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Published in:
Journal of Pathology

Document Version:
Publisher's PDF, also known as Version of record

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The matricellular protein CCN3 regulates NOTCH1 signalling in chronic myeloid leukaemia

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Abstract
Deregulated NOTCH1 has been reported in lymphoid leukaemia, although its role in chronic myeloid leukaemia (CML) is not well established. We previously reported BCR-ABL down-regulation of a novel haematopoietic regulator, CCN3, in CML; CCN3 is a non-canonical NOTCH1 ligand. This study characterizes the NOTCH1–CCN3 signalling axis in CML. In K562 cells, BCR-ABL silencing reduced full-length NOTCH1 (NOTCH1-FL) and inhibited the cleavage of NOTCH1 intracellular domain (NOTCH1-ICD), resulting in decreased expression of the NOTCH1 targets c-MYC and HES1. K562 cells stably overexpressing CCN3 (K562/CCN3) or treated with recombinant CCN3 (rCCN3) showed a significant reduction in NOTCH1 signalling (> 50% reduction in NOTCH1-ICD, p < 0.05). Gamma secretase inhibitor (GSI), which blocks NOTCH1 signalling, reduced K562/CCN3 colony formation but increased that of K562/control cells. GSI combined with either rCCN3 or imatinib reduced K562 colony formation with enhanced reduction of NOTCH1 signalling observed with combination treatments. We demonstrate an oncogenic role for NOTCH1 in CML and suggest that BCR-ABL disruption of NOTCH1–CCN3 signalling contributes to the pathogenesis of CML.

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Keywords: CML; NOTCH1; CCN3; HES1; c-MYC; GSI

Received 15 November 2012; Revised 6 August 2013; Accepted 7 August 2013

No conflicts of interest were declared.

Introduction
Chronic myeloid leukaemia (CML) is a haematopoietic stem cell (HSC) disorder characterized by the constitutive expression of the fusion protein BCR-ABL kinase [1]. Imatinib, the first tyrosine kinase inhibitor (TKI) developed to inhibit BCR-ABL signalling, is used as the front-line therapy for CML [2]. However, a significant subset of CML patients do not respond to imatinib because of intolerance or toxicity or due to drug resistance, and a second-generation TKI is recommended [3,4]. Also, the persistence of the residual BCR-ABL clone even after therapy and its subsequent expansion results in relapses [5]. TKIs can inhibit BCR-ABL activity in CML stem cells but these cells are not addicted to BCR-ABL and BCR-ABL inhibition does not eradicate the leukaemic clone [6]. This has led to widespread interest in identifying the key stem cell regulators that are important in CML pathology [7].

Our group identified the down-regulation of a novel haematopoietic regulator, CCN3, in CML as an initial effector of BCR-ABL activity in a murine CML stem cell model [8]. CCN3 is a member of the CCN family of matricellular proteins that have emerged as key regulators of diverse cellular processes; deregulated expression of CCN proteins is observed in many pathologies including cancer [9]. CCN3 is generally associated with tumour suppressor activity in solid tumours [10–12]. We found that CCN3 was not expressed in cells from CML patients at diagnosis, but expression was restored to normal on response to imatinib [8]. Overexpression of CCN3 in K562 CML cells reduced proliferation, induced apoptosis, and enhanced sensitivity to imatinib [13]; the anti-proliferative effect of CCN3 was associated with decreased phosphorylation of ERK, AKT, and the up-regulation of α6β4 integrin [13,14].

CCN3 is a non-canonical NOTCH1 ligand implicated in the cell fate decisions of HSCs [15,16]. NOTCH1 is activated by receptor–ligand interactions, resulting in the γ-secretase complex-mediated release of its active intracellular domain (NOTCH1-ICD) [17]. The major downstream effector molecules of NOTCH1 include c-MYC [18] and the members of the HES and HEY protein families [19]. Activating mutations of NOTCH1 are observed in nearly 50% of T-cell acute-lymphoblastic leukaemia (T-ALL) and 30% of adult T-cell leukaemia (ATL) patients [20].
NOTCH signalling has been associated with T-cell leukaemias and its role in myeloid malignancies is not well investigated. Since CCN3 is absent in CML, it could be postulated that the regulatory effect of CCN3 on the NOTCH1 signalling pathway is abolished. The objectives of this study were (i) to define the role of NOTCH1 in CML; (ii) to characterize the CCN3–NOTCH1 signalling in CML; and (iii) to examine the anti-proliferative potential of γ-secretase inhibitors (GSIs) which block NOTCH1 signalling in CML.

Materials and methods

Cell lines and reagents

The K562 CML cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany). KCL22s and LAMAs cells were a gift from Professor Junia Melo (University of Adelaide, Adelaide, Australia). CML cell lines (K562, LAMAs, and KCL22s) and K562 cells transfected with the pCMV82 vector encoding full-length CCN3 (K562/CCN3) or empty vector pC6 + (K562/control) were routinely cultured as previously described [14].

Primary CML samples and normal controls

Leukopheresis products were obtained from CML patients at the time of diagnosis and bone marrow from healthy donors was collected as a normal control. Mononuclear cells were prepared using Ficoll Hypaque (GE Healthcare, Buckinghamshire, UK) for 72 h before extracting proteins for western blotting.

BCR-ABL siRNA transfection

K562, KCL22s, and LAMAs cells were transfected with anti-BCR-ABL siRNA or scrambled sequence as previously described [21]. Cells after transfection were incubated at 37°C with 5% CO₂ for 48 h and 72 h post-transfection.

Western blotting

Total proteins [14] and nuclear and cytoplasmatic proteins were extracted from the cells as previously described [22]. Tris acetate gels (3–8%) were used to detect NOTCH1 and BCR-ABL, and 4–12% Bis-Tris gels (Invitrogen, Paisley, UK) for other proteins. Immunoblotting was performed using antibodies to BCR-ABL, NOTCH1, p21, PARP, c-MYC, p27, and TATA box binding protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HES1 (Abcam, MA, USA); and β-actin (Sigma, St Louis, MO, USA). For CCN3 detection, NH5 antibody raised against the C-terminus of CCN3 was provided by B Perbal [22]. Immuno-blots were visualized by enhanced chemiluminescence (ECL plus, Amersham, UK).

Real-time PCR

BCR-ABL and CCN3 primers and probes were used as previously described [8]. NOTCH1 (Hs01062014_m1), 18S rRNA (Hs03928985_g1) primers, and probes were based on TaqMan chemistry (Applied Biosystems, Foster City, CA, USA) and amplification was performed according to the manufacturer’s protocol. The primers for HES1 and c-MYC were designed against Genbank published sequences in association with Primer Express (Applied Biosystems) and were obtained from Eurofins MWG Operon (Huntsville, AL, USA). Amplification for HES1 and c-MYC was performed using the FastStart universal SYBR Green master mix (Roche Applied Science, Mannheim, Germany). All reactions were performed on a 7900HT Fast Real-Time PCR System using ABI sequence detection software v2.3 (Applied Biosystems). After normalizing to the endogenous 18S rRNA control, the relative mRNA fold changes were quantified using the 2−ΔΔCT method [23].

Cell treatment with rCCN3, GSI, and imatinib

K562/control and K562/CCN3 cells (2 × 10⁵ cells/ml) were treated with InSolution™ γ-secretase inhibitor (GSI) X-250 UG (Calbiochem, San Diego, CA, USA) at concentrations of 25, 50, 100, 500, and 1000 nM. For experiments using rCCN3, cells (2 × 10⁵ cells/ml) were grown in Opti-MEM® I Reduced Serum Media (Invitrogen) rather than RPMI supplemented with 10% serum to avoid the possible effect of traces of CCN3 in the serum. K562, KCL22s, and LAMAs cells were treated with 100 ng/ml rCCN3 (Peprotech, London, UK) for 72 h before extracting proteins for western blotting.

For combination treatments of rCCN3 and GSI, cell lines (2 × 10⁵ cells/ml) were treated with 100 ng/ml rCCN3 for 24 h. Following this, GSI was added at 25–1000 nM to the cell culture. Cells were again incubated for 24, 48, and 72 h. For combination treatment with imatinib, cell lines (2 × 10⁵ cells/ml) were treated with 200 nM imatinib along with GSI (25–1000 nM) for 24 and 48 h.

Viability assays

Cell viability was measured using the CellTiter-Glo® assay (Promega, Madison, WI, USA) following the manufacturer’s instructions; luminescence was read using a Tecan plate reader (Tecan Ltd, Switzerland) at 590 nm. The data are represented as luminescence relative to the untreated control. Based on the cell viability measurements, the Chou–Talalay analysis was performed to analyse the effect of GSI in combination with either rCCN3 or imatinib using CalcuSyn software.
A combination index (CI) value of less than 0.9 is synergistic, 0.9–1.1 is additive, and more than 1.1 is indicative of an antagonistic effect between the two drugs in combination [24].

Methylcellulose colony assays
K562/control and K562/CCN3 cells (1 × 10³ cells) were plated in MethoCult H4034 medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 25, 50, 100, 500, and 1000 nM GSI. For combination experiments with rCCN3, K562 cells (1 × 10³ cells) were plated on methylcellulose culture medium containing 100 ng/µl rCCN3 and GSI (above-mentioned concentrations). Parallel assays were conducted with K562 cells with 25–1000 nM GSI without adding rCCN3. For combination treatments with imatinib and GSI, each of the three CML cell lines (1 × 10³) were plated with 200 nM imatinib and increasing concentrations of GSI (25–1000 nm). All assays were performed in triplicates for each treatment. The plates were incubated at 37°C at 5% CO₂ for 7 days in a humidified atmosphere and colonies were counted using an inverted microscope (Olympus, Tokyo, Japan; magnification ×40).

Flow cytometry
K562/control and K562/CCN3 cells (5 × 10⁵) after 24 h of GSI treatment were prepared for propidium iodide staining and analysis using a BD™ LSR II flow cytometer as previously described [13].

Microarray gene expression profiles
To compare and validate our findings, we interpreted the gene expression profiles of CCN3, NOTCH1, HES1, and c-MYC from three CML microarray databases from GEO (accession numbers: GSE24739 [25]; GSE11889 [26]; and GDS838 [27]).

Statistics
Data are presented as mean ± SD. Statistical analyses were performed using the two-tailed Student’s t-test for two-sample tests; for multiple comparisons, two-way ANOVA was used.

Results
Expression of NOTCH1 and CCN3 in CML
We have previously shown down-regulation of CCN3 by BCR-ABL in primary CML CD34+ cells [8]. To determine whether NOTCH1 signalling was dependent on BCR-ABL, K562 cells were transfected with anti-BCR-ABL siRNA and its effect was examined. BCR-ABL knockdown decreased mRNA levels of NOTCH1, HES1, and c-MYC (Figure 1a), and reduced expression of full-length NOTCH1-FL (300 kDa), cleaved NOTCH1-ICD (120 kDa), and NOTCH1 downstream targets c-MYC and HES1 (Figure 1b). Optical densitometry showed an inverse correlation between NOTCH1 and CCN3 expression in CML (Figure 1c). These effects were also observed following BCR-ABL knockdown in KCL22s and LAMAs cell lines (Supplementary Figure 5).

CCN3 reduces NOTCH1 signalling
K562 cells stably expressing CCN3 (K562/CCN3) or transfected with vector control (K562/control) were used to investigate if CCN3 regulates NOTCH1 signalling. NOTCH1 mRNA levels were similar in both cell lines, indicating that CCN3 does not regulate NOTCH1 transcription (Figure 2a). In contrast, NOTCH-FL, NOTCH-ICD, and HES1 protein expression was reduced in K562/CCN3 cells, compared with K562/control, suggesting that CCN3 reduces NOTCH1 signalling in CML and that regulation is post-transcriptional (Figures 2b and 2c). In the nuclear compartment of K562/control cells, increased levels of NOTCH1-ICD and p27 were observed, whereas the
Figure 2. CCN3 reduces NOTCH1 signalling. (a) The mRNA expression of NOTCH1 and CCN3 in K562/CCN3 cells (white bars) relative to K562/control (grey bars). (b) Representative western blot showing NOTCH1-FL, NOTCH1-ICD, and HES1 expression in K562/control and K562/CCN3 cells. (c) Expression of NOTCH1-FL, NOTCH1-ICD, and HES1 levels relative to K562/control cells \((n = 3)\). \(p < 0.05\). (d) Representative western blot showing the expression of NOTCH1-ICD, p21, and p27 in the nuclear and cytoplasmic extracts of K562/control and K562/CCN3 cells. TATA binding protein was used as the nuclear loading control and \(\beta\)-actin was the endogenous control for the cytoplasmic fraction. (e) Expression of NOTCH1-ICD, p21, and p27 in the nuclear fraction of K562/CCN3 cells relative to K562/control cells \((n = 3)\). \(p < 0.05\). (f) Co-immunoprecipitation (Co-IP) showing the association of CCN3 with the extracellular domain of NOTCH1 (NOTCH1-ECD) in K562/CCN3 cells. CCN3 was not bound to the NOTCH1-ICD.

Effect of GSI on K562/control and K562/CCN3 cells

Next we investigated the anti-proliferative effect of GSI, which blocks NOTCH1 signalling. K562/CCN3 cells formed fewer colonies (Figure 3a) and showed a modest increase in sub-G0 events (Figure 3b) compared with K562/control when treated with GSI (25–1000 nM). GSI stabilized NOTCH1-FL in K562/control cells, whereas in K562/CCN3 cells, NOTCH1-FL was present in very low amounts (Figure 3c). NOTCH1-ICD levels were lower in K562/CCN3 than in K562/control cells. In K562/control cells, GSI reduced NOTCH1-ICD levels at high concentrations but these cells still had higher levels of NOTCH1-ICD compared with K562/CCN3. GSI decreased c-MYC levels in both K562/control and K562/CCN3 cells; HES1 expression was significantly reduced in K562/CCN3 cells only (Figures 3d–3g).

Effect of recombinant CCN3 on the NOTCH1 signalling pathway

Previously, we showed that recombinant CCN3 (rCCN3) treatment of K562 cells decreased their cell proliferation and colony formation capacity [13]. Here, we examined if exogenous addition of CCN3 could modulate NOTCH1 signalling. NOTCH1 signalling was significantly reduced with rCCN3 treatment in all CML cell lines (Supplementary Figures 1a, 1b, and 2a–2c). Treatment of K562 cells with GSI alone had no significant effects on cell viability (Supplementary Figure 1c); however, rCCN3 treatment of K562 cells sensitized them to GSI treatment (Supplementary Figure 1d). Pretreatment with rCCN3 induced a synergistic effect with all concentrations of GSI (Chou–Talalay analysis; 0.072–1.044). These observations were supported by colony assays; GSI treatment alone increased the clonogenicity of K562 cells, whereas in combination with rCCN3 there was a significant reduction in colony formation capacity (Figures 4a and 4b). Western blotting showed decreased NOTCH1-ICD, c-MYC, HES1, and induction of PARP cleavage in K562 cells treated with the combination treatment (Figures 4d–4f). A decrease in cell viability and clonogenicity was also observed when KCL22s
Figure 3. Effect of GSI on the proliferation of K562/CCN3 cells. K562/control cells (grey bars) and K562/CCN3 cells (white bars) were treated with GSI (25–1000 nM). (a) Methylcellulose colony formation assay showing the effect of GSI on the clonogenicity of K562/CCN3 cells relative to the corresponding K562/control treatments. Statistical significance was calculated by comparing K562/control and K562/CCN3 with each dose of GSI used. (b) The percentage of sub-G0 events observed with 24 h of GSI treatment. (c) Representative western blot showing the expression of NOTCH1-FL, NOTCH1-ICD, c-MYC, and HES1 after GSI treatment in K562/control and K562/CCN3 cells. (d–g) Densitometry plots showing the relative expression of these proteins in treated cells compared with the untreated. Data represent the mean ± SD of triplicate experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

and LAMAs cells were treated with the rCCN3 and GSI combination (Supplementary Figures 2d–2g).

Effect of combining imatinib and GSI on K562 cells
To examine if there is a synergistic effect of combining imatinib and GSI, K562 cells were treated with imatinib (200 nM) in combination with GSI (25–1000 nM). Imatinib combined with 25 nM GSI induced an approximately 50% reduction in K562 cell viability relative to its use as a single agent (data not shown). GSI had a synergistic effect at concentrations up to 100 nM (25 nM CI 0.52) but was antagonistic at higher concentrations (1000 nM CI 1.51). These observations were substantiated by colony studies (Figure 5a) and similar results were observed in KCL22s and LAMAs cell lines (Supplementary Figures 3a and 3b). Western blotting showed a decrease in NOTCH1 signalling in K562 cells with imatinib treatment and combining GSI.
with imatinib enhanced this effect (Figures 5b–5f). This suggests the potential clinical utility of GSI at low doses.

Correlation between CCN3, NOTCH1, HES1, and c-MYC in CML

We investigated the gene expression levels of CCN3, NOTCH1, HES1, and c-MYC in three CML patients at diagnosis and examined the expression of these genes in three publically available CML microarray studies, two of which compared CML patients with normal donors and one that analysed CML patients following imatinib treatment. Overall, a significant decrease in CCN3 expression was observed in CML compared with normal bone marrow (p < 0.05), with the exception of three patients in the accelerated phase in whom expression was highly variable (Supplementary Figure 4a and Figures 6a and 6b). This is consistent with our previous report showing decreased CCN3 expression in CML patients at diagnosis [8]. One microarray study demonstrated that expression of NOTCH1 and HES1...
Figure 5. Combinatorial treatment of K562 cells with imatinib and GSI. (a) Colony formation capacity of K562 cells (grey bars) when imatinib (200 nM) was used with 25–1000 nM GSI (white bars) ($n = 3$). (b) Representative western blot showing the effect of NOTCH1 signalling in K562 cells treated with the combination of imatinib and GSI. (c–f) Optical densitometry units for NOTCH1-FL, NOTCH1-ICD, c-MYC, and HES1 normalized to β-actin. Data represent the mean ± SD of triplicate experiments. *$p < 0.05$; **$p < 0.01$.

was significantly lower in CML; however, we, along with Bruns et al [26], found no significant difference in the expression of NOTCH1 and HES1. All studies demonstrated that c-MYC was not significantly altered in CML compared with normal. Expression levels of CCN3, HES1, and c-MYC in CML patients in major molecular remission (MMR) were comparable to that of normal bone marrow (Figure 6c), consistent with restoration of normal phenotype in these patients.

We showed that knockdown of BCR-ABL in CML cell lines results in reduced NOTCH1 signalling and therefore hypothesized that NOTCH1 and its downstream targets may be up-regulated in CML. Gene expression levels analysed in three patient samples and microarray studies did not reflect this expectation and generated variable results. In addition, protein levels of NOTCH1, HES1, and c-MYC in CML samples were found to be highly variable (Supplementary Figures 4b–4f). This may be attributed both to the small study size and to the stage of disease that was investigated. While there are limited data available on NOTCH1 expression in CML, a number of studies have found that c-MYC and HES1 expression is unchanged in the chronic phase but expression is increased in accelerated and blast crisis CML [28,29]. As CML cell lines are derived from CML in blast crisis, this is in agreement with our findings and suggests that NOTCH1 signalling may be important in CML disease progression.

Discussion

NOTCH1 signalling has established roles in haematopoiesis, particularly in T-cell commitment and maturation [30]. Pathological roles for NOTCH1 have also been reported in B-chronic lymphocytic leukaemia, where aberrant and constitutively active NOTCH1 signalling is associated with a poor prognosis and drug resistance [31–33]. The role of NOTCH1 in CML is not well defined and there are contradictory reports from several groups. Overexpression of active NOTCH1-ICD in K562 cells caused inhibition of cell proliferation [34], whereas double transgenic mouse models overexpressing Bcr-Abl and Notch1-ICD developed T-ALL, demonstrating a co-operative role for Bcr-Abl and Notch1 [35]. Here, we demonstrate that NOTCH1 signalling in CML is BCR-ABL-dependent, as silencing of BCR-ABL in the K562 CML cell line caused a global reduction of NOTCH1 signalling.

The majority of work on NOTCH1 signalling has focused on activation by canonical ligands of the Delta and Jagged families [36]. However, there are several non-canonical NOTCH ligands that could either activate or inhibit NOTCH signalling [37]. CCN3 is a non-canonical NOTCH ligand and has been previously shown to stimulate NOTCH1 signalling in myoblasts [38], osteoblasts [39], and vascular smooth muscle cells [40]. In contrast, we found that in CML CCN3 inhibits NOTCH1 activity potentially by its association with the extracellular domain of NOTCH1. The low expression of NOTCH1-FL in the presence of CCN3 indicates that CCN3 not only inhibits the production of active NOTCH1-ICD cleaved by the γ-secretase complex, but also has a negative impact on the proteolytic steps that lead to the formation or stability of full-length NOTCH1 receptor. We assayed the nuclear expression of NOTCH1-ICD together with the levels of p21 and p27 in K562/CCN3 cells as BCR-ABL and NOTCH1 deregulate the expression...
of these cell cycle regulators. In CML, BCR-ABL inhibits the nuclear translocation of p27; CD34+ CML progenitor cells have less nuclear p27, which activates the AKT pathway resulting in enhanced cell proliferation [41]. Active NOTCH1 has been shown to permit cell cycle progression into the S-phase by enhancing proteasomal degradation of p27 [42,43]. We found that inhibition of NOTCH1 by CCN3 is associated with increased expression of p27. This suggests that down-regulation of NOTCH1 signalling by CCN3 contributes to the restoration of cell cycle regulation in CML.

Inhibition of the production of active NOTCH1-ICD by GSIs has been shown to induce anti-tumourigenic effects in mouse models of T-ALL [44], breast cancer [45], and pancreatic cancer [46]. We did not find any significant response to GSI used as a single agent in the CML cell lines K562, KCL22s, and LAMAs, consistent with previous reports [47]. However, an anti-proliferative effect of GSI was observed when CCN3 expression was enforced in a CML cell line (K562/CCN3) or when cells were pretreated with rCCN3. GSI also had synergistic activity with imatinib in all three CML cell lines, which may be mediated by the ability of imatinib to induce CCN3 expression in CML. The effect of NOTCH1 inhibition on CML cells has generated conflicting data. K562 cells have been reported to be insensitive to NOTCH1 inhibition [47], and inhibition of NOTCH1 has also been reported to increase proliferation [34]. This could be due to the absence of CCN3 in these systems, as we also found that CML cells did not respond to GSI in liquid cultures and produced increased colony formation of K562 cells when used as a single agent. We suggest from our findings that CML cells are dependent on NOTCH1 activity; however, for GSI to induce anti-proliferative responses in CML, the presence of CCN3 is an essential factor. GSI used in combination strategies was able to reduce HES1 levels and expression of c-MYC. In multiple myeloma mouse models, inhibition of NOTCH1 signalling by GSI-XII induced an anti-tumourigenic effect by down-regulating Hes1 [48]. Expression of HES1 is reported to be high in patients in the CML blast crisis phase compared with the chronic phase; Hes1 interaction with Bcr-Abl is essential for transformation of CML into blast crisis [28]. Similarly, c-MYC is overexpressed in CML and the oncogenic role of c-MYC in the transformation process of CML is well established [29]. We found that the expression of both of these proteins decreased with the combinatorial treatment approaches that we used in our study. CCN3 is a matricellular protein and is expressed in the bone marrow micro-environment [49]. Our study showed that exogenous CCN3 decreased NOTCH1 signalling by modulating the expression of HES1 and c-MYC. This study provides key evidence for CCN3 growth regulatory activity in NOTCH1 signalling in the haematopoietic niche and advocates CCN3 as an essential regulator of normal haematopoiesis.

From this study, supported by in silico analyses, we suggest that the NOTCH1–CCN3 signalling axis plays a key role in myelopoiesis. In CML, BCR-ABL expression causes down-regulation of CCN3 and impacts on NOTCH1 signalling, contributing to the malignant phenotype. This work provides a rational basis for the development of combinatorial therapeutic strategies targeting NOTCH1 in CML, particularly in advanced disease.

**Author contribution statement**

SS, LJC, WHL, and DJS carried out experiments. AEI and LMcC designed the study and analysed data. All authors contributed to drafting the manuscript.
References


SUPPORTING INFORMATION ON THE INTERNET
The following supporting information may be found in the online version of this article.
Figure S1. Recombinant CCN3 reduces NOTCH1 signalling in K562 cells.
Figure S2. Recombinant CCN3 treatment reduces NOTCH1 signalling in KCL22s and LAMAs cell lines.
Figure S3. GSI in combination with imatinib reduces the clonogenicity of KCL22s and LAMAs cells.
Figure S4. NOTCH1, HES1, and c-MYC expression in primary CML samples.
Figure S5. BCR-ABL knockdown in KCL22s and LAMAs cells decreases NOTCH1 signalling.

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