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Charged Amino Acid-rich Leucine Zipper-1 (Crlz-1) as a Target of Wnt Signaling Pathway Controls Pre-B Cell Proliferation by Affecting Runx/CBFβ-targeted *VpreB* and *λ5* Genes*

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The proliferation of pre-B cells is known to further increase the clonal diversity of B cells at the stage of pre-B cells by allowing the same rearranged heavy chains to combine with differently rearranged light chains in a subsequent developmental stage. *Crlz-1* (*charged amino acid-rich leucine zipper-1*) was found to control this proliferation of pre-B cells by working as a Wnt (wingless-related mouse mammary tumor virus integration site) target gene in these cells. Mechanistically, Crlz-1 protein functioned by mobilizing cytoplasmic CBFβ (core binding factor β) into the nucleus to allow Runx (runt-related transcription factor)/CBFβ heterodimerization. Runx/CBFβ then turned on its target genes such as *EBF* (early B cell factor), *VpreB*, and *λ5* and thereby pre-B cell receptor signaling, leading to the expression of *cyclins D2* and *D3*. Actually, the proliferative function of Crlz-1 was demonstrated by not only Crlz-1 or β-catenin knockdown but also Crlz-1 overexpression. Furthermore, the mechanistic view that the proliferative function of Crlz-1 is caused by relaying Wnt/β-catenin to pre-B cell receptor signaling pathways through the regulation of Runx/CBFβ heterodimerization was also verified by employing niclosamide, XAV939, and LiCl as Wnt inhibitors and activator, respectively.

Crlz-1, also called *UTP-3* (U three protein 3) in a bioinformatic search, was originally cloned by a yeast two-hybrid method (1) because of its ability to associate with CBFβ,[3] which has been known to heterodimerize with Runx to increase its DNA binding affinity (2, 3). Previously, we reported that Crlz-1 is expressed specifically in pre-B cells (4) and turned out to be a nuclear protein, thereby mobilizing cytoplasmic CBFβ into nucleus to allow its heterodimerization with nuclear Runx and subsequent transcriptional activation of its target genes by binding to its target DNA site in a form of Runx-CBFβ-Crlz-1 ternary complex (5). In addition, the promoter of *Crlz-1* gene was found to be very strong and regulated by lymphoid enhancer factor-1 (LEF-1) (6), which is a nuclear transcriptional effector of Wnt signaling pathway (7), suggesting that *Crlz-1* might be a Wnt target gene.

Runx/CBFβ has been known to be important in many developmental processes, especially during early B cell development by regulating the expression of *EBF* (8). Furthermore, *VpreB* and λ5 genes for the surrogate light chains of pre-BCR have also been known to be targeted directly and/or indirectly (via EBF) by this Runx/CBFβ transcription factor (9, 10). The early B cell development is checked for a successful rearrangement of μ heavy chain gene segments and its expression at the stage of pre-B cells. Once μ heavy chains are successfully expressed, the signals generated from pre-BCR consisting of μ heavy chains and VpreB and λ5 surrogate light chains allow an initial rapid proliferation of pre-B cells for a while with an allelic exclusion of μ heavy chain gene if necessary. Each of the proliferated pre-B cells then starts to rearrange its own κ or λ light chain gene segments and, with a successful expression of light chains, differentiates into the next stage of IgM-expressing immature B cell (11–14), leading to a greater number of different B cell clones because of their unique combinations of the same μ heavy chains with different light chains and thereby resulting in an even more diverse repertoire of B cells.

Wnt proto-oncogene was originally cloned because of its activation by an mouse mammary tumor virus integration, which causes a mammary tumor in mice (7). Now, its related genes constitute a family and are found to be essential for cellular proliferation and differentiation (15). When Wnt binds to its receptor complex consisting of the Frizzled receptor and its Lrp (low density lipoprotein receptor-related protein) co-receptor, the canonical signaling pathway inhibits the degradation of β-catenin by suppressing the ubiquitination of phosphorylated β-catenin within its destruction complex and consequently causes the destruction complex to be saturated with the accumulating phosphorylated β-catenin and thereby the unphosphorylated form of a newly synthesized β-catenin to accumulate in the cytoplasm and subsequently to translocate into the nucleus (16). Upon nuclear translocation, β-catenin interacts with a member of LEF/TCF (T cell factor) family of transcription factors to influence its target gene expression (17).

In this study, based on the relationship between Wnt/β-catenin, LEF-1, Crlz-1, Runx/CBFβ, and pre-BCR as reported by us and others, we sought to find the roles of Crlz-1 in pre-B

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[3] The abbreviations used are: CBFβ, core binding factor β; pre-BCR, pre-B cell receptor; LEF-1, lymphoid enhancer factor-1.

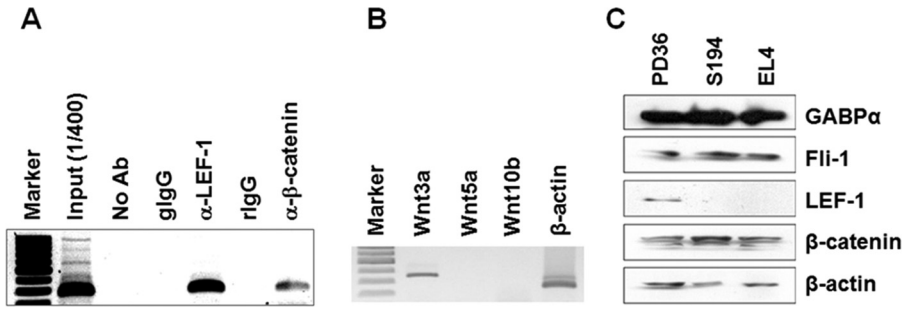

FIGURE 1. ***Crlz-1* is a target gene of Wnt/β-catenin signaling pathway.** *A*, the *Crlz-1* promoter was found to be bound by LEF-1 and β-catenin in PD36 pre-B cells in our ChIP analysis. No antibody (*No Ab*) and isotype-matched IgG antibodies (*gIgG* for goat and *rIgG* for rabbit) were used as negative controls (where α means anti-). *B*, Wnt3a was found to be expressed in PD36 pre-B cells among the three common (Wnt3a, Wnt5a, and Wnt10b) Wnt ligands as examined in an RT-PCR analysis. *C*, among three (GABPα, Fli-1, and LEF-1) transcription factors identified to bind *Crlz-1* promoter in our previous report (6), only LEF-1 was found to be expressed specifically in PD36 pre-B cells as analyzed with Western blotting. β-Catenin was also included in this Western blotting analysis. β-Actin was employed as a positive and/or loading control.

cell proliferation. Actually, *Crlz-1* was found not only to be a bona fide target of canonical Wnt/β-catenin signaling pathway because its promoter was shown to be specifically bound by LEF-1/β-catenin, but also, when expressed, to activate the genes for EBF, as well as VpreB and λ5 surrogate light chains of pre-BCR through the nuclear mobilization of CBFβ and thereby allowance of Runx/CBFβ heterodimerization. Furthermore, Crlz-1 was linked to the transcriptional regulation of *cyclins D2* and *D3*, which are G_1 check point regulators targeted by the pre-BCR signaling during cellular proliferation of pre-B cells (11, 18). We postulate that Crlz-1 would link Wnt signaling to pre-B cell proliferation by mobilizing CBFβ to the nucleus to permit Runx/CBFβ heterodimerization and thereby its subsequent transcriptional activation of *EBF*, as well as *VpreB* and *λ5* surrogate light chain genes of pre-BCR, whose signals would eventually lead to the transcriptional activation of *cyclins D2* and *D3*.

Results

Crlz-1 Is a Target Gene of Wnt/β-Catenin Signaling Pathway; β-Catenin Binds to the Crlz-1 Promoter Together with LEF-1—In our previous report (6), LEF-1 was shown to bind to the distal region of *Crlz-1* promoter and to be critical for the activity of *Crlz-1* promoter. It is well known that LEF-1 acts as a final transcriptional effector with β-catenin as its binding partner in the canonical Wnt signaling pathway (7). Based on these facts, we performed ChIP experiments to see whether the promoter of *Crlz-1* gene was truly bound by β-catenin and thereby a target of Wnt signaling pathway. Actually, β-catenin, as well as LEF-1, was found to be bound to the *Crlz-1* promoter in our ChIP analysis (Fig. 1*A*), attesting that *Crlz-1* is a bona fide Wnt target gene. In addition, Wnt3a among several Wnt ligands examined was found to be expressed in the PD36 pre-B cells (Fig. 1*B*), suggesting an autologous Wnt signaling pathway by this ligand in these pre-B cells. Furthermore, in accordance with the previous report (19), LEF-1 was again confirmed to be expressed specifically in pre-B cells such as PD36 in our case (Fig. 1*C*), explaining partly the pre-B cell-specific expression of *Crlz-1*.

CBFβ, a Binding Partner of Runx, Was Found To Be Localized in the Nucleus of Pre-B Cells and To Be Relocalized to the Cytoplasm When Crlz-1 Was Knocked Down by Its siRNA—In our previous report, we showed that the transfected CBFβ, which was alone localized in the cytoplasm of S194 plasma cells, was mobilized into the nucleus in the presence of co-transfected nuclear Crlz-1 in those plasma cells (5). The mobilized nuclear CBFβ was further shown to associate with the nuclear Runx to form a high affinity heterodimer acting as a transcriptional activator (5). Based on these previous results, we reasoned that endogenous CBFβ might be localized in the nucleus in PD36 pre-B cells where Crlz-1 had been shown to be specifically expressed (4) and thereby that endogenous CBFβ would be relocalized to the cytoplasm when Crlz-1 was knocked down by its siRNA. Indeed, as expected, the endogenous CBFβ was found to be localized in the nuclei of Crlz-1-positive PD36 pre-B cells (Fig. 2*A*, *top panels*, see the cells non-transfected or transfected by scrambled TYE563-siRNA as a negative control) and to be relocalized to the cytoplasm when Crlz-1 was knocked down by transfection of its Cy5-labeled siRNA (Fig. 2*A*, *bottom panels*). In accordance with this CBFβ relocalization by the knockdown of Crlz-1 in PD36 cells, the binding of Runx/CBFβ on its target DNA sites of the *EBF* and *λ5* promoters was found to disappear when Crlz-1 was knocked down by its siRNA transfection in these cells as demonstrated by the disappearance of a ChIP band by anti-CBFβ (Fig. 2*B*), indicating that the binding of Runx/CBFβ on its target site is dependent on the presence of Crlz-1 in PD36 pre-B cells. This release of Runx/CBFβ from its target DNA sites was also observed in our ChIP assays when β-catenin as the upstream signaling effector of Crlz-1 was knocked down by transfecting its own siRNA (Fig. 2*B*).

The Proliferation of Pre-B Cells Was Decreased by the Knockdown of Crlz-1 or β-Catenin as Its Upstream Effector—Pre-B cells, which are generated when μ heavy chain gene has successfully been rearranged to be expressed and assembled with VpreB and λ5 surrogate light chains to form pre-BCR, are known to initially proliferate and subsequently rearrange their κ or λ light chain genes to develop into immature B cells. This initial proliferation of pre-B cells increases further the diversity of antibody repertoire as a characteristic attribute of adaptive immunity. Mechanistically, pre-BCR is reported to give rise to a signal to proliferate during this early pre-B cell development (11).

We hypothesized that Crlz-1, which was shown to be expressed specifically in the pre-B cell stage (4), would regulate

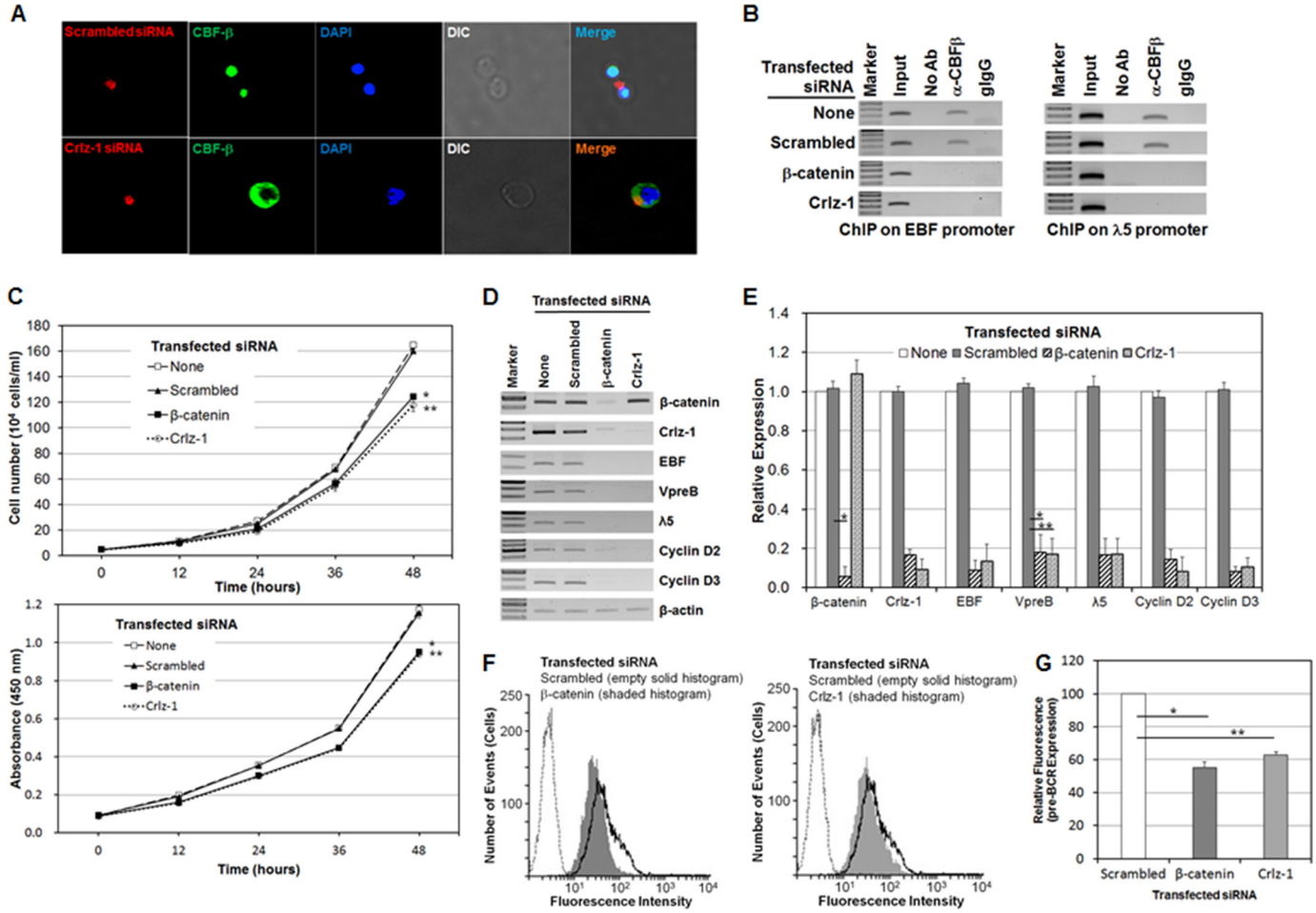

FIGURE 2. **Proliferation of PD36 pre-B cells was decreased by the knockdown of Crlz-1 and thereby the decreased expression of its postulated downstream target genes.** *A*, CBFβ was shuttled back from nucleus to cytoplasm by the knockdown of Crlz-1 in pre-B cells. CBFβ was detected with the primary antibody of anti-PEBP2β (α-CBFβ) and secondary antibody of Alexa-488-labeled donkey anti-goat IgG. Nuclei were counterstained with DAPI. Confocal fluorescence microscopy was employed to capture the images of TYE563 or Cy5 (*red*, siRNA), Alexa 488 (*green*, CBFβ), and DAPI (*blue*, nucleus). The merged fluorescent images are shown on the *right*. The halogen differential interference contrast (*DIC*) images were also included to show the whole cells. *B*, the binding of CBFβ on its target sites of *EBF* and *λ5* promoters was shown to disappear after the knockdown of Crlz-1 and β-catenin in our ChIP assay. *C*, the growth curve of PD36 pre-B cells for a period of 48 h after transfection of Crlz-1 or β-catenin siRNA. The proliferation of cells was assayed not only by counting them after staining with trypan blue (*top panel*), but also by a BrdU incorporation method (*bottom panel*) at the indicated time points. The data from at least three independent transfections were graphed with *error bars* ($\pm$S.E.): *, $p \leq 0.04$ and 0.0003 (*top panel*); *, $p \leq 0.00006$ and 0.0005 (*bottom panel*) for β-catenin *versus* Scrambled siRNA; **, $p \leq 0.03$ and 0.002 (*top panel*), and **, $p \leq 0.00008$ and 0.0004 (*bottom panel*) for Crlz-1 *versus* Scrambled siRNA at 36 and 48 h, respectively. *D* and *E*, the changes of mRNA levels of *EBF*, *VpreB*, *λ5*, and *cyclins D2* and *D3* were determined by RT-PCR after 48 h of siRNA transfections. A representative RT-PCR gel is shown in *D*, and a histogram analysis for the relative expression change of each gene from three repeated transfection experiments is shown with *error bars* ($\pm$S.E.) in *E*. Two cases with the highest and lowest relative expression changes among them were chosen to represent their *p* values: *, $p \leq 0.00009$ in β-catenin; and *, $p \leq 0.0008$, and **, $p \leq 0.0005$ in VpreB. *F*, a representative flow cytometric analysis for the surface expression level of pre-BCR protein, which was measured at 48 h after the β-catenin or Crlz-1 siRNA transfection into PD36 pre-B cells with a flow cytometer. For the comparisons of surface pre-BCR expression levels between Scrambled and β-catenin or Crlz-1 siRNA transfections, their flow cytometric histograms were overlaid, where the ones of Scrambled and β-catenin or Crlz-1 siRNA are indicated by a *solid line* and by *gray shading*, respectively. Isotype control histograms are also included as the *dotted curves*. *G*, flow cytometric analyses for the surface expression of pre-BCR in four repeated sets of siRNA transfections were processed statistically to show a *bar graph* with *error bars* ($\pm$S.E.): *, $p \leq 0.00001$; and **, $p \leq 0.000001$. The fluorescence intensities were integrated to obtain the relative fluorescence for β-catenin or Crlz-1 *versus* Scrambled siRNA transfections. The cells transfected without siRNA (*None*) or with a scrambled siRNA (*Scrambled*) were utilized as negative controls. Also, if necessary, β-actin was employed as a positive and/or loading control.

the proliferation of pre-B cells by mobilizing cytoplasmic CBFβ into nucleus to allow its heterodimerization with Runx (5) and thereby transcriptional activation of its direct and/or indirect target genes such as *EBF* (8), *VpreB*, and *λ5* (9, 10). To prove our hypothesis experimentally, we first decided to check the effects of Crlz-1 knockdown on the pre-B cell proliferation through the pre-BCR signaling pathway. Indeed, to our expectation, the knockdown of Crlz-1 by its siRNA was found to decrease the proliferation of PD36 pre-B cells (Fig. 2*C*). At the molecular levels, in accordance with the shuttling back of CBFβ to cytoplasm by the knockdown of Crlz-1 to cause its separation from nuclear Runx (Fig. 2*A*) and thereby the release of Runx/CBFβ from its target DNA binding sites (Fig. 2*B*), the expression of Runx/CBFβ target genes such as *EBF*, *VpreB*, and *λ5* was shown to be decreased by the knockdown of Crlz-1 as measured by RT-PCR (Fig. 2, *D* and *E*). Furthermore, because the expression of *cyclins D2* and *D3*, which are the critical regulators of G_1 check point of cell division cycle of pre-B cells (11, 18), was also found to be decreased by the Crlz-1 knockdown in these RT-PCR analyses (Fig. 2, *D* and *E*), we postulated that the decreased expression of *VpreB* and *λ5* genes might lead to the decreased pre-BCR assembly and thereby its decreased signaling, eventu-

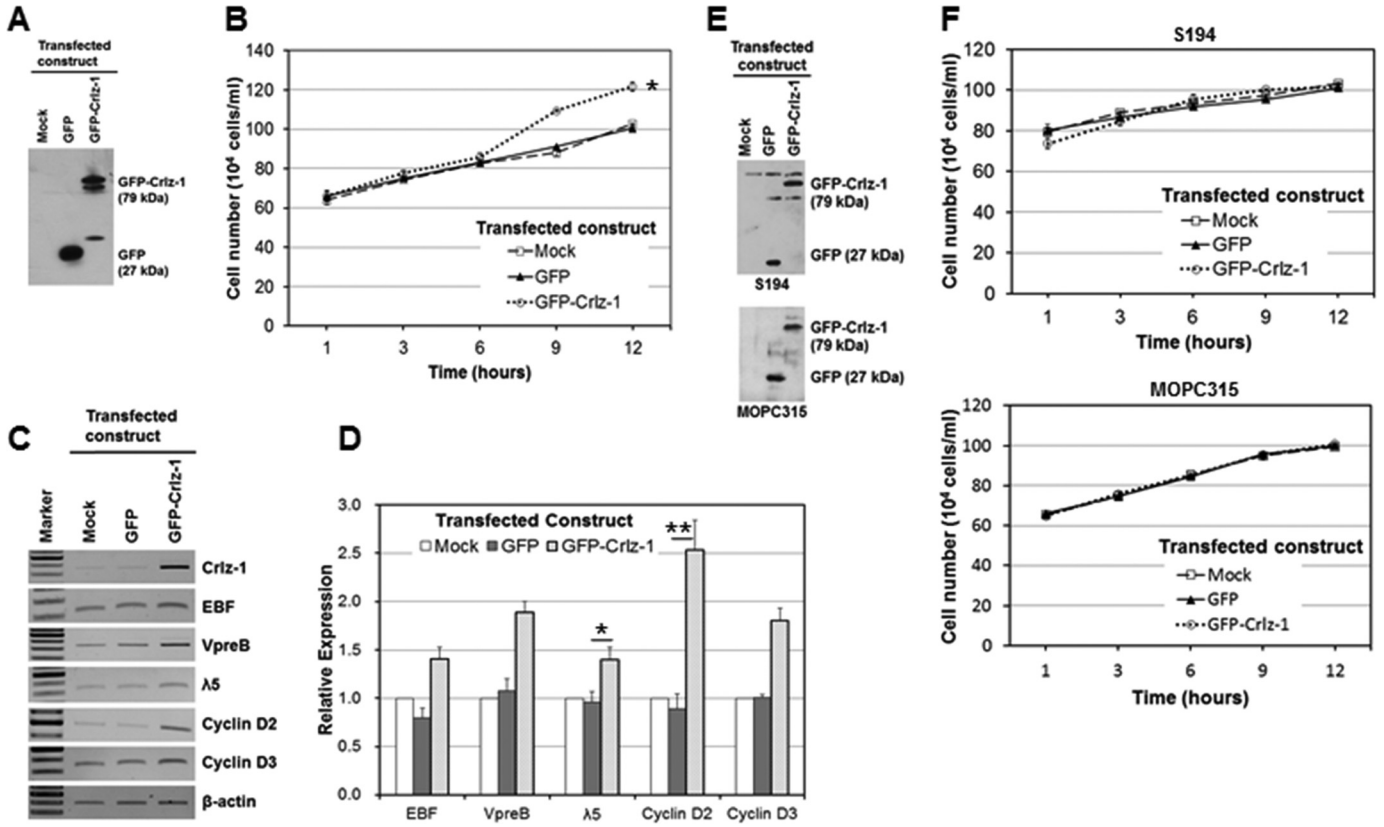

FIGURE 3. **Overexpression of Crlz-1 increased the proliferation of PD36 pre-B cells by increasing the expression of its downstream target genes.** *A*, the overexpression of GFP-Crlz-1 fusion, as well as its control GFP-alone proteins was checked by Western blotting at 12 h after transfections of their expression plasmids into PD36. *B*, the proliferation rate of PD36 cells was graphed by counting cells with trypan blue staining during a period of 12 h after transfection of overexpression plasmid constructs, while the Crlz-1 expression in PD36 cells was stable (see "Discussion"). The data from at least three independent transfections were graphed with *error bars* (±S.E.): *, $p \leq 0.000002$ and 0.001 for GFP-Crlz-1 *versus* GFP transfections at 9 and 12 h, respectively. *C* and *D*, the expression of *EBF*, *VpreB*, *λ5*, and *cyclins D2* and *D3* was examined by RT-PCR at 12 h of plasmid transfections into PD36 cells. A representative RT-PCR gel was shown in *C* and a histogram analysis for relative expression change of each gene from three repeated sets of transfections was graphed with *error bars* (±S.E.) in *D*. Two cases were chosen to represent their *p* values: *, $p \leq 0.05$; and **, $p \leq 0.009$. β-Actin was utilized as a loading control. *E* and *F*, two control plasma cells such as S194 (*top panels*) and MOPC315 (*bottom panels*) were transfected with the expression plasmids and then checked for their expression in a Western blot in *E* as well as for their effects on the cellular proliferation rate in *F*. *Mock* indicates a transfection without any plasmid.

ally leading to the decreased transcription of its target genes such as *cyclins D2* and *D3* (Fig. 2, *D* and *E*). Indeed, as we postulated, a decreased cell surface expression of pre-BCR was demonstrated in the flow cytometric analysis of Crlz-1 siRNA-transfected PD36 pre-B cells (Fig. 2, *F*, *right panel*, and *G*). Taken together, these results obtained by the Crlz-1 knockdown suggested that Crlz-1 might be involved in the regulation of pre-B cell proliferation through the sequential molecular events from the nuclear mobilization of CBFβ and thereby its heterodimerization with Runx to the transcriptional activation of *VpreB* and *λ5* and thereby pre-BCR assembly with its subsequent signaling pathway leading to the transcriptional activation of *cyclins D2* and *D3*. The decreased cellular proliferation by Crlz-1 knockdown could not be explained by the death of cells because any significant cell death was not observed during the experimental period (data not shown).

To verify that the effects of Crlz-1 on pre-B cell proliferation were indeed channeled from the Wnt signaling effector such as β-catenin, the effects of β-catenin knockdown on the cellular proliferation, as well as on the expression of *Crlz-1* and all its subsequent target genes, were also checked. In this β-catenin knockdown experiment, the expression of *Crlz-1* and all its subsequent target genes as identified in Crlz-1 knockdown experiments was also shown to be decreased in the RT-PCR analyses (Fig. 2, *D* and *E*), leading to the decreased proliferation of PD36 pre-B cells (Fig. 2*C*). The decreased cell surface expression of pre-BCR was also demonstrated in the β-catenin siRNA knockdown experiments by the flow cytometric analyses (Fig. 2, *F*, *left panel*, and *G*). All these siRNA transfection experiments certainly demonstrated that the effects of Crlz-1 on the pre-B cell proliferation were caused by its linking of upstream Wnt/β-catenin signaling pathway to the expression of downstream Runx/CBFβ target genes such as *EBF*, *VpreB*, and *λ5* and thereby pre-BCR signaling target genes such as *cyclins D2* and *D3*.

The Proliferation of Pre-B Cells Was Increased by the Overexpression of Crlz-1—To confirm the effects of Crlz-1 on the cellular proliferation of PD36 pre-B cells in the opposite way than the Crlz-1 knockdown method, Crlz-1 was then overexpressed by a transfection of its mammalian expression plasmid (Fig. 3*A*). To the contrary of decreased proliferation by Crlz-1 knockdown, the overexpression of Crlz-1 was found to increase the proliferation of PD36 pre-B cells (Fig. 3*B*). Furthermore, we noticed that Crlz-1 overexpression increased the expression of *EBF*, as well as *VpreB* and *λ5* and thereby *cyclins D2* and *D3* (Fig. 3, *C* and *D*), confirming the results by Crlz-1 knockdown above

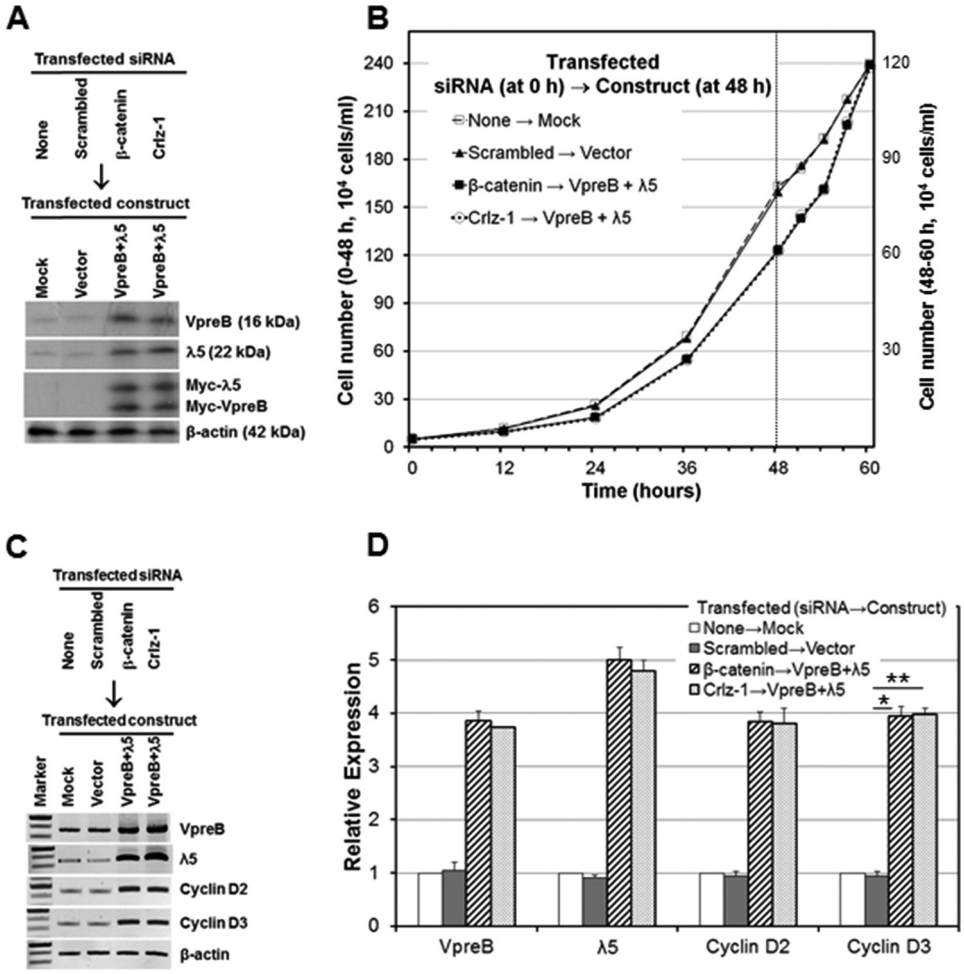

FIGURE 4. **The decreased proliferation of PD36 pre-B cells by the knockdown of Crlz-1 and β-catenin was rescued by the co-overexpression of VpreB and λ5.** The initial transfection of Crlz-1 or β-catenin siRNA into PD36 cells was subsequently followed by the later co-transfection of VpreB and λ5 expression plasmids. The transfection of none or scrambled siRNA followed by mock or plasmid vector transfection was utilized as a rescue control. *A*, Western blots were performed using anti-VpreB and anti-λ5, as well as anti-c-Myc, to identify the overexpressed exogenous Myc-tagged proteins. *B*, growth curves of the coupled knockdown and overexpression experiments were combined to recognize easily the rescue effects. Half of cells at 48 h after siRNA transfection were used to perform the subsequent transfection of expression plasmids to prevent an overconcentrated growth of cells, and thereby, the cellular concentrations before and after transfections of expression plasmids at 48 h are indicated differently on the *left* and *right side axes* of a combined graph. *C*, a representative RT-PCR gel showing the increased expression levels of cyclins D2 and D3 after the coupled knockdown and overexpression experiment. *D*, a histogram analysis for the relative expression change of each gene from three repeated sets of coupled transfections was graphed with *error bars* (±S.E.). *p* values for only cyclin D3 are given: *, $p \leq 0.0001$; and **, $p \leq 0.000003$. β-Actin was utilized as a loading control. *None*, *Scrambled*, and *Mock* are the same as in Figs. 2 and 3.

in the opposite way. However, this proliferative effect of Crlz-1 overexpression was not observed in S194 and MOPC315 plasma cells (Fig. 3, *E* and *F*), justifying that the cellular proliferative effects of Crlz-1 were specific in the context of pre-B cells.

The Decreased Proliferation of Pre-B Cells by the Knockdown of Crlz-1 or β-Catenin Was Rescued by the Co-overexpression of VpreB and λ5 Components of Pre-BCR—To verify the linked signaling pathway from β-catenin to pre-BCR through Crlz-1 with regard to the proliferation of pre-B cells, we planned a rescue experiment in a way that the knockdown for Crlz-1 or β-catenin would be coupled to the overexpression for VpreB and λ5 components of pre-BCR. In one such experiment, the cells were initially transfected by Crlz-1 or β-catenin siRNA to decrease their proliferation and then subsequently by the expression plasmids for VpreB and λ5 to see whether the decreased cellular proliferation could be rescued to catch up the control one of none or scrambled siRNA followed by mock or vector plasmid transfection, respectively. The decreased proliferation of pre-B cells by the initial knockdown of Crlz-1 or β-catenin after 48 h of its siRNA transfection was found to be completely rescued by the subsequent overexpression of VpreB and λ5 after 12 h of the later co-transfection of their expression plasmids (Fig. 4, *A* and *B*). Furthermore, the overexpression of VpreB and λ5 was found to lead to the increased expression of cyclins D2 and D3 (Fig. 4, *C* and *D*), supporting the possibility that the rescue of cellular proliferation was channeled through these proteins.

The Proliferation of PD36 Pre-B Cells Was Decreased or Increased When They Were Treated with Inhibitors or Activator of Wnt Signaling Pathway, Respectively—We utilized niclosamide as an inhibitor of Wnt signaling (20) to see its effects on the expression of *Crlz-1*, which was identified to be a Wnt target gene, and thereby on the cellular proliferation of pre-B cells. The treatment of PD36 pre-B cells with niclosamide led to the decreased expression of *Crlz-1*, and so Runx/CBFβ target genes

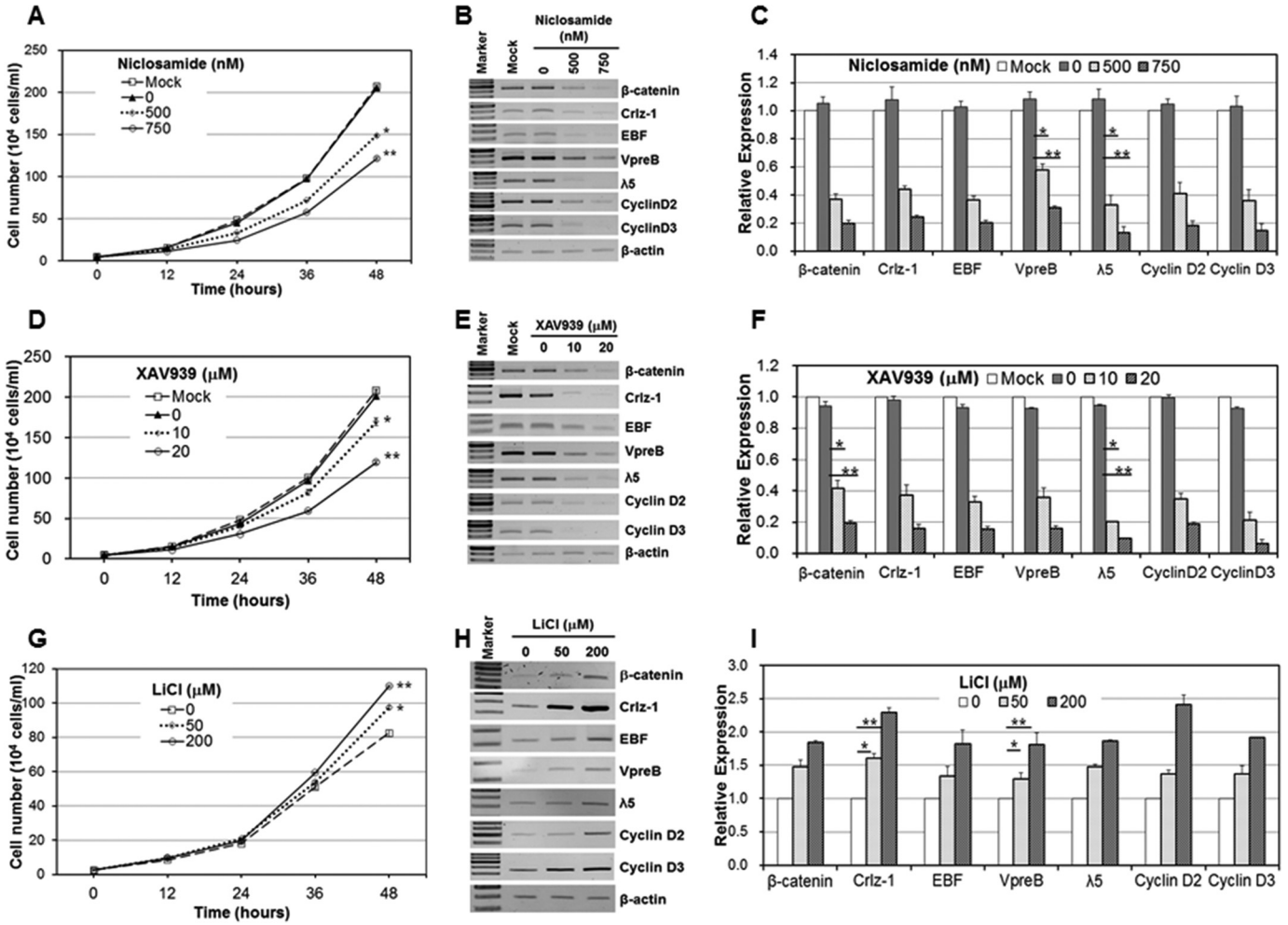

FIGURE 5. **The proliferative effects of Crlz-1 as a linker between Wnt and pre-BCR signaling pathways were demonstrated in PD36 pre-B cells using Wnt inhibitors or activator.** *A*, the proliferation of PD36 pre-B cells was decreased as chased for a period of 48 h after the treatment with niclosamide. The data from at least three independent treatments were graphed with *error bars* (±S.E.): *, $p \leq 0.01$, 0.003 and 0.002 (0 *versus* 500 nM); and **, $p \leq 0.000009$, 0.000001 and 0.000001 (0 *versus* 750 nM) at 24, 36, and 48 h of treatments, respectively. *B* and *C*, the expression of *Crlz-1*, and those of *EBF*, *VpreB*, and *λ5*, as well as those of *cyclins D2* and *D3* were checked by RT-PCR at 48 h after niclosamide treatment. A representative RT-PCR gel was shown in *B*, and a statistical analysis for the relative expression of each gene from three repeated sets of treatments was shown in a *bar graph* with *error bars* (±S.E.) in *C*. Two cases were chosen to represent their *p* values: *, $p \leq 0.002$, and **, $p \leq 0.0001$ in VpreB; and *, $p \leq 0.001$, and **, $p \leq 0.0003$ in λ5. *D–F*, similar results as with niclosamide were seen with XAV939. *D*, growth curve: *, $p \leq 0.08$, 0.01, and 0.001 (0 *versus* 10 μM); and **, $p \leq 0.0006$, 0.00005, and 0.000008 (0 *versus* 20 μM) at 24, 36, and 48 h of treatments, respectively. *E*, a representative RT-PCR gel. *F*, a statistical *bar graph* of three repeated sets of RT-PCR results: *, $p \leq 0.0006$, and **, $p \leq 0.00002$ in β-catenin; and *, $p \leq 0.00000005$, and **, $p \leq 0.00000002$ in λ5. *G–I*, treatment with LiCl has shown the opposite results to those of niclosamide and XAV939. *G*, growth curve: *, $p \leq 0.08$ and 0.002 (0 *versus* 50 μM); and **, $p \leq 0.002$ and 0.0002 (0 *versus* 200 μM) at 36 and 48 h, respectively. *H*, a representative RT-PCR gel. *I*, a statistical *bar graph* of three repeated sets of RT-PCR results: *, $p \leq 0.01$, and **, $p \leq 0.004$ in Crlz-1; and *, $p \leq 0.04$, and **, $p \leq 0.009$ in VpreB. *Mock* is a control experiment without DMSO. β-Actin was utilized as a loading control.

such as *EBF*, *VpreB*, and *λ5* and eventually pre-BCR signaling target genes such as *cyclins D2* and *D3* (Fig. 5, *B* and *C*), leading to the decreased proliferation of pre-B cells (Fig. 5*A*), as did the knockdown of Crlz-1 as well as β-catenin by their siRNA in Fig. 2. Similar results (Fig. 5, *D–F*) were obtained using XAV939 as another Wnt signaling inhibitor (21). In contrast to niclosamide and XAV939, LiCl as an activator of Wnt signaling pathway (22) increased the expression of *Crlz-1* and so *EBF*, *VpreB*, and *λ5* and then *cyclins D2* and *D3* (Fig. 5, *H* and *I*), and thereby the proliferation of pre-B cells (Fig. 5*G*) as the overexpression of Crlz-1 was shown to do in Fig. 3. However, the proliferative effect of LiCl was not observed in S194 plasma cells (data not shown), indicating that the effects were dependent on the context of pre-B cells. Interestingly, the expression of *β-catenin* gene itself was also decreased or increased by these inhibitors and activator, respectively, as assayed by RT-PCR (Fig. 5; see the *top* of each set of gels and the *left* of each set of *bar graphs*), indicating that *β-catenin* gene itself is also a target of Wnt/β-catenin signaling pathway to result in its own autoregulation (23). This phenomenon of β-catenin autoregulation was also verified using normal pre-B cells (see Fig. 7). In the concentration ranges of all these chemicals in the experiments, the death of cells was negligible during the treatment period (data not shown) and thus could be excluded from our interpretation of results.

The Proliferative Function of Crlz-1 as Seen in PD36 Pre-B Cell Line Has Been Verified Using Normal Pre-B Cells—To verify the proliferative function of Crlz-1 in normal pre-B cells, we isolated normal pre-B cells from mouse bone marrows using MACS™ system (Miltenyi Biotec) and performed the same

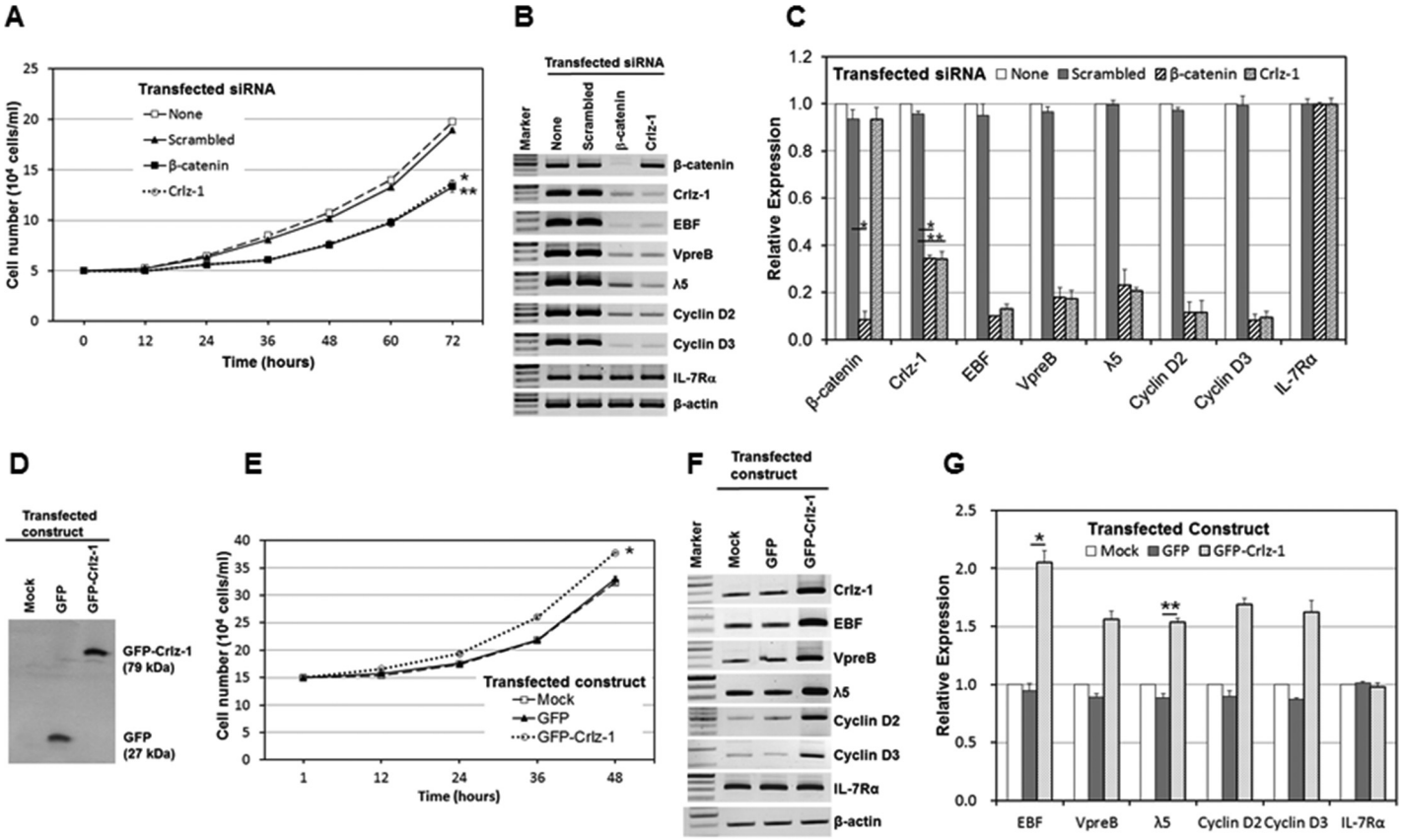

FIGURE 6. **The proliferative function of Crlz-1 was verified in normal pre-B cells by performing the same experiments as in PD36 cells.** *A–C*, results of Crlz-1 or β-catenin siRNA knockdown in normal pre-B cells. *A*, growth curve: *, $p \leq 0.0002$, 0.0002, 0.0002, and 0.00005 for Crlz-1 *versus* Scrambled siRNA; and **, $p \leq 0.0002$, 0.0004, 0.002, and 0.0008 for β-catenin *versus* Scrambled siRNA at 36, 48, 60, and 72 h, respectively. *B*, a representative RT-PCR gel. *C*, a statistical *bar graph* with *error bars* (±S.E.) of three repeated sets of RT-PCR results: *, $p \leq 0.00008$ in β-catenin; and *, $p \leq 0.000004$, and **, $p \leq 0.00005$ in Crlz-1. *D–G*, results of Crlz-1 overexpression in normal pre-B cells. *D*, Western blot showing overexpression at 36 h of transfection. *E*, growth curve: *, $p \leq 0.007$, 0.001, and 0.0009 for GFP-Crlz-1 *versus* GFP transfections at 24, 36, and 48 h, respectively. *F*, a representative RT-PCR gel. *G*, a statistical *bar graph* of three repeated sets of RT-PCR results: *, $p \leq 0.0006$, and **, $p \leq 0.0002$. The growth chase time for normal pre-B cells was prolonged because their growth rate was much slower than the one of PD36 pre-B cell line. *None* or *Mock* is a control transfection without siRNA or any plasmid, respectively.

experiments as those done in PD36 pre-B cells. In this MACS™ system, ~1% of total input bone marrow cells were obtained in the column eluate as pre-BCR-positive cells, whereas ~99% of them were washed away in the flow-through. This percentage of pre-B cells isolated from mouse bone marrows was approximately in a very similar range as seen in the previous flow cytometric sorting of normal pre-B cells (4), suggesting that the isolation of normal pre-B cells in the MACS™ system might also be successful. Furthermore, the successful isolation of normal pre-B cells could also be self-verified because Crlz-1, EBF, VpreB, and λ5, as well as IL-7Rα, all of which are pre-B cell markers, were found to be expressed in the RT-PCR analyses of the isolated normal pre-B cells (Figs. 6 and 7). IL-7Rα acted not only as a pre-B cell marker but also as a non-target gene in these linked signaling pathways.

Consistent with the results of PD36 pre-B cell line, the proliferation of normal pre-B cells was decreased or increased not only by the knockdown (Fig. 6, *A–C*) or overexpression (Fig. 6, *D–G*) of Crlz-1, but also by the treatments with niclosamide (Fig. 7, *A–C*) and XAV939 (Fig. 7, *D–F*) as Wnt signaling inhibitors or LiCl (Fig. 7, *G–I*) as Wnt signaling activator, respectively. Furthermore, in accordance with their effects on the cellular proliferation of normal pre-B cells, the expression of *Crlz-1* and all its downstream target genes as identified using PD36 pre-B cells, which are *EBF*, *VpreB*, and *λ5* as Runx/CBFβ target genes, as well as *cyclins D2* and *D3* as pre-BCR signaling target genes, was decreased or increased not only by the knockdown (Fig. 6, *A–C*) or overexpression (Fig. 6, *D–G*) of Crlz-1 but also by the treatments with niclosamide (Fig. 7, *A–C*) and XAV939 (Fig. 7, *D–F*) or LiCl (Fig. 7, *G–I*), respectively. Transfection of normal pre-B cells with siRNA for β-catenin as an upstream Wnt signaling effector of Crlz-1 also showed similar results (Fig. 6, *A–C*) as obtained using PD36 pre-B cells. All these results obtained using normal pre-B cells definitely verified that Crlz-1 is truly involved in the proliferation of pre-B cells by relaying Wnt/β-catenin to pre-BCR signaling pathways through the regulation of Runx/CBFβ heterodimerization and thereby its transcriptional activity.

Discussion

In the present paper, Crlz-1, which is expressed specifically in the pre-B cell stage during early B cell development (4), was shown to regulate pre-B cell proliferation by linking Wnt/β-catenin with pre-BCR signaling pathways. In addition to such a stage-specific expression of Crlz-1 in pre-B cells for their proliferative regulation, Crlz-1 has also been expressed promi-

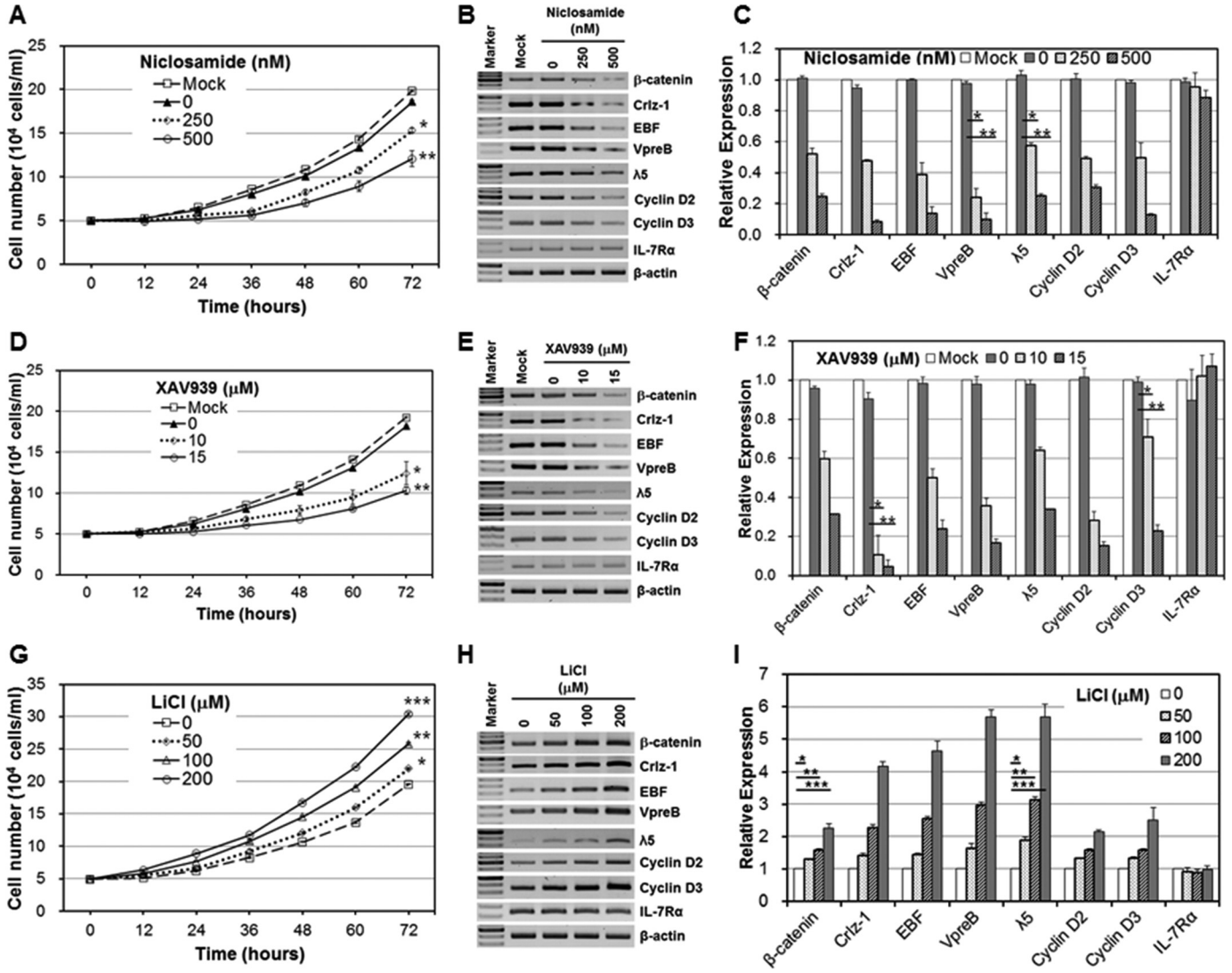

FIGURE 7. **The proliferative function of Crlz-1 as a linker between Wnt and pre-BCR signaling pathways was verified in normal pre-B cells using Wnt inhibitors or activator.** *A–C*, results obtained after treatment with niclosamide. *A*, growth curve: *, $p \leq 0.00005$, 0.002, 0.004, and 0.001 (0 *versus* 250 nM); and **, $p \leq 0.0001$, 0.001, 0.002, and 0.002 (0 *versus* 500 nM) at 36, 48, 60, and 72 h of treatments, respectively. *B*, a representative RT-PCR gel. *C*, a statistical *bar graph*: *, $p \leq 0.0003$, and **, $p \leq 0.00004$ in VpreB; and *, $p \leq 0.0003$, and **, $p \leq 0.00003$ in λ5. *D–F*, results obtained after the treatment with XAV939. *D*, growth curve: *, $p \leq 0.008$, 0.01, 0.02, and 0.02 (0 *versus* 10 μM); and **, $p \leq 0.0002$, 0.000005, 0.00003, and 0.00008 (0 *versus* 15 μM) at 36, 48, 60, and 72 h of treatments, respectively. *E*, a representative RT-PCR gel. *F*, a statistical *bar graph*: *, $p \leq 0.002$, and **, $p \leq 0.00006$ in Crlz-1; and *, $p \leq 0.04$, and **, $p \leq 0.00006$ in cyclin D3. *G–I*, results obtained after treatment with LiCl. *G*, growth curve: *, $p \leq 0.05$, 0.002, 0.0005, and 0.0001 (0 *versus* 50 μM); **, $p \leq 0.001$, 0.00004, 0.00002, and 0.00001 (0 *versus* 100 μM); and ***, $p \leq 0.00007$, 0.000004, 0.000003, and 0.000001 (0 *versus* 200 μM) at 36, 48, 60, and 72 h of treatments, respectively. *H*, a representative RT-PCR gel. *I*, a statistical *bar graph*: *, $p \leq 0.001$, **, $p \leq 0.00002$, and ***, $p \leq 0.0006$ in β-catenin; and *, $p \leq 0.0005$, **, $p \leq 0.00002$, and ***, $p \leq 0.0003$ in λ5. Mock is a control experiment without DMSO. The growth chase time was prolonged for the same reason as mentioned in the legend to Fig. 6.

nently in spermatogonia and Sertoli cells during early testis development (24) and in the germinal center of lymph node,[4] the dark zone of which contains the highly proliferating centroblast B cells (25). Interestingly, the common properties of all these cells and/or tissues appeared to be related to their higher cellular proliferation. In these regards, Crlz-1 could be speculated to be involved in the regulation of cellular proliferation in the contexts of various cell types. However, although the proliferative effects of Crlz-1 in pre-B cells were shown to be caused by linking the canonical Wnt/β-catenin signaling with Runx/CBFβ heterodimerization and thereby transcriptional activation of *EBF* as well as *VpreB* and *λ5* component genes of pre-BCR leading to the transcriptional activation of *cyclins D2* and *D3*, it remains to be elucidated whether Crlz-1 might also be actually involved in the proliferation of other than pre-B cells, and if so, as in pre-B cells, Crlz-1 would also be targeted by Wnt/β-catenin signaling pathway and function by mobilizing CBFβ to the nucleus to allow Runx/CBFβ heterodimerization or by some other ways in the different contexts of those cells. Currently, we are in a progress to define the roles of Crlz-1 in those Crlz-1 expressing cells by generating *Crlz-1* knock-out mice and observing their phenotypes.

Interestingly, Crlz-1 protein turned out to be very unstable when expressed by a transient transfection in pre-B cells (data not shown). Actually, the transiently expressed Crlz-1 protein started to sharply disappear after 12 h of transfection in PD36

[4] J.-H. Pi, S.-Y. Choi, S.-K. Park, and C.-J. Kang, unpublished results.

pre-B cells and could not much be rescued by the addition of MG132, a proteasome inhibitor, indicating that the rapid degradation of transfection-expressed Crlz-1 protein might be caused mainly by some other ways than proteasome. However, the transfection-expressed GFP-alone protein as a control was relatively stable up to 24 h, and its presence could be prolonged by the addition of MG132. Interestingly, the transfection-expressed Crlz-1 proteins in S194 plasma cell line were observed to be relatively stable up to 24 h or up to 48 h in the presence of MG132 (data not shown). In the case of bone marrow-isolated primary normal pre-B cells, which have grown relatively slowly, Crlz-1 protein was noticed to start to decline after 48 h of transfection of its expression plasmid. It might be speculated that Crlz-1 proteins are relatively short-lived in pre-B cells because they are involved in the regulation of short term proliferation of pre-B cells to increase the clonal diversity of B cells during early B cell development.

Other potential information about the functional roles of Crlz-1 in cellular proliferation could be obtained at the molecular level by mapping its various functional domains such as nuclear localization signal and CBFβ-binding domain, which are expected to exist within the protein from our previous and current results. Currently, we are also in the process of mapping these domains. Both the nuclear localization signal and CBFβ-binding domain of Crlz-1 might be responsible for the nuclear mobilization of cytoplasmic CBFβ to allow its heterodimerization with Runx and thereby transcriptional activation of Runx/CBFβ target genes and all their subsequent molecular events and finally consequential proliferation of pre-B cells.

Experimental Procedures

Cell Culture—The cells were maintained at 37 °C in RPMI 1640 medium (Welgene) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 55 μM 2-mercaptoethanol, 1× penicillin/streptomycin (100 units/ml penicillin, 100 μg/ml streptomycin) in an atmosphere of 5% CO_2 saturated with water. PD36 is an Abelson virus-transformed pre-B cell line (26). S194 (TIB-19, ATCC) and MOPC315 (TIB-23, ATCC) are plasmacytoma cell lines. EL4 (TIB-39, ATCC) is a T_H cell line. All the cells are mouse-originated.

Isolation of Normal Pre-B Cells from Mouse Bone Marrows Using MACS™ System—Normal cells from the bone marrows of mice (5-week-old female, FVB strain) were washed with cold PBS containing 1% BSA and 0.05% NaN_3 (PBS-BN1), and filtered through a 70-μm nylon mesh (Falcon). The cells were counted and then cold-centrifuged to be resuspended in Hanks' balanced salt solution with a concentration of 5×10^6 cells/ml. The resuspended cells were incubated at room temperature for 5 min to remove red blood cells by lysis. The cells were then cold-centrifuged to be resuspended in MACS buffer containing 1% BSA (MACS-B) with a concentration of 2×10^8 cells/ml. The resuspended cells were then mixed with 5 μg of preblocking anti-CD16/32 antibodies (553142; BD Biosciences) per 1×10^7 cells and incubated on a rotator at 4 °C for 30 min. After washing the cells twice with PBS containing 0.2% BSA and 0.05% NaN_3 (PBS-BN2) at 4 °C, they were again resuspended using 40 μl of MACS-B buffer per 1×10^7 cells and then mixed with 5 μg of biotin-conjugated rat anti-mouse pre-BCR antibodies (551863; BD Biosciences) per 1×10^7 cells and then incubated on a rotator at 4 °C for 30 min. After washing the incubated cells twice with PBS-BN2, they were then resuspended using 40 μl of MACS-B buffer per 1×10^7 cells, mixed with 10 μl of anti-biotin microbeads (130-105-637; Miltenyi Biotec) per 1×10^7 cells, and incubated on a rotator for 30 min at 4 °C. After washing twice with PBS-BN2, the cells were resuspended with 500 μl of MACS-B buffer per 1×10^8 cells. The resuspended cells were then loaded onto the LS column (130-042-401; Miltenyi Biotec), which had been inserted into the QuadroMACS™ separator and rinsed by flowing 3 ml of MACS-B buffer just before loading. After the loaded cells were flowed through, the column was washed three times using 3 ml of MACS-B buffer each time. The LS column, which was cared not to be dried, was taken out from the magnetic separator to collect pre-BCR-positive cells in a new 15-ml tube by flowing 5 ml of MACS-B buffer. The collected pre-BCR-positive cells were spun down and incubated with 1 ml of RPMI 1640 medium containing 10% bovine calf serum (Welgene) per 1×10^5 cells for 3–4 h to stabilize them before proceeding to the subsequent experiments of transfections or chemical treatments.

Crlz-1 cDNA Cloning and Its GFP Fusion Construction—mRNA was obtained from PD36 pre-B cells using Fast-Track 2.0 (Invitrogen), and then, a λ phage PD36 cDNA expression library was constructed using SuperScript™ Lambda System (GibcoBRL) and Gigapack®III Gold Packaging Extract (Stratagene). A full-length *Crlz-1* cDNA was isolated by screening this PD36 cDNA expression library with a partial *Crlz-1* cDNA fragment generously provided by the Bae lab (1). To construct a mammalian expression plasmid of *Crlz-1*, the NcoI-NotI cDNA fragment from the above isolated *Crlz-1* clone was Klenow-filled and subcloned into the EcoRV site of pCMV-Tag2A (Stratagene) in frame with the codons encoding FLAG tag peptide. To make *GFP-Crlz-1* fusion construct, the *Crlz-1* coding sequence was excised out from the above-mentioned pCMV-Tag2A-based *Crlz-1* construct using EcoRI and SalI and then inserted between EcoRI and SalI sites of pEGFP-C1 (Clontech).

Transient Transfection for Crlz-1 Overexpression or Its siRNA Knockdown—Overexpression of GFP and/or Crlz-1 in the cell lines was made by transfecting the expression plasmids into cells using Neon™ Transfection System (MPK5000; Invitrogen). Briefly, cells in a logarithmic growth phase were washed with PBS and resuspended in 100 μl of Neon resuspension buffer R per 1.2×10^6 (for PD36 or MOPC315) or 1.6×10^6 cells (for S194). An aliquot of 100 μl of resuspended cells was mixed with 0.25 pmol of expression plasmid dissolved in a final volume of 10 μl of Tris-EDTA buffer, where the difference in DNA amount between samples caused by differential molecular weight was adjusted with pBluescript plasmid (Stratagene). 100 μl of cell-DNA mixture was taken into a Neon tip using the Neon pipette with cares to avoid air bubbles. Then the Neon tip with Neon pipette was put into a Neon tube containing 3 ml of Neon electrolytic buffer E2 on the Neon pipette station. The cell-DNA mixture was electrically pulsed twice at 1,400 V (for PD36 cells) or once at 1,350 V (for S194 or MOPC315 cells) with a pulse width of 20 ms. After the pulses, cells in the tip were

immediately transferred into a 6-well plate containing 2 ml of prewarmed culture medium in each well. The transfected cells were checked for their growth rate by directly counting cells after staining with trypan blue or using the BrdU cell proliferation assay kit (6813; Cell Signaling) and then harvested for their Western and RT-PCR analyses.

Transfection of PD36 cells for siRNA knockdown was performed using 1×10^5 cells per 9 μl of Neon resuspension buffer R and 1 μl of 1 μM siRNA (100 nM in a final mix) basically in the same way as described above for the overexpression experiment except for the use of a 10-μl Neon tip. The sequences of siRNA were as follows: Crlz-1, CCU GAA GAC UAA GUA UAA UCU CUA C (sense) and GUA GAG AUU AUA CUU AGU CUU CAG GUA (antisense); β-catenin, sc-29210 purchased from Santa Cruz Biotechnology; and Scrambled control, phos-CUU CCU CUC UUU CUC UCC CUU GUG A (sense) and UCA CAA GGG AGA GAA AGA GAG GAA GGA (antisense).

In the case of primary normal pre-B cells, transfections for both the overexpression and knockdown of Crlz-1 were performed using 3×10^5 (for expression plasmid of 0.25 pmol) or 1×10^5 (for siRNA of 50 nM) normal pre-B cells in a 10-μl Neon tip with a more mild electric pulse of 1200 V. Otherwise, the transfection conditions were the same as those for cell lines.

Rescue of Crlz-1 or β-Catenin siRNA Knockdown-caused Decrease of Cellular Proliferation by the Co-overexpression of VpreB and λ5 Genes—The cells after 48 h of siRNA transfection as described above were divided into 2 equal volumes of 1 ml, each of which was then processed to co-transfect with 0.125 pmol each of *VpreB* (MR220505; OriGene) and *λ5* (MR222045; OriGene) expression plasmids again just as described above. The cells were checked for the rescue of proliferation during the following 12 h.

Treatment of Wnt Inhibitors (XAV939; Niclosamide) or Activator (LiCl)—The 1 M stock solution for each of niclosamide (N3510; Sigma) and XAV939 (sc-296704; Santa Cruz) was initially made and diluted sequentially to 500 μM for niclosamide and 10 mM for XAV939 in DMSO. 2-ml cultures at a concentration of 5×10^4 cells/ml were treated with 3–4 μl of niclosamide or XAV939 solutions consisting of the diluted stock plus adjusting volume of DMSO so that the same final volume of DMSO could be added to each set of parallel cultures. Actually, the final concentrations of DMSO, niclosamide, and XAV939 in each set of parallel cultures corresponded to 0.15–0.2%, 250–750 nM, and 10–20 μM, respectively. For LiCl treatment, the cells were resuspended at a concentration of 2.5×10^4 (for PD36 pre-B cells) or 5×10^4 cells/ml (for normal pre-B cells) in 10 or 2 ml of culture medium, respectively. Each of the resuspended cells was treated with 50–200 μM LiCl (L-8895; Sigma) using 10 mM LiCl stock solution in water.

RT-PCR—Pelleted cells were vortexed vigorously in 1 ml of TRIzol reagent (Invitrogen) and incubated for 5 min at room temperature. After adding 200 μl of chloroform to the TRIzol-treated cells, the mixture was again vortexed and incubated at room temperature for 2–3 min. The resulting solution was centrifuged by 12,000 $\times$ *g* for 15 min at 4 °C. The supernatant was taken and vortexed after an addition of 500 μl of isopropanol, then incubated for 10 min at room temperature, and centrifuged by 12,000 $\times$ *g* for 10 min at 4 °C to precipitate RNA. The resulting RNA precipitate was rinsed carefully with 1 ml of cold 70% ethanol, dried briefly, and dissolved in 50 μl of RNase-free water to obtain a working concentration range (0.5–1 μg/μl for PD36 and 0.05–0.1 μg/μl for normal pre-B cells). Any possible contamination of DNA was removed by treating with 1 unit of RNase-free DNase I (Promega) per 1 μg of RNA for 30 min at 37 °C. RNA was then extracted with phenol-chloroform, precipitated with absolute ethanol, rinsed with cold 70% ethanol, and finally dissolved in 50 μl of DEPC (diethylpyrocarbonate)-treated water. 1 μg of this RNA was reverse-transcribed at 45 °C for 1 h using RT premix (Intron) with adjustment of total reaction volume to 20 μl by adding DEPC-treated water.

After stopping the reaction by heating at 95 °C for 5 min, the volume of cDNA synthesis reaction mixture was adjusted to 100 μl with 80 μl of DEPC-treated water. 5 μl of this 100-μl cDNA sample was added to PCR premix (K-2016; Bioneer) and amplified in a PCR using 1 μl of a pair of gene-specific primers (10 pmol/μl for each of forward and reverse primers) in a final volume of 20 μl by adjusting with water. The PCR conditions were as follows: 5 min at 95 °C for initial denaturation and then cycling 30–45 times for 30 s at 95 °C, 30 s at an appropriate annealing temperature for a pair of gene-specific primers, 45 s at an extension temperature of 72 °C, with an additional final extension at 72 °C for 5 min. The PCR products were electrophoresed on an agarose gel. The sequences of primer pairs used in these PCRs with their corresponding annealing temperatures and the correct sizes of PCR products can be found in Table 1.

Chromatin Immunoprecipitation—ChIP experiments were performed essentially as described previously (4, 27). ChIP DNA samples precipitated by anti-LEF-1 (sc-8591; Santa Cruz), anti-β-catenin (ab32572; Abcam), or anti-PEBP2β (sc-17181; Santa Cruz) were tested to see whether the LEF-1-binding distal region (LEF-1-DR) of *Crlz-1* promoter (6) or Runx/CBFβ-binding regions (EBF1Rbs2 and D′λ5-Box, respectively) of EBF (8) and λ5 (9) promoters were amplified in the PCR using their corresponding primer pairs and annealing temperatures (Table 1). The PCR program consisted of an initial denaturation step at 94 °C for 5 min and then 33–38 cycles of 30 s at 94 °C, 30 s at an appropriate annealing temperature, and 30 s at 72 °C, with an additional final extension at 72 °C for 10 min. Normal rabbit IgG (sc-2027; Santa Cruz) and normal goat IgG (sc-2028; Santa Cruz) were employed as control antibodies.

Western Blotting—The cells were harvested and boiled in an SDS sample buffer and separated on an SDS-PAGE of 8% polyacrylamide gel and transferred to a PVDF membrane (BPS0161; PALL). The primary and secondary antibodies used in our Western blotting experiments were as follows: anti-GFP (ab290), anti-β-actin (ab8227), and polyclonal HRP-conjugated goat anti-rabbit IgG (ab6721), all of which were from Abcam; anti-β-catenin (sc-7199), anti-Fli-1 (sc-356), anti-GABPα (sc-22810), anti-LEF-1 (sc-8591), anti-VpreB (sc-33128), anti-λ5 (sc-33127), and anti-c-Myc (sc-40), all of which were from Santa Cruz; and polyclonal HRP-conjugated goat anti-mouse IgG (A90-116p; Bethyl). ECL reactions were performed with reagents (W100B) from Promega.

TABLE 1
The sequences of PCR primer pairs

Name of primer pair	Forward primer	Reverse primer	Annealing temperature	Expected DNA size
			°C	*bp*
Crlz-1	cta ttt tta gca atc tgg tag gta atg	cat cct gtt ata gaa agg ctt gtt acc	56	356
β-Catenin	tct tgg cta tta cga cag	cct cta tac cac cca ctt	55	446
VpreB	cgt ctg tcc tgc tca tgc tgc	acg gca cag taa tac aca gcc	56	340
λ5	ctt gag ggt caa tga agc tca gaa ga	ctt ggg ctg acc tag gat tg	56	337
EBF	gct gtg gca acc gaa atg ag	ccg tgc ttg gag tta ttg tgg ac	61	217
Cyclin D2	tac ttc aag tgc gtg cag aag gac	tcc cac act tcc agt tgc gat cat	57	489
Cyclin D3	cgt gca aaa gga gat caa gc	aat caa ggc cag gaa gtc g	57	352
Wnt3a	ccc aac ttc tgc gaa cct aa	tct ccg ccc tca agt aag aa	57	347
Wnt5a	ctg gca gga ctt tct caa gg	ctc tag cgt cca cga act cc	60	388
Wnt10b	gga agg gta gtg gtg agc aa	cac ttc cgc ttc agg ttt tc	58	298
IL-7Rα	tta ctt caa agg ctt ctg gag	ctg gct tca acg cct ttc acc tca	58	315
β-Actin	tga acc cta agg cca acc gtg	gca gct cat agc tct tct cca ggg	56	399
	aca ccc cag cca tgt acg ta	ctc ttt gat gtc acg cac ga	57	260
LEF-1-DR (ChIP)	cta tac ata gga ggc tgt ccg cgg c	gtt gtc agc ctc agc ctc ccg gat t	57	259
EBF1Rbs2 (ChIP)	ttt cag aag cct gct gaa gac tga g	cag aaa agg cag gat gcc cat ccc ag	63	225
D′λ5-Box (ChIP)	ggg tta aga cag gca gct gtg ag	caa acc cca ggc tgt ctc tag tt	62	185

Transient Transfection of Crlz-1 siRNA and Immunocytochemistry for the Observation of CBFβ Relocalization—2×10^5 cells of PD36 were transfected with 10 nM of a fluorescently labeled siRNA using 6 μl of Hiperfect (Qiagen) according to the supplier's protocol. The transfected cells were cultured with changes of media at 24 h and harvested at 40 h after transfection. The sequences of TYE563-labeled control siRNA (Integrated DNA Technologies) were TYE563-T*CC UUC CUC UCU UUC UCU CCC UUG UG*A (sense) and TYE563-T*CA CAA GGG AGA GAA AGA GAG GAA GG*A (antisense), where asterisks indicate 3′-thioester bonds. The sequences of Cy5-labeled siRNA for Crlz-1 were Cy5-CCU GAA GAC UAA GUA UAA UCU CUA C (sense) and GUA GAG AUU AUA CUU AGU CUU CAG GUA (antisense).

The harvested cells were mounted onto coverslips by centrifugation at 350 × *g* for 5 min, washed with PBS at room temperature for 5 min, and fixed in 4% paraformaldehyde at room temperature for 20 min. The fixed cells on coverslips were washed twice with PBS, permeabilized with 0.3% Triton X-100 in PBS at 37 °C for 30 min, and washed twice with PBS. The cells were then treated with 1 mg/ml RNase A in 0.5% donkey serum at 37 °C for 30 min and washed with PBS. The washed cells were blocked in 2% donkey serum in PBS at 37 °C for 30 min to 1 h and washed again twice with PBS. For an immunodetection of CBFβ within cells, the cells on coverslip were incubated with the primary antibody of anti-PEBP2β (sc-17181; Santa Cruz) at a concentration of 10–20 μg/ml in the blocking solution at 4 °C overnight. Next day, the cells were washed three times with PBS and incubated with the secondary antibody of Alexa 488-labeled donkey anti-goat IgG (A11055; Invitrogen) at a concentration of 10–20 μg/ml in the same blocking solution at room temperature for 4 h. The cells were then washed three times with PBS, and their nuclei were counterstained with 3 μM of DAPI in PBS containing 0.1% Nonidet P-40 at room temperature for 10 min. Finally, the cells on coverslip were washed once with PBST (PBS with 0.1% Triton X-100), washed three times with PBS, and mounted on slide glasses for an observation by a confocal fluorescence microscope (LSM510-META; Zeiss).

Flow Cytometry—The surface pre-BCR expression after siRNA transfection into PD36 pre-B cells was measured in a flow cytometer (FACSCalibur™; BD Biosciences) and analyzed using a Flowing Software version 2.5.1, which was downloaded free from the University of Turku (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland). The fluorescence labeling of cell surface pre-BCR was performed basically in the same way as described previously (4) except fixing with 0.4% paraformaldehyde before the flow cytometric measurement this time. The antibodies were rat anti-mouse CD16/32 (553142), biotin-conjugated rat anti-mouse pre-BCR (551863), biotin-conjugated rat IgG2aκ isotype control (553928), and PE-conjugated streptavidin (554061), all of which were purchased from BD Biosciences.

Statistical Analysis—A two-tailed Student's *t* test was employed to show the statistical significance of data. $p \leq 0.05$ was considered statistically significant.

Author Contributions—S.-Y. C. performed the experiments in Figs. 2 (*B–G*), 5 (*A–F*), 6 (*A–C*), and 7. S.-K. P. initiated the founding experiments and performed those in Figs. 1 and 2*A*. H.-W. Y. performed the experiments in Figs. 3, 4, 5 (*G–I*), and 6 (*D–G*). J.-H. P. assisted other authors to perform those experiments. C.-J. K. presided all the experimental works and wrote the paper.

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