRL, Carlsbad, CA, USA). Lentivirus particles were produced by co-transfect HEK293T cells with the packaging and vector plasmids. Titres of all lentiviral vectors were determined by transducing HEK293T cells with serial dilutions of vector supernatants. After ultra-centrifugation, the lentivirus titres were $1–10 \times 10^6$ transducing units ml^{-1} ($TU\ ml^{-1}$). K562 cells were infected by the lentivirus particles, then followed limit dilution to obtain the stable cells.

Four–to-six-week-old NOD/SCID mice were obtained from the Laboratory Animal Centre of Chongqing Medical University. All mice were housed in an SPF barrier animal facility before used. After irradiated with 2 Gy X-ray in the Experimental Animal Centre of the Third Military Medical University, 1×10^7 stable cells (K562-Ctrl, K562-siβ1, K562-siβ1 + β1) cells were tail vein injected into lethally irradiated NOD/SCID mice. Then peripheral blood (PB) WBC number was dynamically counted and the mice survival curves were analysis. All animal studies using cell lines were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (Workman et al, 2010).

Soft agar colony assay. After infection, different K562 cells were cultured in a two-layer soft agar for 14 days at 37 °C and 5% CO_2. Washed, single-cell suspension of cells were numerated and plated into 12-well flat-bottomed plates with a total of 2000 cells per well in a volume of 1000 μl per well. After incubation, the colonies ($\geqslant$40 cells for each) were counted. Experiments were performed triplicate at each point.

Real-time RT–PCR. Total RNA was extracted from cultured cells with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. SuperScript II (Takara, Dalian, China) were used for reverse transcription of purified RNA. All gene transcripts were quantified by PCR with thermocycler (Bio-Rad, Hercules, CA, USA) using SYBR reagents (Takara, Dalian, China) according to the manufacturer's instructions. Primer pairs are listed in Table 2.

Western blotting. For western blotting (WB) analysis, cells were collected by centrifugation and lysed in RIPA buffer at 4 °C. Total cell proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a PVDF membrane, immunoreacted with anti-β-arrestin1, anti-β-arrestin2, anti-EZH2, anti-BCR/ABL or anti-β-actin antibodies and then reacted with secondary antibodies, developed image with enhanced chemiluminescence assay (Millipore, Bedford, MA, USA).

Co-immunoprecipitation. For co-immunoprecipitation (Co-IP) assays, whole cell lysates were first pre-cleared with normal IgG and protein A/G plus conjugated agarose beads (Santa Cruz), then incubated with anti-EZH2 antibody (Cell Signaling) or anti-

β-arrestin1 (Santa Cruz) under gentle rocking at 4 °C overnight. Protein A/G plus was added, and the mixture was incubated for further 4 h at 4 °C. Following centrifugation, beads were washed five times with cold PBS and boiled in SDS-loading buffer. The samples were subjected to SDS-PAGE and western blotting analysis.

Immunofluorescence. For immunofluorescence (IF), cultured cells were washed three times in PBS, fixed with freshly prepared 4% formaldehyde in PBS for 15 min at room temperature, and subsequently permeabilized with 0.5% Triton X-100 for 5 min, closed with goat serum for 30 min and incubated with diluted primary antibodies at 4 °C overnight. The next day, cells were incubated for 1 h with the appropriate secondary antibodies conjugated to DyLight. Finally, the specimens were analysed with laser-scanning confocal microscope (Leica, Beusheim, Germany).

Chromatin immunoprecipitation. Chromatin-immunoprecipitation (ChIP) assays were done according to the manufacturer's instruction (Upstate, NY, USA). The presence of the target gene promoter sequences in both the input DNA and the recovered DNA immunocomplexes was detected by quantitative PCR. All the data of quantitative PCR was normalised to those amplified DNA fragments of GAPDH. The levels of BCR/GAPDH or ABL/GAPDH in control cells were viewed as 1.0-fold. The primer pairs for specific promoter regions were from -1000 base pairs to approximately $+500$ base pairs of the transcription start site of the genes. Primer pairs are listed in Table 2.

ChIP-on-chip. ChIP was performed essentially as described previously. The immunoprecipitation DNA including input samples was amplified and labelled by using DSL method. The labelled DNA were dissolved in 80 μl total volume including 3 × SSC, 0.2% SDS, 5 × Denhart's, 25% formamide buffer, hybridised with ChIP-DSL H20K promoter array (Aviva, San Diego, CA, USA) overnight. The hybridised samples were scanned by LuxScan 10KA (CapitalBio, Beijing, China). The image profiling was performed and calculated by LuxScan 3.0 software and Molecule Annotation System (CapitalBio; Jia et al, 2013).

Statistical analysis. One-way ANOVAs were used to statistically analyse the difference among the CML clinical phase patients, and the multiple treatments for cells. And the paired t-test was used to compare the difference between cells with two treatments. Pearson analysis was performed to investigate the correlation of mRNA expression levels of BCR/ABL and β-arrestin1. $P < 0.05$ was considered statistical difference. Statistical analyses were performed using GraphPad Prism 5.0.

Table 2. Primer pairs for this whole manuscript		
	Forward primer (5′–3′)	Reverse primer (3′–5′)
Real-time RT–PCR		
GAPDH	AGAAGGTGGTGAAGCAGGCATC	CGGCATCGAAGGTGGAAGAGTG
β-Arrestin1	CCTGGATGTCTTGGGTCTG	TGATGGGTTCTCCGTGGTA
β-Arrestin2	CAGCCAGGACCAGAGGACA	TGATAAGCCGCACAGAGTT
BCR/ABL	GACATGCCATAGGTAGCAATTTCCC	ACATCACGCCAGTCAACAGTCTGG
ChIP		
GAPDH	AGAAGGTGGTGAAGCAGGCATC	CGGCATCGAAGGTGGAAGAGTG
ABL	TCCAACCCTACTGCTCCCACC	ACCGCCGCCCCTCCCGGCTCTGGTC
BCR	CTGCGAGTTCTGCCAGAGAG	CACCCTCCCCCCGTCCCTGT

RESULTS

Increased β-arrestin1 expression promotes cell progression in CML cells. On the basis of our previous studies (Zou *et al*, 2008; Liu *et al*, 2011), we collected the bone marrow of CML patients in different clinical phases, including 12 cases of CP, 8 cases of AP and 6 cases of BC (Table 1), and tested the expression of β-arrestin1 and β-arrestin2 in mononuclear cells from these samples, respectively. The mononuclear cells from haematological nonmalignant patients ($n = 10$) were used as control. β-arrestin1 and β-arrestin2 mRNA, protein expression were measured by real-time RT–PCR, compared with its respective inner control GAPDH to report the relative fold (Figure 1A), IF (Figure 1B and C) and WB (Figure 1D) with β-actin as reference, respectively. All the observations illustrated that both β-arrestin1 and β-arrestin2 expression were elevated in different clinical phases of CML patients, especially for β-arrestin1. Importantly, the elevated levels of β-arrestin1 expression were positively correlated with the

clinical phases of CML. The enhanced expression of β-arrestin1 and β-arrestin2 were also confirmed by using the CML cell line, K562 (Figure 1E–G). The BCR/ABL expression levels were enhanced with the advanced clinical stages of CML (Figure 1H), which was corresponding with the reports about BCR/ABL. Unexpectedly, the BCR/ABL level was positively correlated with β-arrestin1 (Figure 1I, $R^2 = 0.871$) rather than β-arrestin2 (data not shown). Altogether, we then focused on the role of β-arrestin1 in CML in subsequent studies.

To further determine the role of β-arrestin1 in CML progression, we over-expressed and gene-silenced β-arrestin1 and β-arrestin2 by transducing K562 cells with β-arrestin1 (β1), β-arrestin2 (β2), β-arrestin1-siRNA (siβ1) and β-arrestin2-siRNA (siβ2) lentiviral particles, respectively. Transduction of K562 cells with nonspecific-siRNA lentiviral particles as control (Ctrl). Construction of β1 siRNA, pBS/U6/β1 siRNA and pBS/U6/nonspecific siRNA plasmids were as described previously (Wang *et al*, 2003; Lu *et al*, 2011). The nucleotide sequences carried in the plasmids are 5′-GGAAGCTCAAGCACGAAGACAA-3′ (siRNA
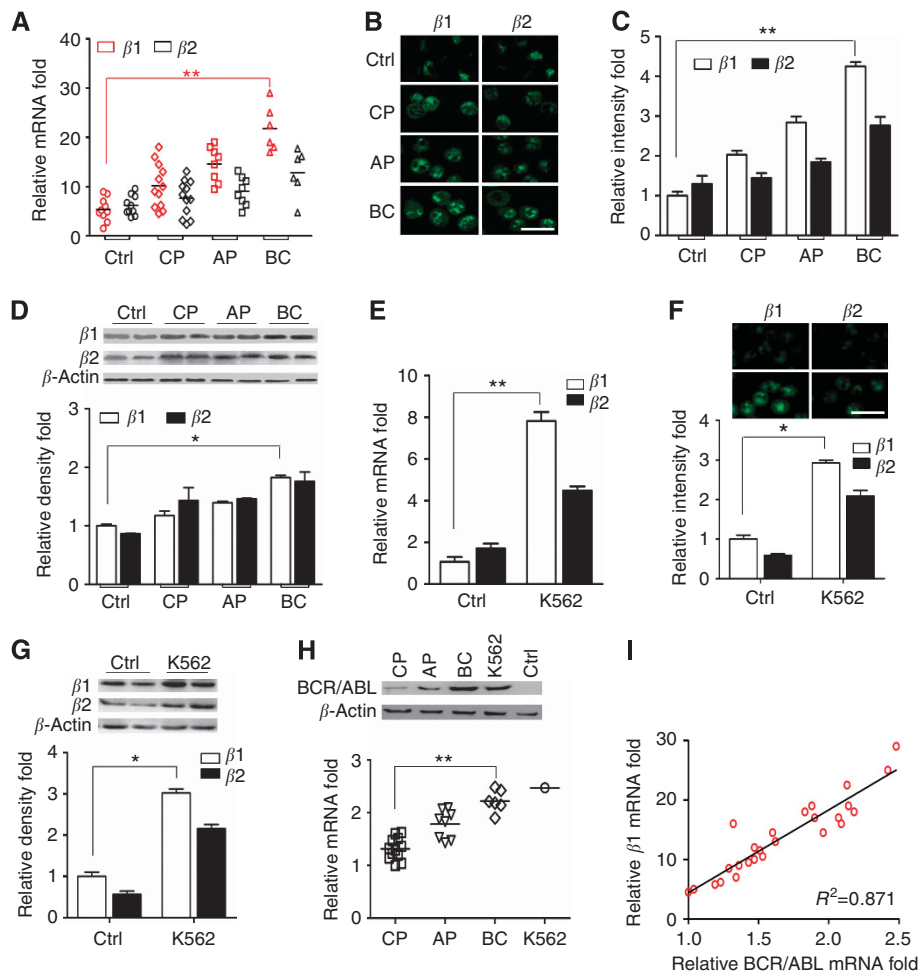

Figure 1. Expression of β-arrestin1 and BCR/ABL is enhanced in CML cells. The mRNA levels of β-arrestin1 and 2 in (**A**) bone marrow cells of newly diagnosed CML patients in different clinical phases and (**E**) K562 cells were analysed by real-time RT–PCR. Representative figures of IF (**B**, **F**) and WB (**D**, **G**) of β-arrestin1 and 2 expression levels in bone marrow cells of CML patients and K562 cells, scale bar, 10 μm. (**C** and **F**) The relative fluorescence intensity fold of β-arrestin1 and β-arrestin2 were quantified by NIS-Elements BR on Nikon software and the intensity of β-arrestin1 in Ctrl was viewed as 1.0-fold. (**D** and **G**) The WB bands of β-arrestin1 and 2 were quantified and normalised to β-actin by Bio-Rad Quantity-One software. (**H**)The mRNA levels of BCR/ABL in CML bone marrow cells and K562 cells were analysed by WB (upper panel) and RT–PCR (lower panel). All the data were calculated and normalised as fold change and the expression of β-arrestin1/GAPDH in Ctrl (haematological nonmalignant patients' cells as control) was viewed as 1.0-fold. The relative mRNA level of BCR/ABL was normalised with its corresponding GAPDH, and the mimimum relative mRNA level of CP group was viewed as 1.0-fold, and β-actin as loading control in WB. (**I**) The Pearson correlation analysis of BCR/ABL and β-arrestin1 mRNA levels ($R^2 = 0.871$) was performed by the use of GraphPad Prism 5.0. Data shown are means ± s.d. of three independent experiments, *$P < 0.05$ and **$P < 0.01$ *vs* the corresponding Ctrl. In **A**, **C**, **D** and **H**, *$P < 0.05$ and **$P < 0.01$ among the groups, respectively. β1 = β-arrestin1; β2 = β-arrestin2.
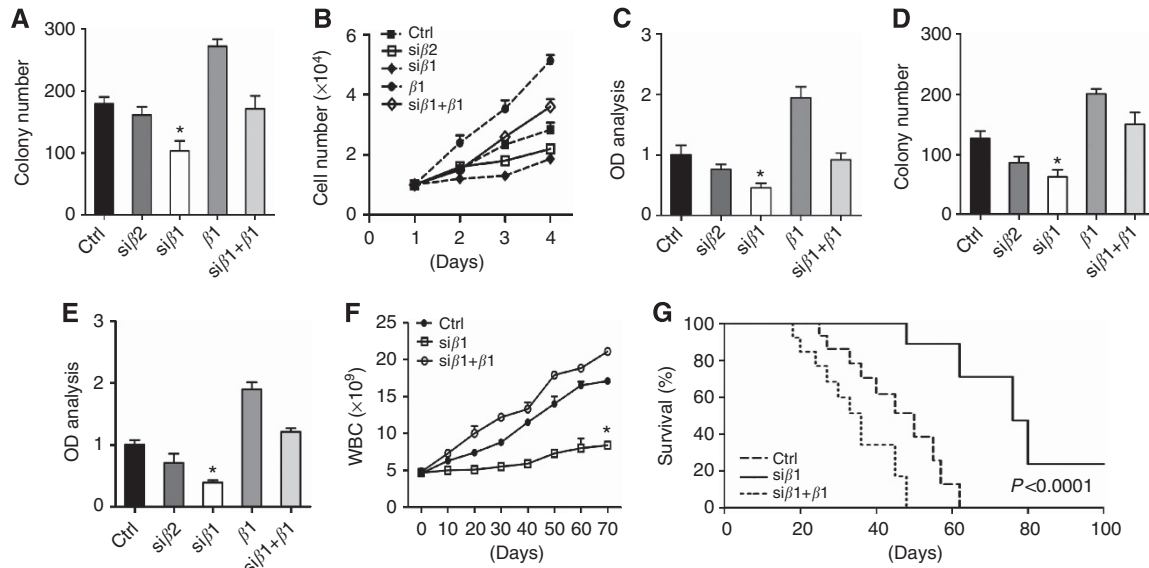
Figure 2. β-Arrestin1 promotes CML cells proliferation *in vitro* and *in vivo*. (**A**) Colony formation in soft agar, (**B**) cell counting and (**C**) MTT assay, shown through optical density (OD) value of 570 nm for 4 days in succession, were performed to analyse the cell proliferation of different stable K562 cells. (**D**) Colony formation and (**E**) MTT assay of transient transducing primary CML cells. Data shown are means ± s.d. of three independent experiments, *$P<0.05$ vs Ctrl. (**F**) Dynamical PB WBC counting and (**G**) survival analysis of CML animal models, $n=10$ in each group. *$P<0.05$, **$P<0.01$ vs Ctrl.
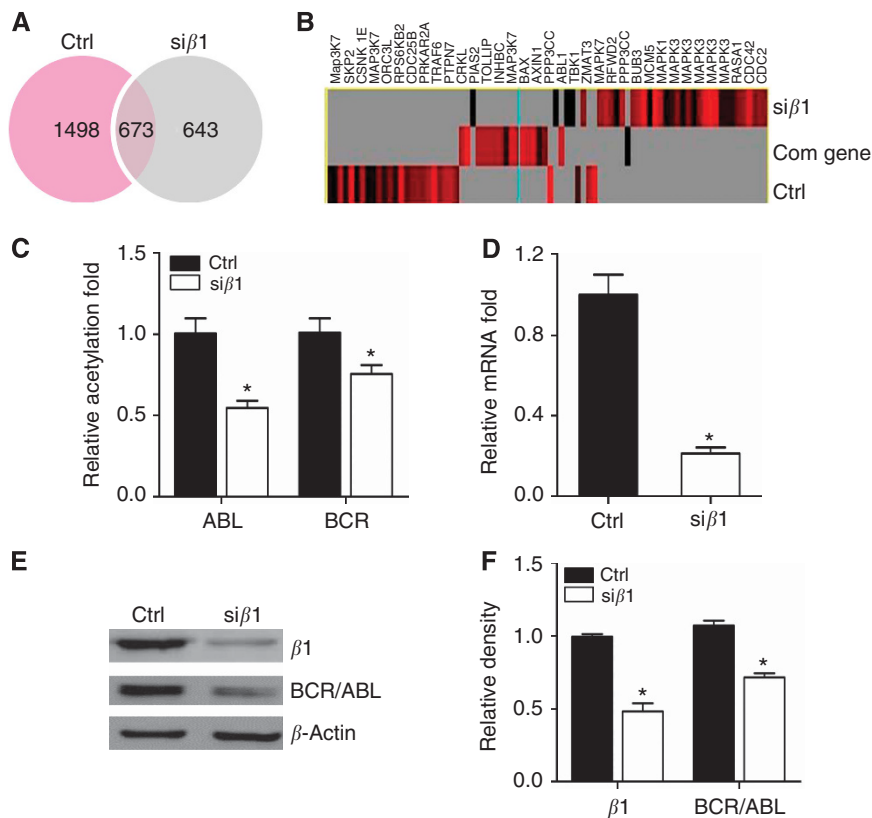
Figure 3. ChIP-on-chip analysis shows β-arrestin1 regulates ABL histone H4 acetylation and affects the expression of BCR/ABL in K562 cells. (**A**) The Venn diagrams show the number of β-arrestin1 mediated histone H4 acetylation modification genes in the indicated K562 cells by using ChIP-on-chip analysis. (**B**) Cluster analysis show the target genes regulated by β-arrestin1. Rows represented the different stable K562 cells. Columns represented the indicated signalling-related genes. The degree of colour saturation reflects the magnitude of acetylation. Black indicates hypoacetylation; red denotes hyperacetylation. (**C**) The acetylation alterations of BCR and ABL were confirmed by H4 ChIP in stable K562-siβ1 cells and K562-Ctrl cells. (**D**) BCR/ABL transcription levels were measured by real-time RT–PCR and normalised as folds with its respective GAPDH in the indicated stable K562-siβ1 (siβ1) and K562-Ctrl cells (Ctrl). (**E**) K562 cells expressing endogenous BCR/ABL was analysed by WB, and β-actin as the loading control. (**F**) The bands of BCR/ABL were quantified and normalised to β-actin by Bio-Rad Quantity-One software. And the data shown are the means ± s.d. of three independent experiments. *$P<0.05$ vs Ctrl. The full colour version of this figure is available at *British Journal of Cancer* online.
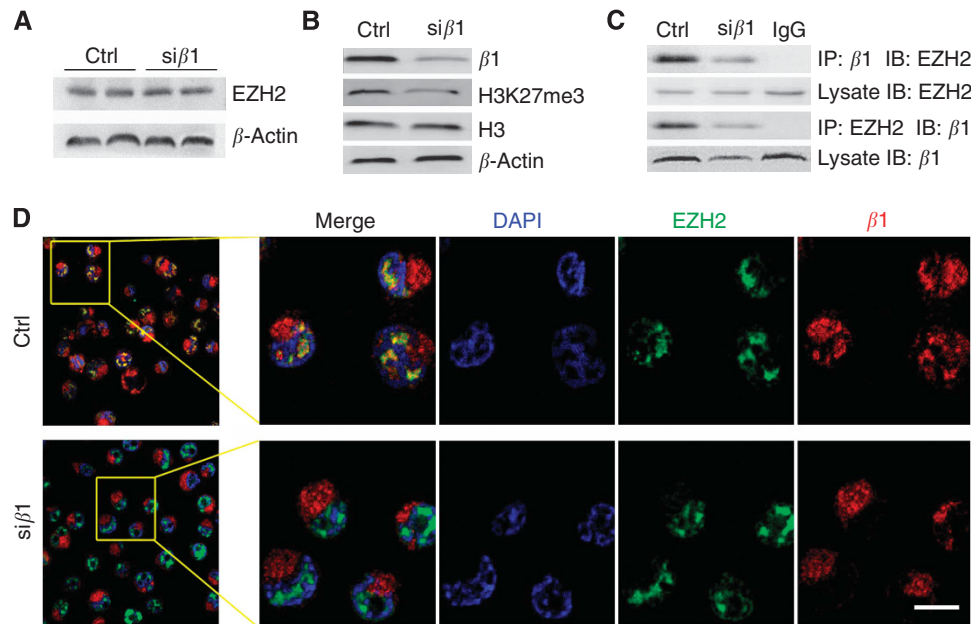
Figure 4. β-Arrestin1 interacts with EZH2 in the nucleus of K562 cells. (A) The expression of EZH2 in stable K562-siβ1 and K562-Ctrl cells was measured by WB with β-actin as the loading control. (B) The expression levels of H3K27me3 were analysed by WB, and H3 as the control. (C) K562 nuclear extracts were immunoprecipitated with EZH2 or β-arrestin1 antibody and the immunocomplexes were analysed in WB using antibodies against EZH2 or β-arrestin1. IgG as the negative control. (D) Confocal visualisation of EZH2 (green) and β-arrestin1 (red) in different stable K562 cells. And single confocal sections were shown. Scale bar, 5 μm. The full colour version of this figure is available at British Journal of Cancer online.

against both splicing isoforms of β-arrestin1 (Parruti et al, 1993). The stable transduced K562 cells were obtained by limited dilution and culture expansion. The expression of β-arrestin1 and β-arrestin2 in stable K562-β-arrestin1 (β1), K562-siβ1 (siβ1), K562-β-arrestin2 (β2), K562-siβ2 (siβ2) and K562-Ctrl (Ctrl) cells were measured by real-time RT–PCR, IF and WB (Supplementary Figure S1) to verify the successful establishment for further experiments. The results from soft agar colony assay showed that over-expression of β-arrestin1 promoted K562 cells proliferation and progression, whereas silencing the β-arrestin1 decreased the clone numbers. Markedly, the inhibition of K562 cell progression of siβ1 could be rescued by β1 (Figure 2A), further supporting the specificity of siRNA and functional conservation of this protein. Both the results from the colony formation assay (Figure 2A), cell growth curves (Figure 2B) and MTT assay (Figure 2C) showed that the growth of K562 cells was depressed when β-arrestin1 was depleted. By contrast, the proliferation of K562 cells increased when β-arrestin1 over-expressed, and β-arrestin1 rescued its depletion. However, the alteration of clone formation, cell growth and proliferation were not significant alteration in K562 cells treated with siβ2 (Figure 2A–C). Similar results were obtained from primary CML patient cells by transiently transducing them with β1, β2, siβ1, siβ2 or Ctrl lentiviral particles (Figure 2D and E).

Subsequently, we first injected the stable K562-Ctrl and K562-siβ1 cells into the tail vein of lethally irradiated NOD/SCID mice, and then injected β1 lentiviral particles to rescue the effect of siβ1, to explore the function of β-arrestin1 in CML in vivo. The PB WBC numbers of mice were dynamically counted and the survival curves were analysed. The results demonstrated that the PB WBC counts were decreased (Figure 2F) and the survival time was extended (Figure 2G) when β-arrestin1 was knocked down, and the effects could be rescued by β-arrestin1, indicating that β-arrestin1 has a crucial role in CML progression.

β-Arrestin1 regulates BCR/ABL expression and H4 acetylation. Considering β-arrestin1 mediated H4 acetylation and regulated transcription of many genes (Kang et al, 2005), ChIP-on-chip for

histone H4 acetylation of whole genome was used for screening the genes, which were regulated by β-arrestin1. The data from ChIP-on-chip illustrated that β-arrestin1 mediated histone H4 acetylation of 1316 genes, including 673 common genes and 643 specific genes in stable K562-siβ1 cells, compared with those in K562-Ctrl cells (Figure 3A). Cluster analysis showed that histone H4 acetylation genes regulated by β-arrestin1 were predominantly involved in MAPK signalling, which was corresponding with the reports (Kang et al, 2005; Lefkowitz and Shenoy, 2005). Importantly, β-arrestin1 regulates the acetylation of ABL gene (Figure 3B). To confirm these results, histone H4 acetylation status was assessed by labelling IP histone H4 with an anti-Ac-lys-12 and lys-16 antibody and IP chromatin with an anti-Ac-H4 antibody. Consistently, histone H4 of either ABL or BCR was hypoacetylated when β-arrestin1 was knocked down (Figure 3C). Here we focused on its role in BCR/ABL expression. Real-time RT–PCR, used to quantify BCR/ABL transcript levels relative to the GAPDH gene, revealed a significant reduction in stable K562-siβ1 cells (Figure 3D). The results showed that BCR/ABL expression was also reduced in stable K562-siβ1 cells by subsequent WB analysis (Figure 3E and F). These results demonstrated that β-arrestin1 could mediate histone H4 acetylation to further affect gene expression.

β-Arrestin1 binds to EZH2 in nucleus to mediate BCR/ABL H4 acetylation and expression. β-Arrestin1 regulates signal transduction and gene transcription mainly through protein–protein interaction (Kang et al, 2005). This prompted us to pay special attention to those factors critically involved in acetylation regulation, and finally selected EZH2, the critical PRC2 member. Among the components of PRC2, the EZH2 enzymatic activity is indispensable for PRC2-mediated gene silencing (Tang et al, 2009). By using Co-IP assay, we found that although there was no correlation between the expression of β-arrestin1 and EZH2 (Figure 4A), the interaction of β-arrestin1 and EZH2 was obviously observed in stable K562-Ctrl cells by using both anti-β-arrestin1 and anti-EZH2 as immunoprecipitation (IP) reaction substrates

(Figure 4C). And the reduced *β*-arrestin1-EZH2 interaction was shown in stable K562-si*β*1 cells with Co-IP assays (Figure 4C). The expression of H3K27me3, the activated form of H3K27, also decreased in K562-si*β*1 stable cells (Figure 4B). The results from immunofluorescence confocal assay also confirmed that the binding of *β*-arrestin1-EZH2 in stable K562-Ctrl cells mainly existed in cell nucleus and nucleoli, and the most binding of *β*-arrestin1 and EZH2 in nucleus was decreased in stable K562-si*β*1 cells compared with those in K562-Ctrl cells (Figure 4D).

To investigate the necessity of *β*-arrestin1-EZH2 formation, further experiments carried on 3-deazaneplanocin A (DZNep), a small-molecule EZH2 inhibitor that disrupts PRC2 and manifests antitumour activity in a variety of cancers (Tan *et al*, 2007; Miranda *et al*, 2009; Momparler *et al*, 2012) to treat stable K562-Ctrl cells, found that DZNep inhibited both the expression of EZH2 and the interaction of *β*-arrestin1-EZH2 without affecting the expression of *β*-arrestin1 (Figure 5A). Moreover, DZNep suppressed the cell growth of K562-Ctrl cells (Figure 5B), extended survival time of mice injected with K562-Ctrl cells through the tail vein (Figure 5C). Consistently, the histone H4 acetylation levels of BCR and ABL (Figure 5D) as well as expression of characteristic BCR/ABL fusion gene (Figure 5E) had also been repressed. Taken

together, these results provide evidence supporting that *β*-arrestin1 interacts with EZH2 to regulate BCR/ABL acetylation and expression, and thus mediates CML progression.

DISCUSSION

β-Arrestin1 has critical roles in epigenetic regulation of gene transcription. In this study, we disclosed that *β*-arrestin1 is critically involved in CML progression. Importantly, *β*-arrestin1 interacted with EZH2, and simultaneously mediated histone H4 acetylation of BCR/ABL, thus regulating CML progression. Collectively, our data reveal an earlier and unexpected role of *β*-arrestin1 in CML progression, as well as being a key regulator of H4 acetylation modification.

Both *β*-arrestin1 and *β*-arrestin2 were previously known as cytosolic signalling regulatory and scaffold proteins. The activation of GPCR recruits both *β*-arrestin1 and *β*-arrestin2 to the cell membrane and interactions of the phosphorylated GPCR and *β*-arrestins induce receptor endocytosis and signal inhibition. However, accumulating evidence also revealed potential functional differences between the two *β*-arrestins subtypes as well as their receptor specificity. For example, previous studies demonstrated
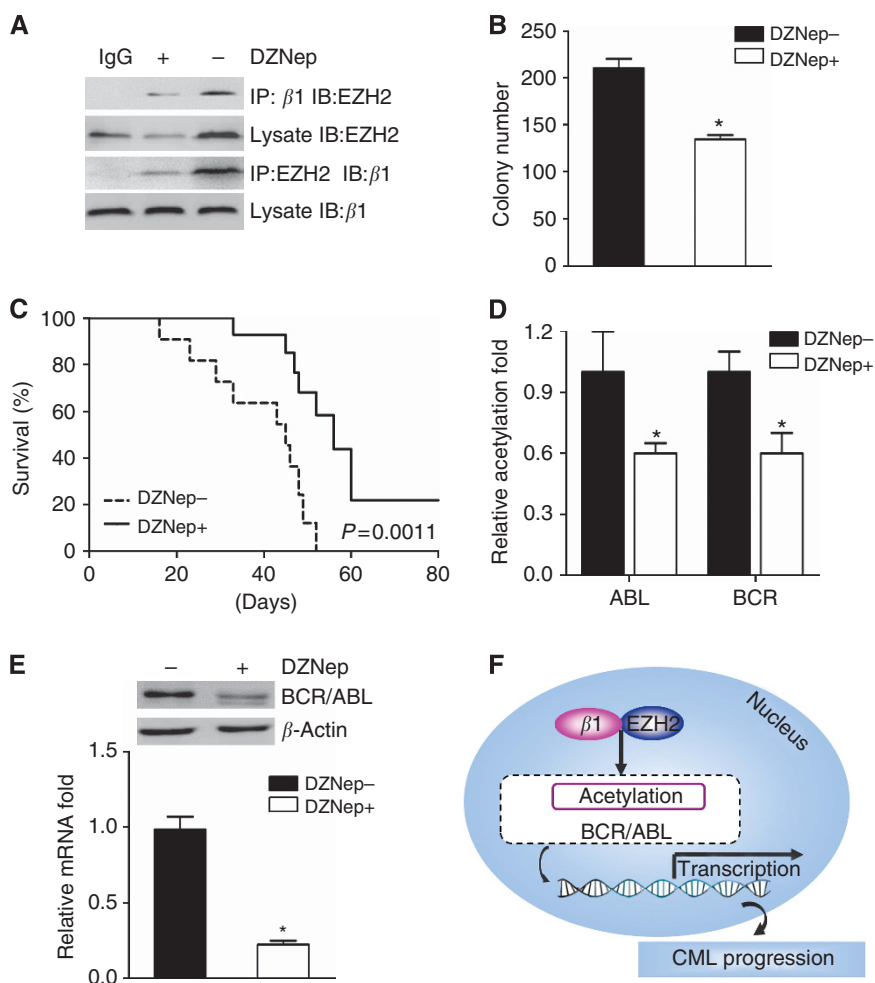

Figure 5. DZNep inhibits *β*-arrestin1-EZH2 binding, alters BCR/ABL gene expression and acetylation and further suppresses K562 cells progression. (**A**) K562 cells were pretreated with or without 2 μM DZNep for 24 h. And then Co-IP was performed to detect the expression of EZH2 and its interaction with *β*-arrestin1. (**B**) Different K562 cells were cultured in a two-layer soft agar for 14 days, and the colonies (⩾40 cells for each) were counted. Experiments were performed triplicate at each point. (**C**) Stable K562-Ctrl cells were injected into the tail vein of lethally irradiated NOD/SCID mice. Ten days later, DZNep was intraperitoneally injected at a dose of 2 mg kg^{-1} daily and the survival of mice was observed. The control mice received PBS. $n = 10$ each group. (**D**) ChIP analysis was used to determine the effects of DZNep in the acetylation of BCR and ABL. (**E**) The expression of BCR/ABL quantified by real-time RT–PCR (lower panel) and WB (upper panel). (**F**) The scheme of this study. *$P < 0.05$ *vs* Ctrl.

that *β*-arrestin1 or *β*-arrestin2 has a vital role in epigenetic regulation of genes (Lefkowitz and Whalen, 2004; Kang *et al*, 2005; Kovacs *et al*, 2009; Yue *et al*, 2009). Although *β*-arrestin2 has been discovered to mediate the initiation and progression of CML (Fereshteh *et al*, 2012), the critical role of *β*-arrestin1 is still lacking. Our results of *β*-arrestin1 in CML, together with previous study of *β*-arrestin2, provide strong evidence supporting that both *β*-arrestin1 and *β*-arrestin2 function as important regulators during CML progression. The different scenarios of *β*-arrestin1 and *β*-arrestin2 in CML could be explained by the functional redundancy between two proteins. *β*-arrestin1 seems to have a more important role during CML progression, probably due to its nuclear localisation ability (Kang *et al*, 2005).

Here, we reported the expression of both *β*-arrestin1 and *β*-arrestin2 in the different clinical phases of CML bone marrow cells in patients. Both the expression of *β*-arrestin1 and *β*-arrestin2 was increased in CML patients and did so in a familiar tendency. However, the enhanced level of *β*-arrestin1 was higher than that of *β*-arrestin2 in the same patient.

Meanwhile, downregulated expression of *β*-arrestin2 had less effect on K562 cells than in K562 cells knocked down *β*-arrestin1 expression (Figure 2). Together with our previous interests in *β*-arrestin1 in malignant tumours (Zou *et al*, 2008; Liu *et al*, 2011) we chose *β*-arrestin1 for further study, and found an epigenetic regulatory role for *β*-arrestin1. The role of *β*-arrestin2 in CML cells should be further investigated in multiple ways, and for a detailed understanding of the molecular mechanisms of CML.

Enhancer of zeste homologue 2 is one of the composed PRC2 parts in the PcG protein family. PcG proteins could bind with DNA and form DNA-protein complexes, and then transduce the signals (Saurin *et al*, 2001; Negre *et al*, 2006; Papp and Muller, 2006). EZH2 is known to be involved in the progression of prostate cancer (Varambally *et al*, 2002), regulation of CD11b in ATRA-induced HL-60 differentiation (Tang *et al*, 2009), some somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-centre origin (Morin *et al*, 2010) as well as EZH2 governor B cell development through histone H3 methylation and IgH rearrangement (Su *et al*, 2003). However, whether EZH2 was involved in CML was unclear. Our findings proved that EZH2 is the scaffold partner of *β*-arrestin1 to form the protein complex to mediate histone H4 acetylation, and thus further regulate CML cells progression. Moreover, we also noticed that recruitment of EZH2 at the promoter regions of BCR/ABL was decreased to some extent when *β*-arrestin1 knocked down (data not shown). The detailed binding information and mechanism of EZH2 with *β*-arrestin1 should be further studied.

In summary, our study unravels the function of *β*-arrestin1 and further illustrates the mechanism by which *β*-arrestin1 participates in epigenetic regulation in haematological malignancies. We have demonstrated that *β*-arrestin1 functions as an important regulator of PcG proteins, which is of great significance for PcG-mediated epigenetic regulation. Future investigations should focus on the better understanding of the mechanisms through which PcG is regulated by *β*-arrestin1 in the context of hematopoietic malignant diseases.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

LZ and PZ designed the study, wrote, edited, and approved the final version of the manuscript. RQ, KL, XQ, and XZ performed the experiments and wrote the manuscript. LW helped in collecting the patients' samples.

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