Dipeptide species regulate p38MAPK–Smad3 signalling to maintain chronic myelogenous leukaemia stem cells

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Understanding the specific survival of the rare chronic myelogenous leukaemia (CML) stem cell population could provide a target for therapeutics aimed at eradicating these cells. However, little is known about how survival signalling is regulated in CML stem cells. In this study, we survey global metabolic differences between murine normal haematopoietic stem cells (HSCs) and CML stem cells using metabolomics techniques. Strikingly, we show that CML stem cells accumulate significantly higher levels of certain dipeptide species than normal HSCs. Once internalized, these dipeptide species activate amino-acid signalling via a pathway involving p38MAPK and the stemness transcription factor Smad3, which promotes CML stem cell maintenance. Importantly, pharmacological inhibition of dipeptide uptake inhibits CML stem cell activity in vivo. Our results demonstrate that dipeptide species support CML stem cell maintenance by activating p38MAPK–Smad3 signalling in vivo, and thus point towards a potential therapeutic target for CML treatment.

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hronic myelogenous leukaemia (CML) arises when the 

BCR-ABL1 oncogene is generated in haematopoietic stem 
cells (HSCs)). Although tyrosine kinase inhibitors (TKIs), such as the first-generation TKI imatinib mesylate (IM) and the second-generation TKIs dasatinib and nilotinib, have markedly improved the prognosis of CML patients, a cure remains elusive). CML stem cells, which are the cellular source of the vast majority of differentiated CML cells, are reportedly 

responsible for the recurrence of CML disease following TKI therapy). Thus, to completely eradicate quiescent CML stem 

cells and CML disease, TKIs may have to be coupled with novel 

therapeutics targeting alternative molecular pathways.

A nutrient supply specifically required for CML stem cell 
maintenance could provide a candidate target for a novel therapy 
capable of eradicating CML stem cells. However, to reduce the 

harmful side effects of such molecular targeting on 

normal haematopoiesis, it is essential to understand the 

altered mechanisms that distinguish CML stem cells from normal HSCs. To pinpoint CML-associated nutrient signalling, we carried out 
global metabolic comparison of normal HSCs with the 

corresponding stages of CML stem cells in tetracycline 
(tet)-inducible CML-affected mice). Our approach allowed us to 

use doxycycline (DOX) withdrawal to synchronize the 

induction of CML disease in these mice via HSC-specific 

activation of the tTA (tetracycline-controlled transactivator) 

protein, and to obtain the most primitive long-term (LT)-CML 

stem cells from the bone marrow (BM) of animals developing 

CML. This strategy of metabolic analysis in a well-characterized 

CML model has uncovered a nutrient signalling pathway that is 

critical for the in vivo maintenance of CML stem cells but not 

normal HSCs.

In mammals, the uptake of small peptides by the Slc15A family 
of oligo/dipeptide transporters provides an effective and energy-
saving intracellular source of amino acids). These transporters 

are encoded by the Slc15A1 (previously designated Pept1), Slc15A2 
(Pept2), Slc15A4 (Pept1) and Slc15A3 (Pept2) genes. Oligo-/dipeptide 

uptake dependent on Slc15A1/2 has been well studied in renal and 

intestinal epithelial cells, but the functions of these 

transporters in haematopoietic cells are obscure. We propose that 

Slc15A2 dipeptide transporter activity sustains CML stem cell 
maintenance by guaranteeing an alternative nutrient supply. Most 

importantly, this survival mechanism apparently does not operate 

routinely in normal HSCs.

CML stem cell maintenance is also influenced by the TGF-β 

pathway, which can either decrease or increase CML stem cell 

numbers in vivo depending on the cellular context). Because 

Smad3, a downstream effector of TGF-β signalling, is a ‘master 

regulator’ of cell fate, it has been of great interest to determine 

whether Smad3 promotes the maintenance of ‘stemness’ in vivo, 

including CML cell stemness. In our study, we provide evidence 

that post-translational modification of Smad3 by non-canonical 

phosphorylation at Ser208 is crucial for CML stem cell activity 
in vivo. Intriguingly, we also demonstrate that this non-canonical 

Smad3 phosphorylation is mediated by dipeptide-triggered 

activation of p38MAPK.

Our results demonstrate that dipeptide species support a 

nutrient signalling mechanism that is required for CML stem cell 

activity in vivo. This novel mechanism has two linked features: (1) 

primitive CML stem cells take up dipeptide species through 

Slc15A2 transporter activity, and (2) these internalized 

dipeptides regulate nutrient signalling pathway(s) through 
p38MAPK-mediated Smad3-Ser208 phosphorylation. Because 

this mechanism does not appear to function in normal HSCs, it 

may be possible to eliminate vulnerable CML stem cells by 

therapeutic targeting of this crucial nutrient signalling pathway, 

offering new hope for reducing CML recurrence in patients.

Results

CML stem cells accumulate several dipeptide species. To 

identify nutrient signalling differences between normal HSCs 

and CML stem cells, we carried out a global metabolic 

comparison of cells isolated from control and tet-inducible 

CML-affected mice. To obtain the latter animals, we crossed 

Tal1-tTA mice with TRE-BCR-ABL1 transgenic mice (FVB/N 

background) to generate Tal1-tTA × TRE-BCR-ABL1 double-

transgenic progeny). When these progeny are subjected to 

DOX withdrawal, synchronous induction of CML disease occurs 

with the generation of CML stem cells. From healthy control 

(Tal1-tTA) and CML-affected (Tal1-tTA + TRE-BCR-ABL1) 

littermates, we isolated the following cell subsets: the 

immature KLS (cKit+ Lineage ‘Sc-1’) population, which 

includes normal HSCs and CML stem cells (also known as 

leukaemia-initiating cells (LiCs)), the committed progenitor 

KLS- (cKit+ Lineage ‘Sc-1’) population and the mature 

Lin+ (Lineage +) population. We then applied metabolomics 
techniques to examine the metabolites of these cells. Although 

quiescent normal HSCs reportedly produce adenosine 5’-triphosphate through anaerobic glycolysis), we observed no 
differences in levels of glucose, fructose 1,6-bisphosphate or pyruvate between normal KLS+ cells and CML-KLS+ cells 

(Fig. 1a; Supplementary Data 1). Adenosine 5’-monophosphate 

levels were slightly higher in CML-KLS+ cells than in normal 

KLS+ cells, but adenosine 5’-triphosphate was not measurable 

by this approach in either population.

When we determined levels of various individual amino acids, 

we observed no differences between normal KLS+ and 

CML-KLS+ cells (Fig. 1b; Supplementary Data 1). However, 

to our surprise, several dipeptide species were markedly increased in 

CML-KLS+ cells compared with normal KLS+ cells, whether 

the latter cells were isolated from healthy littermate (FVB/N) mice 

(Fig. 1c; Supplementary Fig. 1) or from healthy C57BL/6 control 
mice at 8 or 24 weeks of age (Supplementary Data 1). A 
calculation of the ratio of dipeptide levels in CML cells versus 

normal cells at each stage indicated that, compared with most 

Lin+ differentiated CML cells, it is the immature CML-KLS+ 

population that tends to have the largest dipeptide content 

(Fig. 1d). While dipeptides were also elevated in the CML-KLS- 

progenitor population, for reasons elaborated below, we believe 

that this increase was likely due to the increased protein turnover/ 
degradation that is required to support the vigorous proliferation of 

CML progenitors. Although we cannot exclude the 

possibility that there is a systemic increase in dipeptide species 
in the blood of tet-inducible CML-affected mice, we saw no 

obvious correlation between dipeptide levels in the rare 
population of CML-KLS+ cells and the vast majority of 
differentiated CML-Lin- cells (Supplementary Fig. 2). Thus, a 

mechanism(s) intrinsic to CML stem cells may contribute to their 

accumulation of dipeptide species in vivo. We then set out to 
determine why, unlike normal HSCs, quiescent CML stem cells 
store amino acids in dipeptide pools.
Most notable among these was the Slc15A2 gene encoding an oligo-/dipeptide transporter, which quantitative real-time RT–PCR analyses confirmed was highly expressed in LT–CML stem cells compared with not only CML–KLS− progenitors but also normal LT-HSCs (Fig. 2a; Supplementary Data 2).

To perform a functional analysis of whether Slc15A2 activity was in fact implicated in the observed dipeptide accumulation, we first incubated CML–KLS+ cells in vitro with [3H]-labelled glycolysarcosine (GlySar)21,22, which is a dipeptide analogue that cannot be metabolized and acts as a substrate of Slc15A family transporters. Interestingly, CML–KLS+ cells internalized much more [3H]GlySar than did normal KLS− cells, and this uptake was markedly decreased in the presence of the Slc15A2-specific chemical competitor cefadroxil23 (Fig. 2b). We next incubated CML–KLS+ cells with exogenous dipeptide (Ser–Leu) in vitro, followed by metabolomics analysis. Compared with control CML–KLS+ cells, CML–KLS+ cells incubated with Ser–Leu tended to show increased levels of both the Ser–Leu dipeptide and its hydrolysated component amino acids (Ser and Leu) (Fig. 2c).

Importantly, treatment with cefadroxil markedly suppressed these accumulations of Ser–Leu, Ser and Leu, indicating that, even though the internalized dipeptide was rapidly hydrolysod, CML stem cells cultured in vitro still possess intrinsic dipeptide transporter activity. We also evaluated the possibility that defective protein degradation might contribute to the dipeptide accumulation in CML stem cells. Treatment of these cells in vitro with Bortezomib (a 26S proteasome inhibitor) or Bafilomycin A1 (an autophagy inhibitor) tended to decrease individual amino-acid levels (Supplementary Fig. 4). However, in these same cells, treatment with the inhibitors induced only one instance of statistically significant dipeptide accumulation (Supplementary Fig. 5). Thus, a defect in proteasomal degradation or autophagy does not appear to be the major cause of dipeptide accumulation in CML stem cells.

On the basis of these in vitro results, we examined whether cefadroxil could attenuate dipeptide internalization by CML stem cells in vivo. CML-affected mice received oral administration of cefadroxil for 30 days, followed by metabolomics analysis of CML stem cells to measure intracellular dipeptides. Intriguingly, exposure of mice to cefadroxil decreased levels of several dipeptides in immature CML–KLS+ cells, implying impaired uptake of these dipeptide species (Fig. 2d). Combined with our in vitro data, these in vivo results implicate Slc15A2 transporter activity as a major driver of dipeptide accumulation in CML stem cells.

To understand the biological role of dipeptide uptake in CML stem cells, we evaluated how inhibition of dipeptide transporter function affected CML stem cell activity in vitro. Lentiviral transduction of short hairpin RNAs (shRNAs) targeting Slc15A2 messenger RNA (mRNA) also decreased the colony-forming capacity of CML–KLS+ cells but not that of CML–KLS− cells (Fig. 2e). These data suggest that dipeptide uptake through the Slc15A2 transporter maintains CML stem cell activity in vitro.

| CML/normal ratios | KLS+ immature | KLS− progenitor | Lin+ differentiated |
|-------------------|--------------|----------------|--------------------|
| Ala-Leu           | 38.19*       | 3.57*          | 1.30               |
| Ala-Phe           | 25.65*       | 2.26           | 1.41               |
| Ala-Tyr           | 3.15         | 1.45           | 1.00               |
| Ala-Val           | 26.80        | 5.00           | 1.68               |
| Arg-Ala           | 2.44         | 1.85           | 0.33               |
| Asp-Leu           | 5.23*        | (0.05)*        | 2.16               |
| Gin-Leu           | 17.92*       | 4.34           | 2.99               |
| Gly-Leu           | 14.42        | 5.60           | 3.72*              |
| Gly-Phe           | 6.32*        | 8.94*          | 2.21               |
| Ile-Ala           | 10.62*       | 16.58          | 2.52*              |
| Ile-Gln           | 7.23*        | 2.27           | 1.35               |
| Ile-Tyr           | 1.61         | 9.99           | 7.22               |
| Ile-Val           | 10.73*       | 19.28          | 1.61               |
| Leu-Ala           | 50.34*       | 5.69           | 1.26               |
| Leu-Glu           | 6.20*        | 1.98           | 1.54               |
| Leu-Phe           | 1.23         | 2.92*          | 0.79               |
| Leu-Ser           | 18.14*       | 3.34           | 1.84               |
| Leu-Tyr           | 1.88         | 0.99           | 0.89               |
| Phe-Ala           | 7.14*        | 6.36           | 1.92               |
| Phe-Val           | 13.81*       | 6.23           | 1.57*              |
| Ser-Leu           | 10.58        | 4.32           | 3.91               |
| Ser-Phe           | 11.05*       | 7.11*          | 1.31               |
| Ser-Tyr           | 6.87*        | 2.92*          | 2.16               |
| Thr-Phe           | 1.50         | 6.71*          | 2.81               |
| Thr-Val           | 32.43*       | 10.98          | 1.84*              |
| Val-Leu           | 21.58*       | 3.18*          | 1.46               |
| Val-Phe           | 3.33*        | 2.12           | 0.99               |
| Val-Val           | 2.54*        | 4.69*          | 1.08               |
To identify the pathway mediating intracellular nutrient signalling associated with dipeptide uptake, we first investigated whether treatment in vitro of LT-CML stem cells with GlySar or cefadroxil affected signalling via the mTORC1 pathway. We exposed LT-CML stem cells to 5 μM GlySar or 5 μM cefadroxil for 30 min and used the highly sensitive Duolink in situ proximity ligation assay (D-PLA) to evaluate both phosphorylated Raptor-Ser863 and phosphorylated S6 ribosomal protein, which indicate mTORC1 activation. As expected, we found that untreated control LT-CML stem cells exhibited both phospho-Raptor-Ser863 and phospho-S6 (Supplementary Fig. 6). However, after treatment with GlySar or cefadroxil, LT-CML stem cells displayed decreased phosphorylation of Raptor-Ser863 and S6, a result mimicked by treatment with the mTORC1 inhibitor rapamycin (Supplementary Fig. 6). These data indicate that interference with Slc15A2-mediated dipeptide uptake, either by non-metabolizable analogue or chemical competitor, attenuates mTORC1-mediated nutrient signalling in LT-CML stem cells.

AMPK becomes phosphorylated in cells experiencing low energy or nutrient starvation conditions, leading to suppression of the downstream mTORC1 pathway. Treatment of LT-CML stem cells with Metformin, a known activator of AMPK, increases the phosphorylation of both AMPK and Raptor-Ser792, and phospho-Raptor-Ser792 suppresses mTORC1 activity. However, although our treatment of LT-CML stem cells with GlySar or cefadroxil increased phospho-AMPK, these agents did not promote Raptor-Ser792 phosphorylation (Supplementary Fig. 7). Thus, the AMPK is dispensable for the suppression of the mTORC1 pathway seen in LT-CML stem cells experiencing inhibition of Slc15A2-mediated dipeptide uptake.

Smad3–Ser208 phosphorylation supports LT-CML stem cells. Although we found that dipeptides were able to influence nutrient signalling via the mTORC1 pathway, it has been reported that rapamycin treatment does not prolong the survival of CML-affected mice, suggesting that mTORC1 signalling is not crucial for the maintenance of CML stem cells. Because the TGF-β/FOXO–BCL6 signalling pathway is essential for CML stem cell maintenance in vivo, we speculated that there might be a connection between this axis and dipeptide-mediated nutrient signalling that could promote CML stem cell activity in vivo.
To identify the key molecule responsible for the potential cross-talk between nutrient signalling and the TGF-β–FOXO axis, and thereby for CML stemness, we investigated whether Smad2/3, the downstream effectors of TGF-β signalling, were implicated in CML stem cell activity. We found that both Smad2 and Smad3 were phosphorylated at the relevant C-terminal sites in freshly purified LT-CML stem cells (Fig. 3a, top). However, D-PLA analysis revealed that only Smad3 interacted with Foxo3a in these cells, consistent with a previous report\(^\text{13}\) (Fig. 3a, bottom and Fig. 3b). These results suggested that Smad3 might be involved in the TGF-β–FOXO signalling cascade responsible for CML stem cell maintenance. Because Smad3 is a known stemness transcription factor\(^\text{16,34}\), it has been of great interest to determine whether Smad3 promotes the maintenance of CML cell stemness.

We next examined Smad3 phosphorylation sites in freshly purified LT-CML stem cells. Whereas D-PLA detected total phosphorylation of Smad3 at Thr179, Ser204, Ser208, Ser213 and Ser423/425 in TGF-β-treated LT-CML stem cells, as expected (Supplementary Fig. 8), freshly purified LT-CML stem cells showed canonical Smad3 phosphorylation at Ser423/425 and non-canonical phosphorylation only at Ser208 (Fig. 3c,d). Interestingly, although Smad3–Ser423/425 was also phosphorylated in ST-CML stem cells and in CD48\(^+\), multipotent progenitor (MPP; CD135\(^+\) KL\(^+\)) and KL\(^-\) CML cells, Smad3–Ser208...
phosphorylation was unique to the most primitive LT-CML stem cells, as was Smad3–Foxo3a interaction (Fig. 3e–g). These data suggest that phosphorylation of Smad3 at Ser208 may allow LT-CML stem cells to activate Foxo3a, whose transcriptional activity supports CML stem cell maintenance in vivo.

To determine the biological relevance of Smad3 phosphorylation at Ser423/425 and Ser208, we took advantage of two mutant forms of human Smad3 that cannot be phosphorylated: Smad3–3SA, in which Ser423/425 are all converted to Ala, and Smad3–S208A, in which Ser208 is converted to Ala. We infected CML-KLS cells with retroviral vectors expressing either control green fluorescent protein (GFP), Smad3–3SA wild-type (WT), Smad3–3SA or Smad3–S208A, and transplanted these cells into recipient mice (Supplementary Fig. 9). CML stem cell maintenance in vivo was then evaluated by flow cytometry. By 30 days post transplantation, the Smad3 mutations had not affected the size of the GFP+ (Smad3+) CML-KLS+ cell population (Fig. 3h). However, we found a striking decrease in the frequency of the most primitive LT-CML stem cells in recipients transplanted with CML-KLS cells expressing Smad3–S208A (Fig. 3i). Thus, inhibition of non-canonical Smad3–Ser208 phosphorylation in LT-CML stem cells impairs their maintenance in vivo.

Targetting of dipeptide signalling eradicates CML stem cells. We next investigated whether treatment with cefadroxil inhibits the function of CML stem cells and normal HSCs in vitro. Treatment of isolated LT-CML stem cells with cefadroxil significantly reduced their colony-forming capacity (Fig. 5c). To confirm that cefadroxil does not alter the function of normal HSCs, we employed a well-established competitive reconstitution assay.42 Irradiated CD45.2 recipient mice were co-transplanted with 1 × 10^5 unfractionated BM mononuclear cells (MNCs) from healthy CD45.2 mice. These animals then received daily administration of cefadroxil or vehicle for 12 weeks post transplantation. Importantly, there was no decrease in the frequency of donor-derived CD45.1 MNCs in peripheral blood of recipients after 4, 8 or 12 weeks of cefadroxil administration (Fig. 5d,e). Concomitantly, there was a comparable increase in the degree of chimerism originating from donor-derived normal KLS+ cells, whether or not cefadroxil was present (Fig. 5d). Thus, administration of cefadroxil in vivo has no detectable effect on the reconstitutive powers of normal HSCs.

The specificity of dipeptide-induced nutrient signalling to LT-CML stem cells prompted us to investigate whether this pathway might be a possible therapeutic target; that is, whether disruption of dipeptide internalization might lead to the eradication of CML stem cells and a reduction in disease relapse. First, to determine whether the SLC15A2 gene is upregulated in human CML patients as it is in CML-affected mice, we retrieved data on levels of cathepsin K in a mouse model of CML.

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of SLC15A2 mRNA in cells of CML patients listed in a public database gene expression omnibus (GEO: GSE33075). Intriguingly, prior to IM therapy, SLC15A2 mRNA levels were indeed higher in BM leukaemia cells of nine CML patients than in the BM haematopoietic cells of nine healthy individuals (Fig. 6a). However, after IM therapy, SLC15A2 mRNA levels in the same nine CML patients had decreased to a level comparable to that in healthy individuals. To further explore this finding, we returned to our mouse model and compared dipeptide levels in CML-KLS+ cells isolated from CML-affected mice that had received vehicle or IM therapy for 1 month. In line with our observations in human CML patients, metabolomic analysis of these mice indicated that IM treatment tended to decrease dipeptide levels in CML-KLS+ cells (Fig. 6b). In addition, IM treatment of murine LT-CML stem cells in vitro reduced levels of phospho-p38MAPK and phospho-Smad3–Ser208 (Supplementary Fig. 11). These results suggest that the accumulation of dipeptide species may not be the direct cause of TKI-resistance in the CML stem cell population responsible for disease recurrence. However, our findings also suggest that the SLC15A2-mediated nutrient supply we have identified plays a critical role in human CML leukaemogenesis.

However, we cannot exclude the possibility that an unidentified minor population of CML stem cells that is responsible for IM resistance accumulates high levels of dipeptides, and that it is this minor population of CML stem cells that is responsible for IM resistance. Further studies are needed to confirm this hypothesis.

In conclusion, our findings suggest that dipeptide-mediated nutrient signalling plays a critical role in CML stem cell proliferation and survival. Understanding the mechanisms underlying dipeptide accumulation and their role in CML pathogenesis could lead to the development of novel therapeutic strategies for the treatment of this disease.
administration of cefadroxil alone appeared to promote disease development (Fig. 6d, orange line). However, the combined administration of IM plus cefadroxil significantly reduced the recurrence rate of BCR-ABL1+ disease compared with the group treated with IM alone (Fig. 6d, red line).

We then determined whether cefadroxil administration could in fact eradicate primitive CML stem cells in CML-affected mice in vivo. Indeed, the frequency of CML-KLS+ cells among GFP/BCR-ABL1+ CML cells isolated from BM of CML-affected mice was significantly decreased by cefadroxil exposure in vivo (Fig. 6e; Supplementary Fig. 12). Although IM alone also reduced the frequency of CML-KLS+ cells, the combined administration of IM plus cefadroxil had a much greater repressive effect on this population (Fig. 6e; Supplementary Fig. 12). Notably, in serial transplantation experiments, CML-KLS+ cells isolated from cefadroxil-treated CML-affected mice completely lost their ability to drive BCR-ABL1+ disease in new recipients, allowing the animals to survive for over 90 days (Fig. 6f). In contrast, all mice that received CML-KLS+ cells from vehicle-treated CML-affected animals developed BCR-ABL1+ disease and were dead before 80 days, demonstrating that the untreated CML-KLS+ cells had retained their CML-initiating ability. These results indicate that oral administration of cefadroxil to inhibit dipeptide uptake may block nutrient signalling important for the maintenance of CML stem cells in vivo, and further suggest that cefadroxil used in combination with a TKI can improve the survival of CML-affected mice by eradicating CML stem cells.

To investigate the relevance of our findings to human CML therapy, we evaluated the effects of cefadroxil treatment in vitro on CML-LICs obtained from human chronic phase CML patients. We isolated CD34+CD38-Lin- CML-LICs from BM MNCs of three CML patients and treated these cells in vitro with cefadroxil. As expected, cefadroxil suppressed the colony-forming capacity of all three human CML-LIC samples in vitro (Fig. 6g). The co-treatment of human CML-LICs with a combination of a TKI (IM or dasatinib) plus cefadroxil reduced colony formation over the suppressive effect of the TKI alone (Fig. 6h).

Finally, in the same vein, we evaluated whether combined treatment of IM plus one of three clinical grade p38MAPK inhibitors could suppress LT-CML stem cell colony formation in vitro. The p38MAPK inhibitors Ly2228820 (ralimetinib)43, VX-702 (ref. 44) and BIRB796 (doramapimod)45 have already been approved by the FDA (USA) as candidate anti-inflammatory or anti-cancer drugs for patients with rheumatoid arthritis, peripheral artery disease or ovarian cancer. When we treated LT-CML stem cells in vitro with any of these drugs, their colony-forming capacity was significantly decreased (Fig. 7a). The
colonies of healthy donors and nine CML patients pre- and post IM therapy. Data are from a public database (GEO, GSE33075) (P value, one-sided t-test). (b) Metabolomic analyses of dipeptides in CML-KLS cells from CML-affected mice that received either vehicle (male, n = 2; female, n = 4; three experiments) or IM (100 mg kg⁻¹ per day; male, n = 2; female, n = 6; three experiments) for 30 days. Amounts of dipeptides were plotted in whisker boxes. Cross, mean value; horizontal line across box, median value; error bars, maximum and minimum of distribution (P value, vehicle versus Cefa; Welch’s t-test). (c) Quantification of colony-forming capacity of LT-CML stem cells (3% O₂) with either vehicle (−) or 5 μM Cefa (+) for 5 days in the absence or presence of 1 μM IM. Data are the mean colony number ± s.d. (n = 3) and are representative of three experiments (P value, Student’s t-test). (d) Survival rates of CML-affected mice (female) that received vehicle, IM (100 mg kg⁻¹ per day) and/or Cefa (36 mg kg⁻¹ per day) for days 8–90 post transplantation. Results shown are cumulative data obtained from three independent experiments. Statistical differences were determined using the log-rank non-parametric test. (e,f) Eradication of CML stem cells in vivo by cefadroxil. CML-affected mice received vehicle (−), IM and/or Cefa (+) daily for 30 days post transplantation as in (d). (f) Mean frequency ± s.d. of GFP/BCR-ABL1⁺ CML-KLS⁺ cells among total GFP/BCR-ABL1⁺ CML cells (n = 3; P value, Student’s t-test). (f) Survival rate of recipient mice (female) that received serial transplantation of GFP/BCR-ABL1⁺ CML-KLS⁺ cells (3 × 10⁶ cells) from the CML-affected mice in (e) that had been treated with vehicle (female, n = 4) or Cefa (female, n = 5). Mouse survival was monitored for 90 days (P value, log-rank non-parametric test). (g,h) Quantification of colony-forming capacity of human CD34⁺ CD38⁻ Lin⁻ CML-LICs that were treated in vitro with either vehicle (−) or 5 μM Cefa (+) for 5 days, or with vehicle (−) or 5 μM Cefa (+) in the absence or presence of 1 μM IM or 500 nM dasatinib (Dasa) for 3 days. Data shown are the mean colony number ± s.d. (n = 3) (P value, Student’s t-test).

Figure 6 | Inhibition of dipeptide uptake in combination with TKI therapy eradicates CML stem cells in vivo. (a) Relative SLCT15A2 mRNA expression in human CML patients as determined by microarray analysis of nine healthy donors and nine CML patients pre- and post IM therapy. Data are from a public database (GEO, GSE33075) (P value, one-sided t-test). (b) Metabolomic analyses of dipeptides in CML-KLS cells from CML-affected mice that received either vehicle (male, n = 2; female, n = 4; three experiments) or IM (100 mg kg⁻¹ per day; male, n = 2; female, n = 6; three experiments) for 30 days. Amounts of dipeptides were plotted in whisker boxes. Cross, mean value; horizontal line across box, median value; error bars, maximum and minimum of distribution (P value, vehicle versus Cefa; Welch’s t-test). (c) Quantification of colony-forming capacity of LT-CML stem cells (3% O₂) with either vehicle (−) or 5 μM Cefa (+) for 5 days in the absence or presence of 1 μM IM. Data are the mean colony number ± s.d. (n = 3) and are representative of three experiments (P value, Student’s t-test). (d) Survival rates of CML-affected mice (female) that received vehicle, IM (100 mg kg⁻¹ per day) and/or Cefa (36 mg kg⁻¹ per day) for days 8–90 post transplantation. Results shown are cumulative data obtained from three independent experiments. Statistical differences were determined using the log-rank non-parametric test. (e,f) Eradication of CML stem cells in vivo by cefadroxil. CML-affected mice received vehicle (−), IM and/or Cefa (+) daily for 30 days post transplantation as in (d). (e) Mean frequency ± s.d. of GFP/BCR-ABL1⁺ CML-KLS⁺ cells among total GFP/BCR-ABL1⁺ CML cells (n = 3; P value, Student’s t-test). (f) Survival rate of recipient mice (female) that received serial transplantation of GFP/BCR-ABL1⁺ CML-KLS⁺ cells (3 × 10⁶ cells) from the CML-affected mice in (e) that had been treated with vehicle (female, n = 4) or Cefa (female, n = 5). Mouse survival was monitored for 90 days (P value, log-rank non-parametric test). (g,h) Quantification of colony-forming capacity of human CD34⁺ CD38⁻ Lin⁻ CML-LICs that were treated in vitro with either vehicle (−) or 5 μM Cefa (+) for 5 days, or with vehicle (−) or 5 μM Cefa (+) in the absence or presence of 1 μM IM or 500 nM dasatinib (Dasa) for 3 days. Data shown are the mean colony number ± s.d. (n = 3) (P value, Student’s t-test).

Discussion
Although recent technical advances have opened up new ways of investigating metabolites in proliferating mature cancer cells, the metabolic pathways allowing rare cancer stem cells to survive when mature cancer cells cannot are still unknown. In this study, we found that CML stem cells effectively employ dipeptide uptake mediated by Slc15A2 activity to guarantee an alternative nutrient supply and maintain LT survival in vivo.

The results of our study suggest the following model (Fig. 8): (1) CML stem cells accumulate dipeptide species through the Slc15A2 dipeptide transporter; (2) these internalized dipeptides furnish a
nutrient source that leads to the activation of nutrient signalling, including signalling via p38MAPK; (3) p38MAPK-mediated non-canonical phosphorylation of Smad3–Ser208 supports Foxo3a’s known function in supporting CML stem cell activity in vivo. Because normal HSCs do not use this dipeptide-induced nutrient signalling pathway, therapeutic suppression of this novel mechanism should not affect normal HSCs. However, it remains unclear how CML stem cells sense the internalized dipeptides, store them intracellularly and use them as an amino-acid source. For example, protein degradation and/or autophagy pathways may generate dipeptides en route to building up supplies of recycled amino acids. It should also be noted that, although we could not detect an obvious increase in amino acids in CML-KLS cells, including in branched-chain amino acids such as Leu (Fig. 1b; Supplementary Data 1), we cannot exclude the possibility that the downstream nutrient signalling induced by dipeptide uptake includes regulation of the mTORC1 pathway by the cell's amino-acid-sensing machinery\textsuperscript{16–31}. Finally, we should clarify how systemic dipeptide levels in the blood affect CML stem cell activity in vivo. Further investigation should reveal precisely how CML stem cells use dipeptides to ensure LT-CML stem cell maintenance in vivo.

To our knowledge, our study is the first to demonstrate that CML stem cell activity depends on nutrient signalling that regulates post-translational phosphorylation of Smad3 at Ser208. Furthermore, we have shown that dipeptide-induced p38MAPK activation is responsible for this Smad3–Ser208 phosphorylation, and that Foxo3a binds specifically to Smad3 in LT-CML stem cells. Foxo3a is known to cooperate with Smad3 in regulating transcription\textsuperscript{33} and to be required for CML stem cell maintenance\textsuperscript{15,32}. It has also been reported that p38MAPK interacts with Foxo3a and regulates its function\textsuperscript{32}, suggesting a link between the p38MAPK/Smad3–Ser208 axis activated by nutrient signalling and the Foxo3a-mediated pathway supporting CML stem cell activity. Once again, future work should determine how Smad3 interacts with Foxo3a, how phospho-Smad3–Ser208 regulates Foxo3a’s activity and whether this interaction regulates the maintenance of CML stemness in vivo.

Our results also reveal that the antibiotic cefadroxil attenuates dipeptide uptake by CML stem cells and so may be a promising partner in combination therapy with TKIs. However, we still do not understand how dipeptides act as a nutrient source for specific cell types, or how systemic dipeptide distribution contributes to normal health. Indeed, administration of cefadroxil...
in the absence of a TKI appeared to accelerate disease development in CML-affected mice. Thus, any application of transportation inhibitors to CML patients must be carefully considered. Nevertheless, we observed elevated dipeptide uptake only in CML stem cells and not in normal HSCs, and cefadroxil combined with TKI decreased dipeptide uptake and survival of CML stem cells but did not affect normal HSCs (Figs 6e,f and 5d,e). Thus, therapeutic inhibition of dipeptide-induced nutrient signalling may eradicate LT-CML stem cells in vivo with minimal side effects on normal haematopoiesis. Cefadroxil (Duricef; approved by FDA, USA) has long been safely used as a nutrient source specific to CML stem cells, in combination with approved by FDA, USA) has long been safely used as a side effects on normal haematopoiesis. Cefadroxil (Duricef; approved by FDA, USA) has long been safely used as a

**Methods**

**CML mouse models.** Several different mouse models of CML-like disease were employed in this study. First, we used a well-described tetracycline (tet)-inducible CML mouse model8–10,18. Tal1-TTA mice (JAX, #006209)15 and TRE-BCR-ABL1 transgenic mice (JAX, #006207)17, both of the FVB/N genetic background, were purchased from the Jackson Laboratory. Tal1-TTA and TRE-BCR-ABL1 transgenic mice were interbred to generate TRE-BCR-ABL1 double-transgenic mice. These animals were maintained in cages supplied with drinking water containing 20 mg ml⁻¹ DOX (Sigma-Aldrich). At 5–8 weeks after birth, expression of the BCR-ABL oncogene was induced by replacing the DOX-containing drinking water with normal drinking water. Consistent with a previous report8, CML-like disease developed in these recipients by 12–20 days post transplantation. To examine the in vivo effects of the combined administration of IM plus cefadroxil, BCR-ABL1-CML-affected mice received vehicle alone (artificial gastric fluid solution (900 ml ddH₂O containing 2.0 g NaCl, 7 ml conc. HCl and 3.2 g pepsin)), or IM (Gleevec; 100 mg kg⁻¹ per day; Novartis) and/or cefadroxil (36 mg kg⁻¹ per day; Sigma-Aldrich) in vehicle. Treatment was delivered by oral gavage on days 8–9 post transplantation. To examine the effect of Ly2282880 administration alone, BCR-ABL1-CML-affected mice received vehicle alone or Ly2282880 (2.5 mg kg⁻¹ every 3rd day; Axon Medchem) in vehicle, by oral gavage on days 8–60 days post transplantation. To examine the effect of combined administration of dasatinib plus Ly2282880, tet-inducible CML-affected mice received vehicle alone, or dasatinib (5 mg kg⁻¹ per day; Brystol-Meyers Squibb) in vehicle on days 0–30 post DOX withdrawal, and/or Ly2282880 (2.5 mg kg⁻¹ every 3rd day; Axon Medchem) in vehicle on days 7–28 post DOX withdrawal by oral gavage. All animal care in our laboratory was in accordance with the guidelines for animal and recombinant DNA experiments of Kanazawa University.

**Cell sorting.** BM MNCs were isolated from the two hindlimbs of 8–12-week-old (male and female) tet-inducible CML-affected mice (Tal1-TTA & TRE-BCR-ABL1) and 8–12-week-old (male and female) normal healthy littermate mice (described above) (Supplementary Data 1, study 1). We also determined metabolites in 1.0–1.8 × 10⁶ immature KLS⁺ cells isolated from 8-week-old (male and female) C57BL6 congenic (CD45.1) mice (female). For retroviral and lentiviral transductions (see below), KLS⁺ cells and KLS⁻ cells were purified from BM MNCs of 8–12-week-old tet-inducible CML-affected mice (male and female). For HSC-competitive reconstitution assays (see below), normal KLS⁺ cells were purified from BM MNCs of 6-week-old C57BL6 congenic (CD45.1) mice (female). To serial transplantation of CML stem cells (see below), total BM MNCs isolated from the two hindlimbs of BCR-ABL1-CML-affected mice were immunostained with anti-Sca-1(E13–161.7)-PE, anti-CD4(L3T4)-biotin, anti-Gr-1(RB6–8C5)-FITC, and anti-Mac1(M1/70)-FITC (BD Biosciences), visualized with Streptavidin-PE-Cy7 (BD Biosciences). GFP/BCR-ABL1 expressing cells were purified using a FACS Aria III cell sorter (BD Biosciences). For retroviral and lentiviral transductions (see below), KLS⁺ cells and KLS⁻ cells were purified from BM MNCs of the three independent experiments; male and female) or cefadroxil (36 mg kg⁻¹ per day; Sigma-Aldrich) in vehicle. Treatment was delivered by oral gavage for 30 days. For in situ PLA analysis, we plated 1.8–2.0 × 10⁵ cells, KLS⁺-differentiated cells from 8- to 10-week-old CML-affected mice (Tal1-TTA & TRE-BCR-ABL1) on 8–12-week-old normal healthy littermate control (Tal1-TTA) mice (n = 6 mice in each of the two independent experiments; male and female) and 8- to 10-week-old normal healthy littermate control (Tal1-TTA) mice (n = 6 mice in each of the two independent experiments; male and female) and 8- to 10-week-old normal healthy littermate control (Tal1-TTA) mice (n = 6 mice in each of the two independent experiments; male and female) according to a published classification (Lineage + Kit + Sca-1⁻ cells, progenitor KLS⁻ cells (Kit + Lineage + Sca-1⁻ cells) and differentiated Lin⁻ cells according to Supplementary system15. For next-generation RNA sequencing and Duolink in situ PLA analysis (see below), we further purified KLS⁺ cells into the most primitive LT stem cells (CD50⁻/CD135⁺ + CD48⁻/CD135⁻ KLS⁺), ST stem cells (CD50⁻/CD135⁻ KLS⁻), CD48⁺ cells (CD48⁺/CD135⁻/+ KLS⁺) and multipotent progenitor-like (MPP) cells (CD135⁻ KLS⁻)5, as indicated in Supplementary Fig. 3. For retroviral and lentiviral transductions (see below), KLS⁺ cells and KLS⁻ KLS⁺ cells were purified from BM MNCs of the three independent experiments; male and female) or cefadroxil (36 mg kg⁻¹ per day; Sigma-Aldrich) in vehicle. Treatment was delivered by oral gavage for 30 days. 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the radioactivity of [3H]GlySar internalized by cells was measured using a liquid cefadroxil (Sigma-Aldrich) to initiate the transporter reaction. After 60 or 120 min, using Nanodrop (Thermo Fisher Scientific) and an Agilent 2100 Bioanalyzer (Nippon Gene) solution. RNA extraction and sequencing were performed by a mouse genome reference (Mus musculus; mm9, NCBI Build 37) using SeqNova CS by NAnexus Inc. (Mountain View, CA) (https://danexus.com). The next-generation RNA-sequencing data for LT-HSCs and LT-CML stem cells were available from a public database gene expression omnibus (GEO, ID: GSE70031) in NCBI, NIH, USA (http://www.ncbi.nlm.nih.gov/geo/info/Linking.html) (Supplementary Data 2).

Quantitative real-time RT-PCR analysis. Using the RT-PCR kit (Qiagen), RNA samples were purified from 4 to 5 x 10^6 LT stem cells, ST stem cells, CD48^+ KLS cells, MPP and KLS^+ progenitor cells (as defined in Supplementary Fig. 3) isolated from 8 to 10-week-old tet-inducible CML-affect ed (Tg1-T1a^+ TRE-B2 CRABL1^+ mouse and female) mice (n = 6) and 8 to 10-week-old littermate control (Tg1-T1a^- male and female) mice (n = 8) at 5 weeks post DOX withdrawal. RNA samples were reverse transcribed using the Advantage RT-for–PCR kit (Takara Biotechnology). Real-time quantitative PCR was performed using SYBR green Premix EX Taq (Takara) on an Mx3000P Real-Time PCR system (Strategene). The following primers were used: 5'-GCATGCCTCTGGGTGGGTTG-3' and 5'-GGGCGGTGCTGAAAGGATGATG-3' for Pept2 (Sclc15A2), 5'-AGGTCATCTACATTGCAAGCA-3' and 5'-CACCTGATGAAGGATGATGATG-3' for Actb (B2-actin). The reactions were performed in triplicates and data were analyzed and normalized to Actb as the housekeeping gene.

Sclc15A2 transporter activity. Sclc15A2 transporter activity was determined using a well-established assay measuring [3H]GlySar uptake by cells suspended in an acidic transport medium (pH 6.0)^21,22. [3H]GlySar (28.3 μCi/mL, MT-1545) was purchased from Radiochemicals (Perkin-Elmer). Briefly, normal LT-CML stem cells or MPP–KLS^+ cells (1 x 10^6) in 10 μL of SF-03 stem cell medium were suspended in 310 μL [3H]GlySar (178.57 mM−1) transport medium (125 mM NaCl, 4.8 mM KCl, 5.6 mM δ-glucose, 1.2 mM CaCl2–2H2O, 1.2 mM KH2PO4, 1.2 mM MgSO4–7H2O and 25 mM MES, pH 6.0) in the absence or presence of 100 mM cefadroxil (Sigma–Aldrich) to initiate the transporter reaction. After 60 or 120 min, the radioactivity of [3H]GlySar internalized by cells was measured using a liquid scintillation counter.

To examine the uptake of exogenous dipeptide, CML-KLS^+ cells (1 x 10^6) were incubated with 1 μM Ser–Leu and 1 μM Ser–Gly (Roche). The cells were washed and centrifuged to remove unbound ligand, and the cell pellets were immediately fixed with 4% paraformaldehyde for 30 min. For in vitro experiments, LT-CML stem cells were incubated in 3% O2 at 37°C for 30min with the appropriate vehicle (control), 5 μM Li364947 (TGF-β type I receptor kinase ALK5 inhibitor; Merck), 5 μM SB203580 (p38MAPK inhibitor; LC Laboratories), 5 μM GlySar (non-metabolizable dipeptide transporter substrate; Sigma–Aldrich), 5 μM cefadroxil (antibiotic dipeptide transporter competitor; Sigma–Aldrich), 100 nM rnapacycin (mTORC1 inhibitor; Cell Signaling Technologies), 10 μM metformin (AMPK activator; TOCRIS Bioscience) or 300 μM flavopiridol (CDK9 inhibitor; Sigma–Aldrich). Treated cells were fixed with 4% paraformaldehyde for 30 min. In all cases, fixed cells were permeabilized with 0.25% Triton X-100 for 15 min, washed and blocked by incubation in 5% FBS in TBS for 1 h. Blocked cells were incubated overnight at 4°C with the combinations of Abs indicated in Supplementary Table 1. Primary mouse antibody was used at a 1/25 dilution, primary rabbit antibody at 1/50 and primary goat antibody at 1/50. The proximal binding Abs were then detected using these Dylight Abs. Slides were mounted using Duolink in situ mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich), and fluorescent images were acquired by confocal microscopy (FV10i, Olympus) and Photoshop software (Adobe). The number of fluorescent foci per single cell was quantified using Duolink Image Tool software (Olink Bioscience).

As positive and negative controls for Smd3 phospho, we treated LT-CML stem cells in vitro for 30 min in 3% O2 with TGF-β1 (1 ng/mL; R&D Systems) or Ly364947 (5 μM; Merck), respectively. As a negative control for mTORC1 activation, LT-CML stem cells were treated in vitro under the above conditions with rapamycin (100 μM; Cell Signaling Technologies). As a positive control for AMPK activation, LT-CML stem cells were treated in vitro under the above conditions with Metformin (10 μM; Cell Signaling Technologies). As indicated in Fig. 4a and Supplementary Figs 6–8, the appropriate fluorescent foci were detected (or not) in these control experiments. As a technical negative control for D-PLA, we treated LT-CML stem cells with a mouse anti-phospho Ser208 antibody (eBioscience) and confirmed that no fluorescent foci could be detected, as indicated in Fig. 3a–d. As a technical negative control for interaction between Foxo3a and phospho-Smad3–Ser208, we used
Fnox3a<sup>−/−</sup> LT-CML stem cells and confirmed that no fluorescent foci could be detected, as indicated in Fig. 4h.i.

**Colonizing assays.** LT-CML stem cells or LT-Normal HSCs (1 × 10<sup>4</sup>) were co-cultured on OP-9 stromal cells in 3% O2 at 37 °C for 5 days in the presence of either vehicle (control), the dipeptidyl transporter competitor cefadroxil (5 μM), or the p38MAPK inhibitors Ly2228820 (5 μM; Santacruz), VX-702 (5 μM; Santacruz) or BIRB796 (5 μM; Calbiochem). Cells were harvested, washed in PBS and plated in semi-solid methylcellulose containing SCF, interleukin (IL)-3, IL-6 and erythropoietin (Methocult GF M3434; Stem Cell Technologies). After growth for 7 days under hypoxic (3% O2) condition at 37 °C, colony numbers were counted under a light microscope.

For combination treatments of dipeptidyl transporter inhibitor or p38MAPK inhibitor plus TKI, LT-CML stem cells (3 × 10<sup>4</sup>) were plated on OP-9 stromal cells in presence of 0.5 μM BCR-ABL1<sup>-/-</sup> cDNA lentiviral vector (6 mg/μL) alone, or with a combination of Ly2228820 plus IM (1 μM; Axon Medchem) or dasatinib (500 nM; LC laboratories) or with a combination of Ly2228820 plus IM (1 μM; Axon Medchem) and dasatinib (500 nM; LC laboratories). CD3<sup>+</sup> CD34<sup>+</sup> Lin<sup>−</sup> cells were cultured on OP-9 stromal cells under hypoxic (3% O2) conditions. To determine the effects of treatment with cefadroxil (5 μM) alone, or with a combination of cefadroxil plus IM (1 μM; Axon Medchem) or dasatinib (500 nM; LC laboratories), CD3<sup>+</sup> CD34<sup>+</sup> Lin<sup>−</sup> cells were cultured on OP-9 stromal cells under hypoxic (3% O2) conditions. After harvesting in PBS, the colony-forming ability of primitive human CML-LICs was evaluated by culture in semi-solid methylcellulose medium containing SCF, granulocyte–macrophage colony-stimulating factor (CSF), IL-3, IL-6, granulocyte CSF and erythropoietin (Methocult GF <sup>H4</sup>H4435; Stem Cell Technologies). After growth for 7 days at 37 °C under hypoxic (3% O2) conditions, colony numbers were counted under a light microscope.

**Statistical analyses.** Statistical differences were determined using the unpaired Student’s t-test, one-tailed t-test and Welch’s t-test for P values, and a log-rank non-parametric test for survival curves.

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Author contributions

K.N. designed and performed experiments, analysed data and wrote the paper. Y.I., K.L., E.-J.B., H.O. and M.O. performed experiments. J.K. performed bioinformatic analyses. T.I. and Y.K. performed transporter experiments. R.P.M. and S.M.S. performed metabolomics analyses. H.N. provided essential mouse strains. D.-W.K. and Y.T. supervised the research. A.O. and S.-J.K. designed experiments and wrote the paper.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/nc

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