The lysophospholipase D enzyme Gdpd3 is required to maintain chronic myelogenous leukaemia stem cells

Kazuhito Naka✉1, Ryosuke Ochiai2, Eriko Matsubara2, Chie Kondo2, Kyung-Min Yang3, Takayuki Hoshii4, Masatake Araki5, Kimi Araki5, Yusuke Sotomaru6, Ko Sasaki7, Kinuko Mitani7, Dong-Wook Kim8, Akira Ooshima3 & Seong-Jin Kim3,9,10

Although advanced lipidomics technology facilitates quantitation of intracellular lipid components, little is known about the regulation of lipid metabolism in cancer cells. Here, we show that disruption of the Gdpd3 gene encoding a lysophospholipase D enzyme significantly decreased self-renewal capacity in murine chronic myelogenous leukaemia (CML) stem cells in vivo. Sophisticated lipidomics analyses revealed that Gdpd3 deficiency reduced levels of certain lysophosphatidic acids (LPAs) and lipid mediators in CML cells. Loss of Gdpd3 also activated AKT/mTORC1 signalling and cell cycle progression while suppressing Foxo3a/β-catenin interaction within CML stem cell nuclei. Strikingly, CML stem cells carrying a hypomorphic mutation of Lgr4/Gpr48, which encodes a leucine-rich repeat (LRR)-containing G-protein coupled receptor (GPCR) acting downstream of Gdpd3, displayed inadequate disease-initiating capacity in vivo. Our data showing that lysophospholipid metabolism is required for CML stem cell maintenance in vivo establish a new, biologically significant mechanism of cancer recurrence that is independent of oncogene addiction.

1Department of Stem Cell Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8553, Japan. 2Pharmaceuticals and Life Sciences Division, Shimadzu Techno-Research, Inc., 1, Nishinokyo-shimo-cho, Nakagyo-ku, Kyoto 604-8436, Japan. 3Precision Medicine Research Center, Advanced Institute of Convergence Technology, Seoul National University, #C-504, 145, Gwanggyo-ro, Yeongtong-gu, Suwon, Gyeonggi-do 16229, Republic of Korea. 4Department of Molecular Oncology, Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8670, Japan. 5Institute of Resource Development and Analysis, Kumamoto University, 2-2-1, Honjo, Chuo-ku, Kumamoto 860-0811, Japan. 6Natural Science Center for Basic Research and Development, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan. 7Department of Hematology and Oncology, Dokkyo Medical University School of Medicine, 880, Kitakobayashi, Mibu, Shimotsuga-gun, Tochigi 321-0293, Japan. 8Catholic Hematology Hospital, Leukemia Research Institute, The Catholic University of Korea, #222 Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea. 9Department of Transdisciplinary Studies, Graduate School of Convergence Science and Technology, Seoul National University, #C-504, 145, Gwanggyo-ro, Yeongtong-gu, Suwon, Gyeonggi-do 16229, Republic of Korea. 10MedPacto Inc., 92, Myeongdal-ro, Seocho-gu, Seoul 06668, Republic of Korea. ✉email: kanaka55@hiroshima-u.ac.jp
Glycerophospholipids (phospholipids) organise the lipid bilayer in a cell’s plasma membrane. These phospholipids are also a source of lipid mediators such as prostaglandins and leukotrienes, which play essential roles in immune responses, inflammation, and cancer development. Phospholipids contain two hydrophobic fatty acid chains at the sn-1 and sn-2 sites, and one hydrophilic polar base (choline, serine, inositol, or ethanolamine) at the sn-3 site (Fig. 1a, upper right). Since the 1950s, it has been known that glycerol 3-phosphate (G3P) is first converted into lysophosphatidic acids (LPAs) and then into various phospholipids via the Kennedy pathway (the so-called de novo pathway), as illustrated in Fig. 1a. To supply a wide variety of lipid molecules, the fatty acid chains and polar bases of phospholipids undergo exchange via the Lands’ cycle (remodelling pathway) to create distinct types of lysophospholipids. These lysophospholipids can then be recycled back into LPAs through the activities of three lysophospholipase D enzymes, namely Autotaxin (ATX), GDPD3 (also termed GDE7), and GDPD1 (GDE4), which have the ability to specifically hydrolyse the polar base at the sn-3 site of a lysophospholipid.

Lysophospholipids have only one fatty acid chain at the sn-1 or sn-2 site and so are more water-soluble than phospholipids, allowing them to act as lipid second messengers mediating regulatory signalling. These molecules convey their signalling functions by binding to certain LPA receptors (LPA1–LPA6) belonging to the G-protein coupled receptor (GPCR) family. However, the precise roles of lysophospholipid metabolism in cancer cells in general, and in cancer stem cells in particular, are not yet understood. It is known that ATX-disrupted mice exhibit embryonic lethality, and that enforced expression of ATX or LPARs promotes the initiation and metastasis of breast cancer in transgenic mouse strains. In humans, our knowledge is even more limited, although aberrant expression of phosphatidylinositol-3-kinases (PCs) has been detected in patients with colorectal cancer, triple-negative breast cancer, or lung adenocarcinoma.

Chronic myelogenous leukaemia (CML) stem cells are the hierarchal apex cells in CML disease. CML stem cells have both the capacity to self-renew and to produce mature CML cells. Despite their expression of the BCR-ABL oncogene, CML stem cells have been reported to maintain their stemness in an oncogene-independent manner, the mechanism of this maintenance is unknown. Although the advent of tyrosine kinase inhibitors (TKIs) has dramatically improved the prognosis of CML patients, CML stem cells are untouched by TKI treatment and survive to cause the relapse of CML disease. A cure for CML therefore remains elusive.

The oncogene-independent survival of CML stem cells has spurred many researchers to search for CML stem cell-specific vulnerabilities in the metabolic pathways controlling their energy production, amino acid acquisition, and lipid mediator generation. For instance, activation of the PPAR-mediated signalling pathway by its agonist pioglitazone can reduce CML stem cells in an oncogene-independent manner, and (2) the retroviral BCR-ABL1 transduction CML cell model, termed the retro-CML-affect ed mouse model, designated herein as retro-CML mice. The latter mutants were derived by bone marrow transplantation (BMT) of murine HSCs that were retrovirally transduced with the BCR-ABL1-ires-EGFP gene, as reported in our earlier study.

For this study, we used two CML mouse models: (1) SCL-TTA x TRE-BCR-ABL1 double transgenic CML mice, the so-called tet-inducible CML-affect ed mouse model, designated herein as tet-CML mice; and (2) the retroviral BCR-ABL1 transduction CML cell model, termed the retro-CML-affect ed mouse model, designated herein as retro-CML mice. The latter mutants were derived by bone marrow transplantation (BMT) of murine HSCs that were retrovirally transduced with the BCR-ABL1-ires-EGFP gene, as reported in our earlier study.

For this study, we used two CML mouse models: (1) SCL-TTA x TRE-BCR-ABL1 double transgenic CML mice, the so-called tet-inducible CML-affect ed mouse model, designated herein as tet-CML mice; and (2) the retroviral BCR-ABL1 transduction CML cell model, termed the retro-CML-affect ed mouse model, designated herein as retro-CML mice. The latter mutants were derived by bone marrow transplantation (BMT) of murine HSCs that were retrovirally transduced with the BCR-ABL1-ires-EGFP gene, as reported in our earlier study.

For this study, we used two CML mouse models: (1) SCL-TTA x TRE-BCR-ABL1 double transgenic CML mice, the so-called tet-inducible CML-affect ed mouse model, designated herein as tet-CML mice; and (2) the retroviral BCR-ABL1 transduction CML cell model, termed the retro-CML-affect ed mouse model, designated herein as retro-CML mice. The latter mutants were derived by bone marrow transplantation (BMT) of murine HSCs that were retrovirally transduced with the BCR-ABL1-ires-EGFP gene, as reported in our earlier study.

For this study, we used two CML mouse models: (1) SCL-TTA x TRE-BCR-ABL1 double transgenic CML mice, the so-called tet-inducible CML-affect ed mouse model, designated herein as tet-CML mice; and (2) the retroviral BCR-ABL1 transduction CML cell model, termed the retro-CML-affect ed mouse model, designated herein as retro-CML mice. The latter mutants were derived by bone marrow transplantation (BMT) of murine HSCs that were retrovirally transduced with the BCR-ABL1-ires-EGFP gene, as reported in our earlier study.

For this study, we used two CML mouse models: (1) SCL-TTA x TRE-BCR-ABL1 double transgenic CML mice, the so-called tet-inducible CML-affect ed mouse model, designated herein as tet-CML mice; and (2) the retroviral BCR-ABL1 transduction CML cell model, termed the retro-CML-affect ed mouse model, designated herein as retro-CML mice. The latter mutants were derived by bone marrow transplantation (BMT) of murine HSCs that were retrovirally transduced with the BCR-ABL1-ires-EGFP gene, as reported in our earlier study.
Glycerol 3-phosphate (G3P)  

\[ HO-PO_2^- \]

\[ H_2O_3P \]

\[ PO_3H_2 \]

Kenedy pathway  
(de novo pathway)

\[ HO-PO_2^- \]

\[ R1'-CO \]

Lysophosphatidic acids (LPAs)  

Gdpd3  

Lands' cycle  
(remodeling pathway)

\[ HO-P-O-R3' \]

Phospholipids  
(PC, PS, PE, PI, etc.)

R1, R2: Saturated or unsaturated alkyl group  
R3: Polar base (choline, serine, ethanolamine, inositol, etc.)

Lyosphospholipids  
(LPC, LPS, LPE, LPI, etc.)

**Fig. 1** Gdpd3 is implicated in CML disease initiation in vivo.  
(a) Diagram of pathways of lysosphospholipid biosynthesis. G3P is converted into LPAs, and LPAs are then converted into phospholipids by catalysing hydrolysis (magenta dotted line). (PC Phosphatidylcholine, PS Phosphatidylserine, PE Phosphatidylethanolamine, PI Phosphatidylinositol, LPC Lysophosphatidylcholine, LPS Lysophosphatidylserine, LPE Lysophosphatidylethanolamine, LPI Lysophosphatidylinositol).  
(b) qRT-PCR determination of Gdpd3 mRNA expression in LT-stem (LT), CD48, MPP, and LK cells (see Supplementary Fig.2) isolated from Gdpd3\(^{+/+}\)/tet-CML-affected (SCL-tTA\(^{+}\)TRE-BCR-ABL1\(^{+}\)) mice (one male, six females) or normal littermate (SCL-tTA\(^{−}\)) mice (four males, four females). Data are the mean ratio ± s.d. of transcript levels normalised to Actb (n = 3 biologically independent samples) (P-value, unpaired two-sided Student's t-test).  
(c) Quantitation of colony-forming capacity of haematopoietic stem/progenitor (LSK) cells that were transduced with/without Cy3-labelled siRNA targeting mouse Gdpd3 mRNA (mGdpd3 siRNA #1 or #3). Cy3\(^{+}\) and Cy3\(^{−}\) CML-LSK cells were purified at 3 days post-transduction and plated in semi-solid methylcellulose medium. Data are the mean colony number ± s.d. (n = 3) and are representative of biologically independent three experiments (P-value compared with control, unpaired two-sided Student’s t-test).  
(d) Quantitation of colony-forming capacity of haematopoietic stem/progenitor (LSK) cells that were isolated from normal Gdpd3\(^{+/+}\)/tet-CML (one male, six females) and Gdpd3\(^{−/−}\)/tet-CML (one female) mouse and cultured in semi-solid methylcellulose medium under hypoxic (3% O\(_2\)) conditions. Data are the mean colony number±s.d. (n = 3) and are representative of biologically independent two experiments (P-value, unpaired two-sided Student’s t-test).  
(e) Absolute numbers of LT-CML stem cells isolated from the two hind limbs of Gdpd3\(^{+/+}\)/tet-CML-affected (SCL-tTA\(^{+}\)TRE-BCR-ABL1\(^{+}\)) mouse and cultured in semi-solid methylcellulose medium under hypoxic (3% O\(_2\)) conditions. Data are the mean colony number±s.d. (n = 3) and are representative of biologically independent two experiments (P-value, unpaired two-sided Student’s t-test).  
(f) Quantitation of colony-forming capacity of LT-CML stem cells that were isolated from a Gdpd3\(^{+/+}\)/tet-CML-affected mouse (one male) and a Gdpd3\(^{−/−}\)/tet-CML-affected mouse (one male) and co-cultured on an OP-9 stromal cell layer under hypoxic (3% O\(_2\)) conditions. Data are the mean colony number±s.d. (n = 3) and are representative of biologically independent three experiments (P-value, unpaired two-sided Student’s t-test).  
(g) Survival rates of Gdpd3\(^{+/+}\)/tet-CML-affected mice (18 males, eight females, n = 26 biologically independent samples) and Gdpd3\(^{−/−}\)/tet-CML-affected mice (19 males, four females, n = 23 biologically independent samples) after induction of CML by Dox withdrawal (P-value, Log-rank non-parametric test).
a previously reported Gdpd3-deficient mutant, our Gdpd3−/− mice were born at the expected Mendelian ratio and appeared healthy (data not shown). Thus, the Gdpd3-mediated lysophospholipid metabolic pathway is dispensable for normal mouse development and survival. With respect to haematopoiesis, whereas the red blood cell (RBC) count and haematocrit (HCT) appeared to increase in Gdpd3−/− mice compared to Gdpd3+/+ mice, no difference was observed in white blood cell (WBC) count (Supplementary Table 1). In addition, no differences were observed in CPU-GM, BFU-E, or CFU-mix populations of BMMNCs, or in the colony-forming capacity of LT-CML cells isolated from normal healthy Gdpd3+/+ and Gdpd3−/− littermate mice (Fig. 1d, Supplementary Fig. 4). In serial BMT experiments, normal LS cells from Gdpd3−/− mice maintained their BM reconstitution capacity through the first- and second-rounds of such transplantation in vivo (Supplementary Fig. 5).

We next employed our Gdpd3−/− tet-CML mouse model to evaluate Gdpd3’s function in CML stem cells. The absolute number of LT-CML stem cells was modestly higher in such transplantation in vivo (Supplementary Fig. 5). The frequency of BrdU appeared to increase in Gdpd3−/− mice compared to Gdpd3+/+ mice; however, no consistent trend in BM (Supplementary Fig. 7). Accordingly, the cell cycle distribution of BrdU+ cells compared to BM and spleen of recipients bearing the mutant cells was similar in both BM and spleen (Supplementary Fig. 7). These data suggest that, even though the loss of Gdpd3+ function in CML stem cells.

Consistent with the accelerated initiation of CML disease in the spleen was strikingly elevated among Gdpd3−/− mice compared to Gdpd3+/+ mice (Fig. 1e). Unexpectedly, Gdpd3−/− LT-CML stem cells displayed a higher colony-forming capacity than Gdpd3+/+ LT-CML stem cells (Fig. 1f). Consistent with this finding, Gdpd3−/− tet-CML mice developed CML disease more rapidly than Gdpd3+/+ tet-CML mice, usually within three months of CML induction by Dox withdrawal (Fig. 1g). However, this enhanced disease-initiating capacity had attenuated by six months post-Dox withdrawal such that more Gdpd3−/− tet-CML mice survived longer than did Gdpd3+/+ tet-CML mice. These results suggest that, even though the loss of Gdpd3 in CML stem cells initially aggravates CML disease, these cells eventually lose the ability to differentiate into mature CML cells.

To examine the self-renewal capacity of Gdpd3-deficient CML-LSK cells in vivo, we employed our retro-CML model mice. Consistent with the accelerated initiation of CML disease observed in our tet-CML model, recipient mice transplanted with Gdpd3−/− retro-CML-LSK cells developed CML disease more rapidly than recipients transplanted with Gdpd3+/+ retro-CML-LSK cells (Fig. 2a). However, to our surprise, serial BMT experiments indicated that Gdpd3−/− retro-CML-LSK cells isolated from primary recipients showed a marked decrease in disease-initiating capacity in second-round recipients compared to Gdpd3+/+ retro-CML-LSK cells (Fig. 2b). We next examined the effect of Gdpd3 loss on the absolute number and frequency of BCR-ABL1/EGFP+ CML-LSK cells in first- and second-round BMT recipients. No significant differences were observed in either the frequency or the absolute number of BCR-ABL1/EGFP+ CML-LSK cells in BM of first-round recipients (Fig. 2c; Supplementary Fig. 6a). However, in spleen, the absolute number of CML-LSK cells was dramatically increased in first-round recipients, even though we could not detect a difference in the frequency of these cells (Fig. 2d; Supplementary Fig. 6b). This elevated absolute number of CML-LSK cells in spleen resulted from a higher number of total splenocytes in Gdpd3−/− retro-CML mice compared to Gdpd3+/+ retro-CML mice (data not shown). When we examined second-round recipients, however, we found that CML-LSK cells were dramatically decreased in both BM and spleen of recipients bearing the mutant cells (Fig. 2e, f; Supplementary Fig. 6c, d). These data prompted us to compare the cell cycle distribution of Gdpd3+/+ and Gdpd3−/− CML-LSK cells isolated from retro-CML mice subjected to in vivo BrdU incorporation assays. The frequency of BrdU+ S phase cells in the spleen was strikingly elevated among Gdpd3−/− CML-LSK cells compared to Gdpd3+/+ CML-LSK cells (Fig. 2g, h), with a similar trend in BM (Supplementary Fig. 7). Accordingly, the frequency of BrdU− G0/G1 cells among Gdpd3−/− CML-LSK cells was reduced compared to Gdpd3+/+ CML-LSK cells. These results demonstrate that Gdpd3 deficiency promotes the cell cycle progression of CML stem/progenitor cells, and thus breaks the quiescence essential for maintaining the stemness of CML stem cells in vivo.

LPAs and lipid mediators are decreased in Gdpd3−/− CML cells.

Given that the Gdpd3 gene encodes a lysophospholipase D enzyme that hydrolyses lysophospholipids into LPAs, we investigated levels of various LPAs predicted to be products of Gdpd3 activity in BM cells of normal WT, Gdpd3+/+ tet-CML and Gdpd3−/− tet-CML-affected mice. Interestingly, an examination of normal WT BMMNCs and CML BM cells showed that the presence of CML disease dramatically altered the levels of many LPAs (Fig. 3a). Several LPAs, including LPA20:4 (arachidonate), were increased in Gdpd3+/+ CML BM cells compared to normal WT BMMNCs, whereas only LPA18:1 (oleate) was decreased in this experiment. In contrast, most of these same LPAs tended to decrease in both BMMNCs and LS cells isolated from Gdpd3−/− tet-CML mice compared to those from Gdpd3+/+ tet-CML mice (Fig. 3a, b). Thus, in general, loss of Gdpd3 enzymatic activity decreases LPA levels in the CML context.

We next performed a sophisticated global lipidomics analysis of 196 lipid mediators and discovered that several non-LPA lipid mediators were also markedly decreased in Gdpd3−/− tet-CML BM cells compared to Gdpd3+/+ tet-CML BM cells (Fig. 4). Notably, prostaglandin D2 (PGD2) and PGF2, which were increased in Gdpd3+/+ tet-CML BM cells compared to normal WT BMMNCs, appeared to be decreased in Gdpd3−/− tet-CML BM cells compared to Gdpd3+/+ tet-CML BM cells (Fig. 4a). In the same vein, levels of eicosanoid and docosanoid fatty acids, such as 5-oxo-6,8,9,11Z,14Z-eicosatetraenoic acid (5-KETE), 15-hydroxy-5Z, 8Z,11Z,13E,17Z-eicosapentaenoic acid (15-HEPE), and 17-hydroxydocosahexaenoic acid (17-HDHA), tended to be lower in Gdpd3−/− tet-CML BM cells than in Gdpd3+/+ tet-CML BM cells (Fig. 4b). Lyso-PAF (platelet-activating factor) was higher in Gdpd3−/− tet-CML BM cells than in normal WT BMMNCs, but decreased in Gdpd3−/− tet-CML BM cells compared to Gdpd3+/+ tet-CML BM cells (Fig. 4c). Taken together, these data suggest that Gdpd3 deficiency decreases many important lipid mediators in CML cells in vivo.

Loss of Gdpd3 activates the AKT/mTORC1 pathway.

To understand how Gdpd3 deficiency affects downstream signalling pathways, we first investigated the AKT/mTORC1 pathway in LT-CML stem cells isolated from Gdpd3+/+ and Gdpd3−/− CML mice. Interestingly, levels of phosphorylated AKT and S6 ribosomal protein were increased in Gdpd3−/− LT-CML stem cells compared to Gdpd3+/+ LT-CML stem cells, indicating that Gdpd3 acts to suppress the AKT/mTORC1 pathway in Gdpd3−/− LT-CML stem cells (Fig. 5a, b; Supplementary Fig. 8a, b). We then examined the subcellular localisation of Foxo3a, whose export from a cell’s nucleus to its cytoplasm is induced by activated AKT. We observed that, while Foxo3a was located within the nuclei of Gdpd3−/− LT-CML stem cells as expected, Foxo3a pressed Foxo3a import into the nucleus in primitive LT-CML stem cells in vivo.

TKI treatment is more effective in the absence of Gdpd3.

Although the discovery of TKIs has dramatically improved the prognoses of CML patients, the insuperable problem remains that
TKI therapy does not kill the CML stem cells responsible for disease relapse. To determine whether Gdpd3-mediated lysophospholipid metabolism was necessary for disease-relapsing capacity in CML stem cells, we transplanted recipient mice with Gdpd3<sup>+/+</sup> or Gdpd3<sup>−/−</sup> retro-CML-LSK cells in a first-round of BMT and treated the animals with the TKI dasatinib. Disease relapse was significantly decreased in treated recipients bearing Gdpd3<sup>−/−</sup> retro-CML-LSK cells compared to those that received Gdpd3<sup>+/+</sup> retro-CML-LSK cells (Fig. 6a). Thus, Gdpd3 expression in CML stem cells confers resistance to TKI therapy.

To determine if this increased survival of BMT recipients bearing Gdpd3<sup>−/−</sup> retro-CML-LSK cells was due to an inability to produce mature leukaemia cells, we used flow cytometry to examine the frequency of BCR-ABL1/EGFP<sup>+</sup> mature leukaemia cells in peripheral blood (PB). At 16–24 days post-BMT, we found no apparent differences in leukaemia cell numbers between Gdpd3<sup>+/+</sup> retro-CML-affected and Gdpd3<sup>−/−</sup> retro-CML-affected mice that had not been treated with dasatinib (Fig. 6b). In contrast, at 40–60 days post-BMT in mice treated with dasatinib, the frequency of BCR-ABL1/EGFP<sup>+</sup> leukaemia cells was significantly decreased in PB of Gdpd3<sup>−/−</sup> retro-CML-affected mice compared to PB of Gdpd3<sup>+/+</sup> retro-CML-affected mice (Fig. 6c). Flow cytometry following a t-SNE (t-Distributed Stochastic Neighbour Embedding) algorithm analysis indicated that PB samples from untreated control and Gdpd3-deficient retro-CML-affected mice both contained BCR-ABL1/EGFP<sup>+</sup>

Fig. 2 Gdpd3 maintains the self-renewal capacity of CML stem cells in vivo. a Survival rates of retro-CML-affected mice in a primary BMT experiment that received BCR-ABL1/EGFP-transduced LSK cells from either Gdpd3<sup>+/+</sup> mice (one male, one female) or Gdpd3<sup>−/−</sup> mice (two females). Results shown are cumulative data obtained from two biologically independent experiments (P-value, Log-rank non-parametric test). b The survival rate of second-round BMT recipient mice that received serial transplantation of BCR-ABL1/EGFP<sup>+</sup> CML-LSK cells (3 × 10<sup>4</sup> cells) isolated from either the Gdpd3<sup>+/+</sup> retro-CML-affected mice (14 females) or Gdpd3<sup>−/−</sup> retro-CML-affected mice (15 females) as in a. Results shown are cumulative data obtained from three biologically independent experiments. Mouse survival was monitored for 90 days. (P-value, Log-rank non-parametric test) c-f Absolute numbers of BCR-ABL1/EGFP<sup>+</sup> CML-LSK cells isolated from c, e BM of the two hind limbs and d, f spleen of Gdpd3<sup>+/+</sup> retro-CML-affected mice and Gdpd3<sup>−/−</sup> retro-CML-affected mice after c, d a first-round or e, f second-round of serial BM transplantation. Data are the mean absolute numbers ± s.d. of BCR-ABL1/EGFP<sup>+</sup> LSK cells. n numbers in a-f indicate biologically independent mouse numbers. (P-value, unpaired two-sided Student’s t-test). (See also Supplementary Fig. 6).

g, h Cell cycle distribution of CML-LSK cells in the spleen of Gdpd3<sup>+/+</sup> retro-CML-affected mice (three females) or Gdpd3<sup>−/−</sup> retro-CML-affected mice (three females) that were intraperitoneally administered BrdU for 3 h after a first-round of BMT as in a. Results are the mean frequency ± s.d. of G0/G1, S-phase CML-LSK cells, BrdU<sup>+</sup> S-phase CML-LSK cells, and G2/M phase CML-LSK cells (n = 3 biologically independent samples) (P-value, unpaired two-sided Student’s t-test). (See also Supplementary Fig. 7). h Representative dot plots of BrdU incorporation and DNA content for the CML-LSK cells in g.
Fig. 3 The loss of the Gdpd3 gene decreases LPAs in CML cells in vivo. a, b Global lipidomics analyses (see Methods and Supplementary Method 1) for the indicated LPAs in a BMMNCs (1 × 10^6) isolated from healthy 8-wk-old normal C57BL/6 mice (two males, four females), Gdpd3^+/+ tet-CML-affected mice (five females), or Gdpd3^−/− tet-CML-affected mice (one male, three females), and in b CML-LSK cells (5 × 10^5) isolated from Gdpd3^+/+ tet-CML-affected mice (four males and 11 females), or Gdpd3^−/− tet-CML-affected mice (six males, ten females), at 5 weeks post-Dox withdrawal. Data are the mean concentration (ng ml^{-1}) ± s.d. (n = 3 biologically independent samples) (P-value, unpaired one-sided Student’s t-test). The X-axis labels represent LPA [carbon number] unsaturated bond number of the fatty acid chain in the LPA.

leukaemia cells that were distinct from the populations of normal myelogenous cells, T cells and B cells in PB of normal healthy WT mice (Supplementary Fig. 9a, b, c). Thus, Gdpd3^−/− retro-CML-LSK cells do have the ability to give rise to BCR-ABL1/EGFP-expressing mature leukaemia cells. However, although BCR-ABL1/EGFP^+ leukaemia cells were still present in PB of dasatinib-treated Gdpd3^+/+ retro-CML-affected mice (Supplementary Fig. 9c), they were dramatically decreased in PB from dasatinib-treated Gdpd3^−/− retro-CML-affected mice (Supplementary Fig. 9d). Thus, in the absence of Gdpd3, TKIs are more effective in reducing disease relapse caused by CML stem cells.

To investigate whether GDPD3 expression was altered in human CML patients, we retrieved data on levels of GDPD3 mRNA in cells of imatinib-treated CML patients listed in a public database GEO (GEO: GSE12211)30. Intriguingly, for five out of the six CML patients examined, GDPD3 mRNA levels were indeed higher in CD34^+ cells isolated after imatinib therapy than in CD34^− cells isolated before treatment (Fig. 6d). These results implicate GDPD3 in the survival of human CML stem/progenitor cells.

We next examined the in vitro effects of siRNA targeting of human GDPD3 mRNA in the K562 human CML cell line as well as in primary human CD34^+ CML cells isolated from BM of a chronic phase CML patient. We transduced K562 cells or primary CD34^+ CML cells with FITC-labelled hGDPD3 siRNA#1 or #2 and purified cells containing FITC-hGDPD3 siRNAs by cell sorting (Supplementary Fig. 10). Although siRNA-mediated repression of hGDPD3 mRNA decreased the colony-forming capacity of K562 cells in vitro, the suppressive effect was only subtle (Fig. 6e). In contrast, the colony-forming capacity of human CD34^+ cells transduced with hGDPD3 siRNA was quite dramatically reduced (Fig. 6f). When we repeated this experiment in the presence of imatinib, we observed some colony formation by imatinib-resistant CD34^+ cells that had not been transduced with hGDPD3 siRNA. Intriguingly, the combination of imatinib plus hGDPD3 siRNA effectively suppressed colony formation by these resistant cells (Fig. 6g). These results reinforce our hypothesis that GDPD3 may contribute to the maintenance of primitive CML cells (rather than to the differentiation of mature CML cells) in humans as well as in mice.
Fig. 4 Lysophospholipase D Gdpd3 is required for the production of lipid mediators in CML cells in vivo. Global lipidomics analyses (see “Methods”, Supplementary Method 2, 3) for the lipid mediators in BMMNCs (1 × 10^7) isolated from healthy 8-wk-old normal C57BL/6 mice (two males, four females), or Gdpd3+/+ tet-CML-affected mice (five females), or Gdpd3−/− tet-CML-affected mice (one male, three females), at 5 weeks post-Dox withdrawal. Data are the mean average±s.d. of peak area normalised to IS (internal standard) area. a, Prostanoids, b, eicosanoids and docosanoid, and c Lyso-PAF were indicated.

Loss of Gdpd3 results in aberrant expression of GPCR genes. To understand how Gdpd3 deficiency impairs the self-renewal capacity of CML stem cells in vivo, we compared gene expression profiles of murine Gdpd3−/− LT-CML stem cells and Gdpd3+/+ LT-CML stem cells. RNA-Seq followed by gene ontology (GO) term enrichment analyses indicated that several GPCR family genes, including Lpar4/Gpr23, Ltb4r1/Gpr16, Gpr82, and Gpr84, were decreased in Gdpd3−/− LT-CML stem cells compared to Gdpd3+/+ LT-CML stem cells (Fig. 7a; Supplementary Fig. 11a, b). Interestingly, Lpar4/Gpr23 encodes a GPCR that binds to LPAs which are the predicted products of the Gdpd3 enzyme. Thus, a defect in lysophospholipid hydrolysis due to loss of Gdpd3 activity might affect Lpar4/Gpr23 expression. Strikingly, we found that mRNA levels of Lgr4/Gpr48, which encodes a leucine-rich repeat (LRR)-containing GPCR, were also significantly lower in Gdpd3−/− LT-CML stem cells than in Gdpd3+/+ LT-CML stem cells (Fig. 7a). Although the LRR-containing GPCR family member Lgr5/Gpr49 is reportedly responsible for the maintenance of intestinal stem cells and cancer stem cells, the biological function of Lgr4/Gpr48 is not yet fully understood. These data therefore prompted us to investigate the role of Lgr4/Gpr48 in CML stem cells.

We transduced tet-CML-LSK cells with siRNAs targeting Lgr4/Gpr48 mRNA and found that the colony-forming capacity of these cells was reduced (Fig. 7b; Supplementary Fig. 12). Because conventional Lgr4/Gpr48−/− mice are embryonic lethal, we used a mouse strain bearing a hypomorphic mutation of Lgr4 established by gene-trap (Gt) methodology. Levels of Lgr4 mRNA in these mutants were decreased to 10% of levels in WT mice. We then generated Lgr4−/− and Lgr4Gt/Gt tet-CML and retro-CML mice using the protocols described above. Absolute numbers of LT-CML stem cells were comparable between Lgr4+/+ and Lgr4Gt/Gt tet-CML mice, whereas the frequency of these cells was modestly increased in Lgr4Gt/Gt tet-CML mice (Fig. 7c; Supplementary Fig. 13a). The colony-forming capacity of LT-CML stem cells isolated from Lgr4Gt/Gt tet-CML-affected mice decreased slightly compared to that of cells from Lgr4−/− tet-CML-affected controls (Fig. 7d). To evaluate the self-renewal capacity of these CML-initiating cells in vivo, we performed BMT of LSK cells from Lgr4Gt/Gt or Lgr4−/− retro-CML mice into irradiated recipients. Animals that received Lgr4−/− retro-CML-LSK cells developed CML disease just as retro-CML-LSK cells developed reduced disease-initiating capacity in transplanted recipients (Fig. 7e). At 20 days post-transplantation,
the frequency and an absolute number of BCR-ABL1/EGFP+ LSK cells in Lgr4+/+ and Lgr4Gt/Gt retro-CML mice were comparable (Fig. 7f; Supplementary Fig. 13b). However, at 90 days post-transplantation, the frequency and an absolute number of BCR-ABL1/EGFP+ LSK cells had decreased significantly in Lgr4Gt/Gt retro-CML mice compared with Lgr4+/+ retro-CML mice (Fig. 7f; Supplementary Fig. 13b). In the same vein, the frequency of BCR-ABL1/EGFP+ mature CML cells was markedly reduced in Lgr4Gt/Gt retro-CML mice compared with Lgr4+/+ retro-CML mice (Fig. 7g). Thus, whereas Lgr4 is dispensable for the actual development of CML stem/progenitor cells, our results indicate that Lgr4 is important for their LT maintenance.

Gdpd3 is essential for binding Foxo/β-catenin within nucleus. Previous reports have demonstrated that Lgr4/Gpr48 functions as a receptor for R-spondins in the Wnt/β-catenin signalling pathway37-39, and that the Cnmb1 gene encoding β-catenin is crucial for the disease-relapsing capacity of CML stem cells40. We previously showed that Foxo3a is also essential for the self-renewal capacity of CML stem cells20. Lastly, Foxo3a and β-catenin are known to cooperatively regulate the metastasis of colon cancer cells41. This collection of facts prompted us to investigate if Gdpd3 or Lgr4, or both play a role in the nuclear interaction between Foxo3a and β-catenin in primitive LT-CML stem cells. We used the highly sensitive Duolink® in situ PLA technology to reveal binding between Foxo3a and active β-catenin within cell nuclei. As expected, we readily detected Foxo3a/β-catenin interaction in the nuclei of Gdpd3+/+ LT-CML stem cells (Fig. 8a; Supplementary Fig. 14). However, no interaction between Foxo3a and active β-catenin was observed in the nuclei of LT-CML stem cells isolated from Gdpd3−/− tet-CML mice. Thus, Gdpd3 is involved in regulating the binding of Foxo3a to active β-catenin in LT-CML stem cells. Intriguingly, no Foxo3a/β-catenin binding was observed in LT-CML cells isolated from Lgr4Gt/Gt tet-CML mice, suggesting a potential mechanistic link.

Previous work has determined that β-catenin is activated in CML stem cells by PGE2 via EP124. We had already shown that PGE2 was decreased in Gdpd3−/− CML BM cells compared to Gdpd3+/+ CML BM cells (Fig. 4a). Thus, we focused on the effect of enforced PGE2 treatment in vitro on the interaction between Foxo3a and β-catenin in LT-CML stem cells. PGE2 treatment did not elevate Foxo3a/β-catenin binding in Gdpd3+/+ LT-CML stem cells (Fig. 8b; Supplementary Fig. 15). Indeed, PGE2 treatment only slightly increased this interaction in the nuclei of Gdpd3−/− LT-CML stem cells. In contrast, PGE2 treatment dramatically increased Foxo3a/β-catenin interaction in the nuclei of Lgr4Gt/Gt tet-CML stem cells, exposing an interesting difference between Gdpd3−/− LT-CML stem cells and Lgr4Gt/Gt LT-CML stem cells that remains under investigation. In any case, our data collectively establish that Gdpd3 and lysophospholipid metabolism play multiple key roles in the maintenance of the stem cells driving CML.

Discussion

Although signalling pathways related to lipid mediators are known to be involved in regulating CML stem survival21-24, prior to our work, there had been little understood about how lipid biogenesis contributes to the self-renewal capacity of CML stem cells. In this study, we have shown that CML-LSK cells lacking the gene involved in lysophospholipid metabolism is largely responsible for the maintenance of CML stem cells in vivo. As illustrated in Fig. 8c, it is well known that Gdpd3 hydrolyses lysophospholipids back into LPAs8,9. Phospholipids are produced from LPAs via the Kennedy pathway (de novo pathway)4 and are then converted into various lysophospholipids via the Lands’ cycle (remodelling pathway)3 (see also Fig. 1a). These mechanisms sustain the production of pleiotropic lysophospholipids and phospholipids, which contain many different combinations of fatty acid chains and polar bases. Lysophospholipase D activity appears to mainly contribute to lysophospholipid metabolism via LPA recycling. With respect to signalling downstream of Gdpd3-mediated lysophospholipid metabolism, we have shown that the AKT/mTORC1 pathway becomes more highly activated in Gdpd3−/− LT-CML stem cells compared with Gdpd3+/+ LT-CML stem cells (Fig. 5a, b). In addition, Gdpd3 deficiency triggers...
Fig. 6 Gdpd3 is implicated in TKI-resistance in mouse and human CML stem cells. a Survival rates of retro-CML-affected mice in a primary BMT experiment that received BCR-ABL1/EGFP-transduced LSK cells from either Gdpd3+/+ (two males, two females) or Gdpd3−/− (three males, one female) as in Fig. 2a and were treated with dasatinib (5 mg kg⁻¹ per day) for days 8–60 post-transplantation. Results shown are cumulative data obtained from two independent experiments (n = 16 biologically independent recipient mice). Mouse survival was monitored for 90 days. (P-value, Log-rank non-parametric test). b Frequency of BCR-ABL1/EGFP⁺ leukaemia cells at 16–24 days post-transplantation in PB from retro-CML-affected mice that received BCR-ABL1/EGFP-transduced LSK cells from Gdpd3+/+ mice (four females) or Gdpd3−/− mice (four females) as in Fig. 2a and were not treated with TKI (n = 4 biologically independent samples). Data are the mean frequency (%) ± s.d. of BCR-ABL1/EGFP⁺ leukaemia cells (P-value, unpaired two-sided Student’s t-test). c Frequency of BCR-ABL1/EGFP⁺ leukaemia cells at 40–60 days post-transplantation in PB from retro-CML-affected mice that received BCR-ABL1/EGFP-transduced LSK cells from Gdpd3+/+ mice (four females) or Gdpd3−/− mice (four females) as in Fig. 2a and were treated with dasatinib as in a (n = 4 biologically independent samples). Data are the mean frequency (%) ± s.d. of BCR-ABL1/EGFP⁺ leukaemia cells (P-value, unpaired two-sided Student’s t-test). d Relative Gdpd3 mRNA expression in CD34⁺ cells from CML patients pre- and post-imatinib (IM) therapy as determined by microarray analysis. Data are from a public database (GEO, GSE12211) (n = 6 biologically independent samples) (P-value, unpaired two-sided Student’s t-test). e–g Quantitation of the colony-forming capacity of e K562 human CML cells and f, g primary human BM CD34⁺ CML cells that were transduced with/without FITC-labelled siRNA targeting human GDPD3 mRNA (hGDPD3 siRNA #1 or #2). FITC⁺ and FITC⁻ CML cells were purified at 3 days post-transduction and plated in semi-solid methylcellulose medium with/without imatinib (1 μM) for 14 days. Data are the mean colony number ± s.d. (n = 3). (P-value compared with control, unpaired two-sided Student’s t-test). The relevant FACS data are shown in Supplementary Fig. 10. Results are representative of three biologically independent trials.
Fig. 7 Lgr4/Gpr48 is involved in CML stem cell self-renewal in vivo. a RNA-Seq determinations of mRNA levels of the indicated GPCR family genes in LT-CML stem cells isolated from Gdpd3+/+ tet-CML-affected mice (22 males, 12 females) and Gdpd3−/− tet-CML-affected mice (five males, five females). Results are expressed as FPKM (see Methods). Data are the mean FPKM ± s.d. (n = 3 biologically independent samples) (P-value, unpaired two-sided Student’s t-test). Results of the MA-plot and GO term enrichment analyses for these RNA-Seq data are shown in Supplementary Fig. 11a, b. b Quantitation of the colony-forming capacity of CML-LSK cells that were isolated from BM of the two hind limbs of Lgr4+/+ tet-CML-affected mice (four males, one female) and Lgr4Gt/Gt tet-CML-affected mice (five females) (n = 5 biologically independent samples). Data are the mean absolute numbers ± s.d. of LT-CML stem cells (P-value, unpaired two-sided Student’s t-test). The relevant FACS data are shown in Supplementary Fig. 12. c Absolute numbers of LT-CML stem cells isolated from BM of the two hind limbs of Lgr4+/+ or Lgr4−/− tet-CML-affected mice (four males, one female) and Lgr4Gt/Gt tet-CML-affected mice (five females) (n = 5 biologically independent samples). d Absolute numbers of LT-CML stem cells that were isolated from an Lgr4Gt/Gt retro-CML-affected mouse (one male) and analysed as in Fig. 1f. Data are the mean colony number ± s.d. (n = 3) and are representative of three biologically independent experiments. (P-value compared with control, unpaired two-sided Student’s t-test). Results are expressed as FPKM (see Methods). Data are the mean FPKM ± s.d. (n = 3 biologically independent samples) (P-value, unpaired two-sided Student’s t-test). e Survival rates of retro-CML-affected mice that received BCR-ABL1/EGFP-transduced LSK cells from Lgr4+/+ mice (one male, one female) or Lgr4Gt/Gt mice (two females). Results shown are cumulative data from two independent experiments (n numbers indicate biologically independent recipient mouse numbers). Mouse survival was monitored for 90 days. (P-value, Log-rank non-parametric test). f Absolute numbers of BCR-ABL1/EGFP+/− CML-LSK cells isolated from BM of the two hind limbs of Lgr4+/+ retro-CML-affected mice and Lgr4Gt/Gt retro-CML-affected mice after first-round BMT. Data are the mean absolute numbers ± s.d. of BCR-ABL1/EGFP+/− LSK cells (n numbers indicate biologically independent sample numbers) (P-value, unpaired two-sided Student’s t-test). (See also Supplementary Fig. 13b). g Frequency of BCR-ABL1/EGFP+ leukaemia cells post-BMT in PB of retro-CML-affected mice that received BCR-ABL1/EGFP-transduced LSK cells from Lgr4+/+ mice (three females, n = 3 biologically independent samples) or Lgr4Gt/Gt mice (seven females, n = 7 biologically independent samples) as in Fig. 7e. Data are the mean frequency ± s.d. of BCR-ABL1/EGFP+ cells (P-value, unpaired two-sided Student’s t-test).
cell division in CML stem cells and breaks the quiescence necessary to sustain CML stemness in vivo (Fig. 2g, h). Thus, our data indicate that Gdpd3 is a key suppressor of the AKT/mTORC1 pathway in CML stem cells, repressing their proliferation despite their expression of the oncogene.

Our findings are consistent with reports stating that CML stem cells maintain their survival in an oncogene-independent manner, and support our hypothesis that Gdpd3-mediated lysophospholipid metabolism is crucial for this maintenance in vivo.

Our gene expression profiling studies showed that the expression of several GPCR genes was significantly decreased in Gdpd3−/− LT-CML stem cells compared to Gdpd3+/+ LT-CML stem cells. The GPCRs constitute the prototypical seven-transmembrane receptor family. GPCRs regulate diverse signal transduction pathways and so are attractive pharmacological targets. Drugs inhibiting some GPCRs have now been approved by the FDA.

Importantly, GPCR signalling has been implicated in the maintenance of embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, somatic stem cells, and cancer stem cells. More specifically, the GPCR Gpr84 is involved in regulating acute myelogenous leukaemia (AML) stem cells, and the GPCR Lgr5/Gpr49 contributes to the maintenance of intestinal stem cells and cancer stem cells.

Our data indicate that Lgr4/Gpr48 is essential for the disease-initiating capacity of CML stem cells in vivo (Fig. 7e). At this time, the molecular mechanism by which Gdpd3 regulates the transcription of GPCR mRNAs remains under investigation. However, it is possible that lysophospholipid metabolism as shaped by Gdpd3 regulates Lgr4/Gpr48 mRNA levels in CML stem cells, which in turn might support the self-renewal capacity of these cells and thus their maintenance in vivo (Fig. 8c).

We found that several non-LPA lipid mediators, including PGE2, eicosanoids, docosanoid, and Lyso-PAF, appeared to decrease in Gdpd3−/− CML-BM cells compared to Gdpd3+/+ CML-BM cells (Fig. 4), implicating Gdpd3-mediated lysophospholipid metabolism in their production. Perhaps of relevance, our RNA-Seq analysis indicated that Alox-15 expression was increased in Gdpd3−/− CML-BM cells compared to Gdpd3+/+ PT-CML stem cells (Supplementary Fig. 11c). The Alox-15 gene encodes an arachidonate 15-lipoxygenase that is important for the maintenance of murine CML stem cells. A reduction in levels of lipid mediators caused by Gdpd3 deficiency might result in decreased Alox-15 mRNA in CML stem cells, compromising their survival.

We were interested to note that enforced PGE2 treatment in vitro rescued Foxo3a/β-catenin interaction in Lgr4Gt/Gt LT-CML stem
cells but not in Gdpd3−/− LT-CML stem cells. These differential responses to PGE2 are probably due to differing levels of intracellular lipid components, since Gdpd3−/− stem cells alter their production of several LPAs and lipid mediators, leading to the activation of the downstream AKT/mTORC1 pathway. In contrast, production of several LPAs and lipid mediators, leading to the support of CML stemness in vivo.

To facilitate the molecular mechanisms by which Gdpd3-mediated lysosphospholipid metabolism and TGFβ-FOXO signalling cooperate to support CML stemness in vivo.

**Methods**

**CML mouse models.** A knockout mouse strain in which the Gdpd3 (NM_024228) gene was disrupted was generated by Setsuuroto Inc. (Tokushima, Japan) using a genome-editing technique. In brief, in vitro fertilised zygotes (C57BL6 x C57BL6) were electroporated with 100 ng µl−1 recombinant Cas9 protein (Alt-R® S. Pyrogen free, 1074181, Integrated DNA Technologies, Inc., Coralville, IA, USA), 100 ng µl−1 crRNA (Alt-R® CRISPR-Cas9 crRNA, Integrated DNA Technologies), and 100 ng µl−1 tracrRNA (Alt-R® CRISPR-Cas9 tracrRNA, Integrated DNA Technologies). The target sequence of Gdpd3 crRNA was 5′-GAGGAGGAACAGATGACACAGGAG-3′ (Supplementary Fig. 3a). Electroporated zygotes were transferred into the oviduct of pseudopregnant female mice, and the mutant progeny were born on E19. Genotyping was carried out by hot-start PCR of genomic DNA from mouse tail tips.

**β-Actin, TRE-BCR-ABL1 double transgenic mice as described26.** These animals were designated as tet-CML-affected mice in this study. To establish our BCR-ABL1 transduction/transplantation-based CML model (retro-CML-affected mice), normal haematopoietic stem/progenitor (LSK) cells were purified for serial transplantation of CML-LSK cells (see below), MNCs isolated from the two hind limbs and spleen of retro-CML-affected mice were first isolated by incubation with anti-FcγRIIa/Ib mAbs (a normal healthy C57BL/6 mouse (negative control), were stained with anti-CD48-APC-Cy7 mAb (clone: A2F10, lot # 13-1351-85, BD Biosciences), anti-CD135/Flk2/Flt3-biotin (dilution 1:200, clone: 2B8, BD Biosciences), and anti-cKit-APC (dilution 1:200, clone: 5C7, BD Biosciences). CD150+ BMMNCs from C57BL/6 mice (6–8 wk old) were first isolated by binding with anti-FcγRIIa/Ib mAbs visualised using Streptavidin-PE-Cy7 (dilution 1:400, cat # 553759, lot # 611257, BD Biosciences), CD150+ CD48+ CD135+ LSK (Lineage− sca-1+ cd48−) cells were purified using a FACS Aria III cell sorter (S/N, P648201002) with BD FACSdiva software ver. 6.1.3 (BD Biosciences). To purify WT haematopoietic stem/progenitor (LSK) cells, MNCs isolated from WT, Gdpd3−/−, and Lgr4/Gpr48−/− mice (6–8 wk old) were first isolated by binding with anti-FcγRIIa/Ib mAbs (a normal healthy C57BL/6 mouse (negative control), were stained with anti-CD48-APC-Cy7 mAb (clone: A2F10, lot # 13-1351-85, BD Biosciences), anti-CD135/Flk2/Flt3-biotin (dilution 1:200, clone: 2B8, BD Biosciences), and anti-cKit-APC (dilution 1:200, clone: 5C7, BD Biosciences). CD150+ BMMNCs from C57BL/6 mice (6–8 wk old) were first isolated by binding with anti-FcγRIIa/Ib mAbs (a normal healthy C57BL/6 mouse (negative control), were stained with anti-CD48-APC-Cy7 mAb (clone: A2F10, lot # 13-1351-85, BD Biosciences), anti-CD135/Flk2/Flt3-biotin (dilution 1:200, clone: 2B8, BD Biosciences), and anti-cKit-APC (dilution 1:200, clone: 5C7, BD Biosciences). CD150+ CD48+ CD135+ LSK (Lineage− sca-1+ cd48−) cells were purified using a FACS Aria III cell sorter (S/N, P648201002) with BD FACSdiva software ver. 6.1.3 (BD Biosciences).
anti-Gr-1-APC (dilution 1:500, Clone # RB6-8C5, Cat. # 17-5931-82, Lot # E07334-1630, ebioscience) mAbs. BCR-ABL1/EGFP® cells were evaluated using a FACS Aria III instrument and the tenascin-rearranged in flow™ (build number 10.6.1) software. BCR-ABL1/EGFP® cells in PB isolated from a Gdpd3+/−, Gdpd3−/−, Lgr4+/−, and Lgr4+/− retro-CML-affecte mous were evaluated using a FACS Aria III instrument. The absolute number of CML-LSK cells in the spleen and BM of a retro-CML-affecte mous was calculated as [total number of MNCs isolated from the BM of the two hind limbs × frequency of CML-LSK cells (%) × 1/100]. The absolute number of LT-CML stem cells in BM of a tet-CML mous was calculated as [total number of MNCs isolated from the BM of two hind limbs × frequency of CD150+ CD48− CD135− LSK cells (%) × 1/100]. For analysis of flow cell counts, PB from the postorbital vein was analysed by a particle counter PCE-310 (ERMA Inc., Tokyo, Japan).

Lipidomics. For lipidomics analyses, total BMMNCs (1 × 10^7) were isolated from Gdpd3+/− tet-CML-affecte mous (five females), Gdpd3−/− tet-CML-affecte mous (one male and three females) at 5-week post-Dox withdrawal as described above. Total BMMNCs (1 × 10^7) were also isolated from 6- to 8-week old WT C57BL/6 mous (two males and four females). CML-LSK cells (5 × 10^5) were isolated from Gdpd3+/− tet-CML-affecte mous (four males and 11 females), or from Gdpd3−/− tet-CML-affecte mous (six males and ten females), at 5 weeks post-Dox withdrawal as described above. Cell pellets were frozen at −80 °C immediately after centrifugation. Lipidomics analyses were performed by Shimadzu Techno-reseach Inc. (Kyoto, Japan). LPA's were measured using a NexeraX2 system (Shimadzu Corporation, Kyoto, Japan) and Triple Quad 5500 (Sciex, Framingham, MA, USA) (Source Data file, Supplementary Method 1). Lipid mediators were measured using a NexeraX2 system and an LC-MS-8050 liquid chromatograph-mass spectrometer system (Shimadzu) (Source Data file, Supplementary Method 2, 3). Data were compiled using the LipidMediator LC/MS/MS Method Package (Ver. 3) (Shimadzu). Lipidomics data analyses were performed using Traverse MS Ver. 1.2.7. (Reifsys Inc, Tokyo, Japan).

RNA sequencing and bioinformatics. LT-LK cells were isolated from Gdpd3+/− and Gdpd3−/− mice and Gdpd3−/− tet-CML-affecte mous were directly sorted into 500 μl isogene solution (Nippon Gene, Co. Ltd., Toyama, Japan). RNA extraction and sequencing were performed by DNAFORM (Yokohama, Japan). The RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies). All RNA samples had an RNA integrity number of >7.5 and 150 bases were generated using HiSeq X Ten (Illumina). Sequence reads in Fastq format were assessed for quality using FastQC. Filtered reads were mapped to the reference genome related to the species using STAR v2.6.1a alignment software. Gene expression levels were measured with the Bioconductor package DESeq2 (version 1.20.0)52 (https://bioconductor.org/packages/release/bioc/html/DES0eq2.html) and sorted using an Agilent 1000 Bioanalysis Agio2 (Agio2, New Jersey). The RNA sequencing, bioinformatics, and sorted RNA sequencing data are available in the public database GEO (ID: GSE194424) in NCBI, NLM, USA (https://www.ncbi.nlm.nih.gov/geo/).

Quantitative real-time RT-PCR analysis. The RNase kit (QIAGEN, Venlo, Netherlands) was used to purify RNA samples from LT-LK stem cells (4 × 10^5) isolated from six Gdpd3+/− tet-CML-affecte mous (SCL-241® TRE-BCR-ABL1® mous) and eight healthy littermate control (SCL-241® TRE-BCR-ABL1® mous) mice at 5 weeks post-Dox withdrawal as described above. These cells were sorted using an Agilent 1000 Bioanalytical BaseMean FoldChange, P-value (two-tailed Wald test)53, and adjusted P-value (P-value) (http://www.screener.org/doc/index.html). MA-plots were created using the Beoke library (version 0.13.0) (https://www.screener.org/doc/index.html). GO enrichment analyses were performed using the DAVID Bioinformatics Resources 6.8, (http://david.abcc.ncifcrf.gov/).

Cell cycle analysis. To determine the cell cycle status of CML-LSK cells in vivo, retro-CML-affecte mous were intraperitoneally administrated BrDU (100 mg·kg⁻¹ of body weight in saline; Sigma) for 3 h. CML-LSK cells were recovered as described above, and stained with anti-BrDU-APC (dilution 1:500). Cell cycle was measured using an Agilent 1000 Bioanalytical BaseMean FoldChange, P-value (two-tailed Wald test)53, and adjusted P-value (P-value) (http://www.screener.org/doc/index.html). MA-plots were created using the Beoke library (version 0.13.0) (https://www.screener.org/doc/index.html). GO enrichment analyses were performed using the DAVID Bioinformatics Resources 6.8, (http://david.abcc.ncifcrf.gov/).
Pharmingen™). The cell cycle distribution of CML-LSK cells was determined using a FACS Aria III instrument.

**Fluorescence immunostaining.** For immunostaining, LT-CML stem cells that were freshly isolated from tetr-X100 affected mice were immediately fixed with 4% paraformaldehyde for one hour. Fixed cells were permeabilised with 0.25% Triton-X100 for 15 min, washed, and blocked by incubation in 2% BSA in TBS for one hour. Blocked cells were incubated overnight at 4°C with rabbit anti-phospho-AKT (Ser473) (dilution 1:50, Cat. # D9E, Cell Signalling Technology, Danvers, MA, USA), rabbit anti-phospho-S6 ribosomal protein (Ser235/236) (dilution 1:50, Clone # D8E, Cat. # 4665S, Lot # 5, Cell Signalling Technology, Danvers, MA, USA), rabbit anti-phospho-β-catenin (dilution 1:25, Clone 8E7, Cat. # 05-665, Lot # 2700799 and 3270747, Millipore, Temecula, CA, USA) mAbs. Primary antibodies (Abs) were blocked with the DNA marker DAPI (Sigma). Stained slides were mounted using ProLong® Diamond® (Thermo Fisher Scientific, Waltham, MA, USA), and fluorescent images were acquired using confocal microscopy (FV10i, Olympus Corporation, Tokyo, Japan) and Photoshop software (CS4 Ver11.0.2, Adobe).

**Duolink® in situ proximity ligation assay.** To examine interactions between Foxo3a and active β-catenin, we used the Duolink® in situ PLA system (Merck, Kenilworth, NJ, USA). LT-CML stem cells that were freshly isolated from tetr-X100 affected mice were immediately fixed with 4% paraformaldehyde for 60 min. For PGE2 treatment, LT-CML stem cells were incubated in 4% O2 at 37°C for 2 h with the appropriate vehicle control or 10 μM PGE2 (Item: 14010, Batch:0533515-998, Thermo Fisher Scientific, Ca, USA). LT-CML stem cells were incubated overnight at 4°C with rabbit anti-Foxo3a IgG (dilution 1:200, Cat. # A11030, Lot # 833292, Molecular Probes, Oregon, OR, USA), AlexaFluor 546-conjugated goat anti-rabbit IgG (dilution 1:200, Cat. # A21245, Lot # 927083, Molecular Probes® antibodies (Abs)). Nuclei were stained with the DNA marker DAPI (Sigma). Stained slides were mounted using ProLong® Diamond® (Thermo Fisher Scientific, Waltham, MA, USA), and fluorescent images were acquired by confocal microscopy (FV10i, Olympus) and Photoshop software (CS4 Ver11.0.2, Adobe).

**Statistical analyses and reproducibility.** For RNA-sequencing in Fig. 7a and Supplementary Figs. 1a–c, FPKM, BaseMean, FoldChange, P-value (two-tailed Wald test) and adjusted P-value (two-tailed Wald test) were determined by the Bioconductor package DESeq2 (ver.1.20.0) (https://bioconductor.org/packages/release/bioc/html/DESeq2.html)52. For the Kaplan-Meier analyses for Fig.7a, b, the P-value (two-tailed Wald test)52,53 were determined by the Survival packages/release/bioc/html/DESeq2.html)52. For the Kaplan-Meier analyses for Supplementary Figs. 1, 3b, 4a–c, 5a, b, 6a–d, 7, 11a–c, 13a, b, and Supplementary Table 1 have been provided as Source Data file. Source data are provided with this paper.

Received: 12 January 2020; Accepted: 26 August 2020; Published online: 17 September 2020

**References**

1. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nature Rev. Mol. Cell Biol.* 9, 112–124 (2008).

2. Serhan, C. N. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 510, 92–101 (2014).

3. Röhrig, F. & Schulze, A. The multifaceted roles of fatty acid synthesis in cancer. *Nat. Rev. Cancer* 16, 732–749 (2016).

4. Kennedy, E. P. & Weiss, S. B. The function of cytidine coenzymes in the biosynthesis of phospholipids. *J. Biol. Chem.* 222, 193–214 (1956).

5. Lands, W. E. Metabolism of glycerolipids: a comparison of leithyglycerolipid synthesis. *J. Biol. Chem.* 231, 883–888 (1958).

6. Shimou, H. & Shimizu, T. Acyl-CoA lysophospholipid acyltransferases. *J. Biol. Chem.* 284, 1–5 (2009).

7. Tokumura, A. et al. Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J. Biol. Chem.* 277, 39436–39442 (2002).

8. Ohshima, N. et al. New members of the mammalian lysophosphatidylcholine phosphodiesterase family: GDE4 and GDE7 produce lysophosphatic acid by lysophosphatic acid D. *J. Biol. Chem.* 290, 4260–4271 (2015).

9. Rahman, I. A. et al. Calcium-dependent generation of N-acylethanolamines and lysophosphatidic acids by glycerophosphodiesterase GDE7. *Biochem. Biophys. Acta 1861*, 1881–1892 (2016).

10. Yung, Y. C., Stoddard, N. C. & Chun, J. LPA receptor signaling: pharmacology, physiology, and pathophysiology. *J. Lipid Res.* 55, 1192–1214 (2014).

11. Kihara, Y., Mizuno, H. & Chun, J. Lysophospholipid receptors in drug discovery. *Exp. Cell Res.* 333, 171–177 (2015).

12. Liu, S. et al. Expression of autotaxin and lysophosphatidic acid receptors increases mammary tumorigenesis, invasion, and metastases. *Cancer Cell* 15, 539–550 (2009).

13. Moolenaar, W. H. & Perrasik, A. Insights into autotaxin: how to produce and present a lipid mediator. *Nat. Rev. Mol. Cell Biol.* 12, 674–679 (2011).

14. Hirai, T. et al. Accumulation of arachidonic acid in human plasma lysophosphatidylinositol at the outer edge of colorectal cancer. *Sci. Rep.* 6, 29935 (2016).

15. O'Hare, T., Zabriksie, M. S., Eiring, A. M. & Deininger, M. W. Pushing the limits of targeted therapy in chronic myeloid leukaemia. *Nat. Rev. Cancer* 12, 513–526 (2012).

16. Corbin, A. S. et al. Human chronic myeloid leukaemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J. Clin. Invest. 121*, 396–401 (2011).

17. Saulele, S., Richter, J., Hochhaus, A. & Mahon, F. X. The concept of treatment-free remission in chronic myeloid leukaemia. *Leukemia* 30, 1638–1647 (2016).

18. Holyoake, T. L. & Vetrie, D. The chronic myeloid leukaemia stem cell: stemming the tide of persistence. *Blood* 129, 1595–1606 (2017).

19. Prost, S. et al. Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature 525*, 380–383 (2015).

20. Chen, Y., Hu, Y., Zhang, H., Peng, C. & Li, S. Loss of the β-catenin, and how they behave. *Nature Rev. Mol. Cell Biol.* 9, 112–124 (2008).

21. Prost, S. et al. Prostaglandin E1 and its analog Misoprostol inhibit human CML stem cell renewal via EP4 receptor activation and repression of AP-1. *Cell Stem Cell* 21, 359–373 (2017).

22. Naka, K. et al. TGF-β-FoxO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature 463*, 676–680 (2010).
26. Nakai, K. et al. Dipeptide species regulate p38MAPK-Smad3 signalling to maintain chronic myelogenous leukaemia stem cells. Nat. Commun. 6, 8039 (2015).

27. Huetter, C. S., Zhang, P., Van Etten, R. A. & Tenen, D. G. Reversibility of acute B-cell leukaemia induced by BCR-ABL1. Nat. Genet. 24, 57–60 (2000).

28. Koscsmier, S. et al. Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL1 leukemogenesis.

29. Wijayatunge, R. et al. Deficiency of the endocytic protein Hip1 leads to decreased G6pd3 expression, low phosphocholine, and kypholordosis. Mol. Cell. Biol. 38, e00385–18 (2018).

30. Brunnert, D. et al. Early in vivo changes of the transcriptome in Philadelphia chromosome-positive CD34+ cells from patients with chronic myelogenous leukemia following imatinib therapy. Leukemia 23, 983–985 (2009).

31. Igarashi, H., Akahoshi, N., Ohno-Nakanishi, T., Yasuda, D. & Ishii, S. The lysophosphatic acid receptor LPA4 regulates hematopoiesis-supporting activity of bone marrow stromal cells. Sci. Rep. 5, 11410 (2015).

32. Barker, N. et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 457, 608–611 (2009).

33. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459, 262–265 (2009).

34. Battle, E. & Clevers, H. Cancer stem cells revisited. Nat. Med. 23, 1124–1134 (2017).

35. Mazerbourg, S. et al. Leucine-rich repeat-containing, G protein-coupled receptor 4 null mice exhibit intrauterine growth retardation associated with embryonic and perinatal lethality. Mol. Endocrinol. 18, 2241–2254 (2004).

36. Hoshii, T. et al. LGR4 regulates the postnatal development and integrity of male reproductive tracts in mice. Biol. Reprod. 76, 303–317 (2007).

37. Carmon, K. S., Gong, X., Lin, Q., Thomas, A. & Liu, Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling. Proc. Natl. Acad. Sci. U.S.A. 105, 11452–11457 (2011).

38. de Lau, W. et al. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signaling. Nature 476, 293–297 (2011).

39. Glinka, A. et al. LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling. EMBO Rep. 12, 1055–1061 (2011).

40. Heidell, F. H. et al. Genetic and pharmacologic inhibition of β-catenin targets induces β-catenin-resistant stem cell lesions in CML. Cell Stem Cell 10, 412–424 (2012).

41. Tenbaum, S. et al. β-catenin confers resistance to PI3K and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer. Nat. Med. 18, 892–901 (2012).

42. Flock, T. et al. Selectivity determinants of GPCR-G-protein binding. Nature 545, 317–322 (2017).

43. Hauser, A. S. et al. Pharmacogenomics of GPCR Drug Targets. Cell 172, 41–54 e19 (2018).

44. Shimada, I., Ueda, T., Kofuku, Y., Eddy, M. T. & Wuthrich, K. GPCR drug candidates: integrating solution NMR data with crystal and cryo-EM structures. Nat. Rev. Drug Discov. 18, 59–82 (2019).

45. Doze, V. A. & Perez, D. M. GPCRs in stem cell function. Prog. Mol. Biol. Transl. Sci. 115, 175–216 (2013).

46. Choi, H. Y. et al. G protein-coupled receptors in stem cell maintenance and somatic reprogramming to pluripotent or cancer stem cells. BMB Rep. 48, 68–80 (2015).

47. Dietrich, P. A. et al. GPR84 sustains aberrant β-catenin signaling in leukemic stem cells for maintenance of MLL leukemiaogenesis. Blood 124, 3284–3294 (2014).

48. Hashimoto, M., Yamashita, Y. & Takemoto, T. Electroporation of Cas9 protein/sgRNA into early pronuclear zygotes generates non-mosaic mutants in the mouse. Dev. Biol. 418, 1–9 (2016).

49. van der Maaten, L. & Hinton, G. Visualizing Data using t-SNE. J. Mach. Learn. Res. 9, 2579–2605 (2008).

50. Yamada, M. et al. A comprehensive quantification method for eicosanoids and related compounds by using liquid chromatography/mass spectrometry with high speed continuous ionization polarity switching. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 995–996, 74–84 (2015).

51. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 (2013).

52. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with Deseq2. Genome Biol. 15, 550 (2014).

53. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B (Methodol.) 57, 289–300 (1995).

54. Salik, B. et al. Targeting RSPO3-LGR4 signaling for leukemia stem cell eradication in acute myeloid leukemia. Cancer Cell 38, 263–278.e6 (2020).

Acknowledgements

We thank D.G. Tenen for the SCL–IT4 and TRE–BCR-ABL1 transgenic mouse strains. H. Nakatani for the C57BL/6–CD45.1 mouse strain, H. Matsushima and Y. Yamashita for genome-editing, K. Watanabe for lipidosics, Y. Takegami and N. Tominaga for RNA-Seq, M. Iwamoto for Lgr4Gt/Gt mouse strain, H. Honda for BCR-ABL1 cDNA, and T. Kitamura for Plat-E retroviral packaging cells. K.N. was supported by a Grant-in-Aid for Scientific Research (B) (KAKENHI Grant Numbers 17H0357800 and 20H0351700) from MEXT, the Government of Japan, and a grant from the Princess Takamatsu Cancer Research Fund (Grant Number 17-24920). S.-J.K. was supported by a grant from the Korea Health Technology R&D Project through the National Cancer Center (HAI17C0037), the Ministry of Health and Welfare, Republic of Korea.

Author contributions

K.N. designed and performed experiments, analysed data, and co-wrote the paper. R.O. and C.K. performed lipidosics analyses. K.-M.Y. performed bioinformatic analyses. T.H., M.A. and K.A. provided the Lgr4Gt/Gt mouse strain. K.M. and K.S. provided viable BM-MNCs from a human CML patient. Y.S. supported animal experiments. D.-W.K. supported CML research. A.O. and S.-J.K. designed experiments and co-wrote the paper.

Competing interests

The authors declare the following competing interests: R.O., E.M. and C.K. are employees of Shimadzu Techno-research, Inc. S.-J.K. has personal financial interests as a shareholder in MedPacto Inc. All other authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials disclosed in the manuscript apart from those disclosed.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-18491-9.

Correspondence and requests for materials should be addressed to K.N.

Peek review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020