Adrenomedullin-CALCRL axis controls relapse-initiating drug tolerant acute myeloid leukemia cells

Clément Larrue1,2, Nathan Guiraud1, Pierre-Luc Mouchel1,3, Marine Dubois1, Thomas Farge1, Mathilde Gotanègre1, Claudie Bosc1, Estelle Saland1, Marie-Laure Nicolau-Travers1,3, Marie Sabatier1, Nizar Serhan1, Ambrine Sahal1, Emeline Boet1, Sarah Mouche2, Quentin Heydt1, Nesrine Aroua1, Lucille Stuani1, Tony Kaoma4, Linus Angenendt5, Jan-Henrik Mikesch5, Christoph Schliemann5, François Vergez1,3, Jérôme Tamburini2,6,7,8, Christian Récher1,3,8 & Jean-Emmanuel Sarry1

Drug tolerant/resistant leukemic stem cell (LSC) subpopulations may explain frequent relapses in acute myeloid leukemia (AML), suggesting that these relapse-initiating cells (RICs) persistent after chemotherapy represent bona fide targets to prevent drug resistance and relapse. We uncover that calcitonin receptor-like receptor (CALCRL) is expressed in RICs, and that the overexpression of CALCRL and/or of its ligand adrenomedullin (ADM), and not CGRP, correlates to adverse outcome in AML. CALCRL knockdown impairs leukemic growth, decreases LSC frequency, and sensitizes to cytarabine in patient-derived xenograft models. Mechanistically, the ADM-CALCRL axis drives cell cycle, DNA repair, and mitochondrial OxPHOS function of AML blasts dependent on E2F1 and BCL2. Finally, CALCRL depletion reduces LSC frequency of RICs post-chemotherapy in vivo. In summary, our data highlight a critical role of ADM-CALCRL in post-chemotherapy persistence of these cells, and disclose a promising therapeutic target to prevent relapse in AML.
Despite improvements in the complete response rate obtained after conventional chemotherapy, the overall survival of acute myeloid leukemia (AML) patients is still poor, due to frequent relapses caused by chemotherapy resistance. Although novel targeted therapies are holding great promises, eradicating drug tolerant/resistant AML cells after chemotherapy remains the major challenge in the treatment of AML. AML arises from self-renewing leukemic stem cells (LSCs) that can repopulate human AML when assayed and xenografted in immunocompromised mice. Even though these cells account for a minority of leukemic burden, gene signatures associated with a stem cell phenotype or function have an unfavorable prognosis in AML. Clinical relevance is evidenced by the enrichment in LSC-related gene signatures in AML specimens at the time of relapse compared to diagnosis. While it has been initially shown that LSCs may be less affected by chemotherapy than more mature populations, recent works demonstrated that the anti-AML chemotherapy cytarabine (AraC) might deplete the LSC pool in patient-derived xenograft (PDX) models. These results suggest the coexistence of two distinct LSC populations, one chemosensitive and thus eradicated by conventional treatments and one that is chemoresistant, persists and might induce relapse in AML (relapse-initiating drug-resistant leukemic stem cells, RIC). Thus, a better phenotypical and functional characterization of RICs is crucial for the development of new AML therapies aiming at reducing the risk of relapse.

Although it was first proposed that the LSC-compartment was restricted to the CD34+CD38− subpopulation of human AML cells, several studies subsequently demonstrated that LSCs are phenotypically heterogeneous when assayed in NSG mice. These observations highlight that more functional studies are needed to better and more fully characterize LSCs, particularly under the selection pressure imposed by chemotherapy. Eradicating LSCs without killing normal hematopoietic stem cells (HSCs) depends on the identification of therapeutically relevant markers that are overexpressed in the AML compartment. In recent years, tremendous research efforts led to the identification of several cell surface markers such as CD47, CD123, CD44, TIM-3, CD25, CD32, or CD93 discriminating LSCs from HSCs. In addition, it has been proposed that LSCs also have both a specific decrease in their reactive oxygen species (ROS) content and an increase in BCL2-dependent oxidative phosphorylation (OxPHOS), revealing a vulnerability that can be exploited by treatment with BCL2 inhibitors such as venetoclax. This is consistent with several studies demonstrating that mitochondrial OxPHOS status contributes to drug resistance in myeloid leukemia. Taken together, these results suggest that specific characteristics of LSCs can be exploited to develop targeted therapeutic approaches.

Here, we report that the cell surface G protein-coupled receptor (GPCR) family calcitonin receptor-like receptor (CALCRL) and its ligand adrenomedullin (ADM) are expressed in AML cells and that CALCRL sustains LSC function. High expression of both CALCRL and ADM is predictive of an unfavorable prognosis in a cohort of 179 AML patients. Moreover, depletion of CALCRL abrogates leukemic growth and dramatically induces cell death in vivo. Transcriptomic and functional analyses show that CALCRL drives E2F1, BCL2, and OxPHOS pathways involved in the chemoresistance. Furthermore, we observed that cell surface expression of CALCRL is enriched after AraC treatment in PDX models as well as after intensive chemotherapy in AML patients. Limiting dilution analyses coupled to genetic manipulation demonstrates that RICs are critically dependent on CALCRL for their maintenance. Altogether, our findings demonstrate that CALCRL is a new RIC player with a critical effector role in both their stemness and chemoresistance, and thus is a relevant target to eradicate this specific cell population.

Results

The receptor CALCRL and its ligand adrenomedullin are expressed in AML cells and associated with a poor outcome in patients. Using a clinically relevant chemotherapeutic model, we and others previously demonstrated that LSCs are not necessarily enriched in post-AraC residual AML, suggesting LSCs include both chemosensitive and chemoresistant stem cell subpopulations. In order to identify new vulnerabilities in the chemoresistant LSC population that might be responsible for relapse, we analyzed transcriptomic data from three different studies that (Fig. 1a and Table S1): (i) identified 134 genes overexpressed in functionally defined LSC compared with a normal HSC counterpart (Eppert et al., 2011; GSE30377); (ii) uncovered 114 genes of high expression associated with poor prognosis in AML (the Cancer Genome Atlas, AML cohort, 2013); and (iii) selected 536 genes overexpressed at relapse compared to pairwise matched diagnosis samples (Hackl et al., 2015; GSE66525). Surprisingly, we found one unique gene common to these three independent transcriptomic datasets: CALCRL, encoding the G protein-coupled seven-transmembrane domain receptor poorly documented in cancer that has been recently described as associated with a poor prognosis in AML. Using four independently published cohorts of AML patients (TCGA AML cohort; GSE12417; GSE14468; BeatAML cohort), we observed that patients with high CALCRL expression had a shorter overall survival (Fig. 1b and Fig. S1a) and are more refractory to chemotherapy (Fig. S1b) compared to patients with low CALCRL expression. This correlated with a higher expression in complex versus normal karyotypes (Fig. S1c). Furthermore, CALCRL gene expression was significantly higher at relapse compared to diagnosis in patients treated with intensive chemotherapy (Fig. 1c). CALCRL expression was also higher in the leukemic compartment compared with normal hematopoietic cells, and more specifically in the LSC population as both functionally (Fig. 1d) and phenotypically (Fig. S1d) defined, compared with the AML bulk population. Interestingly, CALCRL expression was higher in the more immature AML subtypes according to FAB stratification, suggesting that CALCRL is a marker of cell immaturity (Fig. S1e). Using flow cytometry, we determined that CALCRL was expressed at the cell surface (Fig. S1f), more markedly in leukemia compared to normal CD34+ hematopoietic progenitor cells (Fig. S1g; see Table S2 for mutational status of patients). Of note, CALCRL expression did not correlate with any most-found mutations (Fig. S1h). Next, we assessed the expression of ADM, a CALCRL ligand already described in several solid cancers. The ADM gene is overexpressed in AML cells compared to normal cells (Fig. S1j, k), although its expression is not altered in AML patients at relapse compared to diagnosis (Fig. S1l) and is not linked to mutational status (Fig. S1i). Using a combination of western blotting, confocal microscopy, and RNA microarray, we have established that CALCRL, its three co-receptors RAMP1, RAMP2 and RAMP3, as well as ADM (but not GCRP, another putative CALCRL ligand) are expressed in all the tested AML cell lines and primary AML samples (Fig. S1m–r). Moreover, analysis of three independent cohorts (TCGA, Verhaak et al. and BeatAML) confirmed that only ADM was highly expressed in primary samples compared to CALCA and CALCB (two genes encoding GCRP) that were not expressed or poorly expressed (Fig. S1s). Next, we addressed the impact of CALCRL and ADM protein levels at diagnosis on patient outcome. Using IHC analyses, we observed that increasing protein levels of CALCRL or ADM were associated with decreasing complete remission rates, inferior 5-year overall survival and event-free survival (EFS) in a cohort of 179 intensively treated AML patients (Fig. 1e, f). When patients were clustered into 4 groups according to CALCRL and ADM expression (low/low vs low/high vs high/
low vs high/high; Fig. 1G and Table S3), we observed that the CALCRL\textsuperscript{high}/ADM\textsuperscript{high} group was associated with the lowest overall survival and that high expression of only CALCRL or ADM also correlated to reduced EFS and complete remission rate (Fig. 1h). Next, we addressed the protein level of CGRP using IHC analyses and we detected a slight diffuse signal of this protein, suggesting a paracrine secretion of CGRP in AML. However, we demonstrated that protein levels of CGRP had no impact on 5-year overall survival and EFS (Fig. S2a–c), indicating that ADM was likely the main driver of CALCRL activation in AML.

All these data supported the hypothesis that the ADM-CALCRL axis is activated in an autocrine-dependent manner and associated with a poor prognosis in AML.

The CALCRL-ADM axis is required for cell growth and survival. Next, we investigated the impact of deregulated CALCRL-ADM axis in cell proliferation and survival. CALCRL depletion was associated with a decrease in blast cell proliferation (Fig. 2a) and an increase in cell death (Fig. 2b) in three AML (MOLM-14, OCI-AML2, OCI-AML3) cell lines. Furthermore, ADM-targeting
shRNA (Fig. S3a) phenocopied the effects of shCALCRL on cell proliferation and apoptosis in MOLM-14 and OCI-AML3 cells (Fig. S3b, c). In order to confirm these results in vivo and control the invalidation of the target over time, we have developed tetracycline-inducible shRNA models. First, we established control shRNA or shCALCRL were expressed in MOLM-14, OCI-AML2, or OCI-AML3 cell lines. (a) Graph shows cell number of MOLM-14, OCI-AML2, or OCI-AML3. Three days after transduction, cells were plated at 0.3 M cells/ml (D0) and cell proliferation was followed using trypan blue exclusion (n = 4 independent experiments for OCI-AML2 and n = 6 independent experiments for MOLM-14 and OCI-AML3, two-way ANOVA). (b) Graph shows the percentage of Annexin-V+/7-AAD+ cells 4 days after cell transduction (n = 3 independent experiments for MOLM-14 and OCI-AML3 and n = 4 independent experiments for OCI-AML2, unpaired t-test). (c) Investigation of the role of CALCRL on leukemic cell growth in vivo in a context of engrafted cells. (d) Leukemia burden measured using mCD45.1+/hCD33+/hCD33+/AnnV− markers (unpaired t-test).  

**Fig. 2 CALCRL depletion reduces AML cell growth in vitro and in vivo.** a-c Control shRNA or shCALCRL were expressed in MOLM-14, OCI-AML2, or OCI-AML3 cell lines. (a) Graph shows cell number of MOLM-14, OCI-AML2, or OCI-AML3. Three days after transduction, cells were plated at 0.3 M cells/ml (D0) and cell proliferation was followed using trypan blue exclusion (n = 4 independent experiments for OCI-AML2 and n = 6 independent experiments for MOLM-14 and OCI-AML3, two-way ANOVA). (b) Graph shows the percentage of Annexin-V+/7-AAD+ cells 4 days after cell transduction (n = 3 independent experiments for MOLM-14 and OCI-AML3 and n = 4 independent experiments for OCI-AML2, unpaired t-test). (c) Investigation of the role of CALCRL on leukemic cell growth in vivo in a context of engrafted cells. (d) Leukemia burden measured using mCD45.1+/hCD33+/hCD33+/AnnV− markers (unpaired t-test). e Mice survival monitoring (Log-rank (Mantel-Cox) test). f Investigation of the role of CALCRL on leukemic cell growth in vivo in a context of engrafted cells. g Measurement of Leukemia burden (unpaired t-test). h Mice survival monitoring (Log-rank (Mantel-Cox) test). Data are mean ± s.e.m.
CALCRL is required for leukemic stem cell maintenance. As CALCRL expression is linked to an immature phenotype and CALCRL-depletion impaired AML cell growth, we next aimed to investigate the role of CALCRL in LSC biology. First, we analyzed for gating strategy, demonstrating the requirement of CALCRL in preserving the function of LSCs.

Depletion of CALCRL alters cell cycle and DNA repair pathways in AML. To examine regulatory pathways downstream of CALCRL, we generated and performed comparative transcriptomic and functional assays on shCTR vs shCALCRL MOLM-14 cells. CALCRL knockdown was associated with a significant decrease in the expression of 623 genes and an increase in 278 genes (FDR < 0.05) (Fig. 4a; see Data Source document). Data mining and western blotting analyses showed significant

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**Fig. 3 CALCR is required for leukemic stem cell maintenance.** a Barplot shows the percentage of CALCRL-positive cells in (n = 11 primary AML samples) in cells classified as HSC-like, progenitor-like, GMP-like, Promonocyte-like, Monocyte-like, or cDC-like malignant cells (Van Galen et al. 2019). b Heatmap shows the percentage of CALCRL-positive cells in each patient individually. c SPRING visualization of single-cell transcriptomes. Points are color-coded by indicated cell-type annotations. d Phenotypic distribution and number of CALCRL-positive cells. e GSEA of stem cell signatures functionally identified by Eppert et al. or phenotypically defined by Gentles et al. or Ng et al. was performed using transcriptomes of cells expressing low (blue) vs high (red) levels of CALCRL gene (TCGA, AML cohort, Enrichment Score based on a Kolmogorov-Smirnov statistic). f Primary AML samples or cells from primary mice were collected and treated ex vivo with siCTR or siCALCR and transplanted in limiting doses into primary or secondary recipients. Human marking of >0.1% was considered positive for AML engraftment except for AML#31 for which the cut-off was 0.5% because the sample was hCD33− (Poisson statistic). g Engraftment results. Data are mean ± error bars (upper and lower limit, Poisson statistic).
depletion in genes involved in cell cycle and DNA integrity pathways (Fig. 4b, c), and a reduction in protein level of RAD51, CHEK1, and BCL2 in shCALCRL AML cells (Fig. 4d). This was associated with an accumulation of cells in the G1 phase (Fig. 4e, f). Interestingly, enrichment analysis showed that depletion of CALCRL affects the gene signatures of several key transcription factors such as E2F1, P53, or FOXM1 described as critical cell cycle regulators (Fig. 4g). We focused on the E2F1 transcription factor, whose importance in the biology of leukemic stem/progenitors cells has recently been shown32. We first confirmed that CALCRL depletion was closely associated with a significant decrease in the activity of E2F1 (Fig. 4h). As E2F1 activity is mainly regulated by CDK-cyclin complexes, we assessed the protein expression of these actors after CALCRL downregulation. We observed a decrease in both the phosphorylation of Rb and the expression of cyclins A, B1, D1, and E, reflecting the cell cycle arrest in cells depleted for CALCRL (Fig. 4i).

Then, we demonstrated that the knockdown of E2F1 affected protein expression of RAD51, CHK1, but not BCL2 (Fig. 4j), inhibited cell proliferation (Fig. 4k), cell cycle progression (Fig. 4l, m),...
and induced cell death in both MOLM-14 and OCI-AML3 (Fig. 4n). We further investigated whether CALCRL might regulate the proliferation of primary AML cells. Interestingly, CALCRL protein level positively correlated with clonogenic capacities in methylocellose (Fig. 4o). Moreover, the depletion of CALCRL in primary samples decreased the number of colonies (Fig. 4p), and BCL2 and RAD51 protein levels (Fig. 4q). All these results suggest that CALCRL has a role in the proliferation of AML blasts and controls critical pathways involved in DNA repair processes.

**CALCRL downregulation sensitizes leukemic cells to chemotherapeutic drugs.** Based on putative targets of CALCRL such as BCL2, CHK1, or FOXM1, we hypothesized that CALCRL was involved in chemoresistance. Accordingly, CALCRL depletion sensitized MOLM-14 and OCI-AML3 cells to AraC and idarubicin as assessed by the reduction in cell viability (Fig. 5a) and the induction of cell death (increased Annexin-V staining, Fig. 5b); and increased cleavage of apoptotic proteins Caspase-3 and PARP, Fig. 5a). Furthermore, depletion of ADM or E2F1 also sensitized AML cells to these genotoxic agents (Fig. 5c). These results indicate that DNA repair pathways were affected when the ADM-CALCRL axis was impaired in AML.

**Downregulation of CALCRL sensitizes leukemic cells to chemotherapy in vivo.** We xenografted NSG mice with AML cell lines transduced with inducible shCALCRL demonstrating the same chemosensitization profile than constitutive shRNAs (Fig. S5d, e). After full engraftment, CALCRL was depleted by doxycycline and mice were treated with 30 mg/kg/day AraC for 5 days (Fig. 6a). While AraC alone had no effect on AML proliferation, AraC in combination with shCALCRL significantly reduced the total number of blasts (Fig. 6b), induced a higher rate of cell death (Fig. 6c), and prolonged survival of mice (Fig. 6d) compared to others conditions. Furthermore, MOLM-14 cells expressing shCTR and treated with vehicle or AraC were FACS-sorted and plated in vitro for further experiments. Interestingly, after 1 week of in vitro culture, human AML cells from AraC-treated mice were more resistant to AraC (IC50: 1 μM for vehicle group vs 5.40 μM for AraC-treated group) and idarubicin (IC50: 31.98 nM for vehicle group vs 111.3 nM for AraC-treated group) (Fig. 6e). Next, we observed that AML cells treated with AraC in vivo had higher protein expression levels of CALCRL, and a slight increase in RAD51 and BCL2, whereas CHK1 was similar to untreated cells (Fig. 6f). To evaluate the role of CALCRL in this chemoresistance pathway in vivo, we depleted CALCRL in these cells. Knockdown of CALCRL by two different shRNAs sensitized cells to AraC and idarubicin compared to shCTR in cells treated with vehicle (Fig. 6g) or AraC alone (Fig. 6h). Remarkably, the IC50 of AraC and idarubicin in AraC-treated cells in vivo and transduced with shCALCRL was also decreased, demonstrating that CALCRL participated to chemoresistance pathways in AML. We further aimed to determine what ligand might be involved into this chemoresistance pathway. First, we depleted RAMP1, RAMP2, or RAMP3 using shRNAs (Fig. S6a) and observed that only RAMP2 was necessary to sustain the growth of MOLM-14 cells (Fig. S6b). Consistent with this result, RAMP2 downregulation induced cell death and sensitized AML cell to AraC and Ida (Fig. S6c). We also showed that only exogenous ADM1-52 and ADM13-52, but not CGRP, were able to decrease AraC-induced cell death in MOLM-14 cell line, highlighting that the CALCRL-driven chemoresistance is mainly due to the ADM ligand (Fig. 6d). We used a PDX model treated with a CGRP inhibitor, olcegepant, in association with AraC to confirm these results (Fig. S7a). We showed that CGRP inhibition did not affect leukemic burden and did not sensitize to AraC in vivo (Fig. S7b). Interestingly, AraC upregulated CALCRL (Fig. S7c-e), confirming our in vitro observations in vivo in PDX model. As previously described and as a positive control of the in vivo activity of olcegepant, we showed that this drug efficiently decreased the percentage of murine CMP but not LSK and GMP subpopulations (Fig. S7f-h). Altogether, these experiments indicate that ADM was the main ligand of CALCRL in AML.

Because mitochondrial metabolism has emerged as a critical regulator of cell proliferation and survival in basal and chemotherapy-treated conditions in AML, we analyzed the impact of CALCRL depletion on mitochondrial function. GSEA showed a significant depletion in the gene signature associated with mitochondrial oxidative metabolism in the shCALCRL MOLM-14 cells (Fig. S8a and Table S4). Mitochondrial oxygen consumption...
chemotherapy may represent an innovative therapeutic approach in cells untreated or treated 24 h with Ara-C (1 µM). Mitochondria remain functional (Fig. S8b). We also consistently observed rescue almost entirely inhibited basal apoptosis induced by the depletion of CALCRL and by the combination with AraC or idarubicin (Fig. S8k).

Overall, these results suggest that CALCRL mediates the chemoresistance of AML cells.

Depletion of CALCRL in residual disease after AraC treatment impedes LSC function. To address the role of CALCRL in response to chemotherapy in primary AML samples, we used a clinically relevant PDX model of AraC treatment in AML12. After engraftment of primary AML cells, NSG mice were treated for 5 days with AraC and sacrificed at day 8 to study the minimal residual disease (MRD; Fig. 7a). We analyzed 10 different PDX models and stratified them according to their response to AraC as low (fold change, FC AraC-to-Vehicle <10) or high (FC > 10) responders (Fig. 7b). The percentage of cells positive for CALCRL in the AML bulk was approximately doubled in the low responder group compared to the high responder group (3.6% vs 7.8%; p=0.0434; Fig. 7d).

As it has been reported that BCL2 controlled the oxidative status in AML cells, we investigated its role downstream of CALCRL. We showed that upon AraC treatment, the overexpression of BCL2 in MOLM-14 cells (Fig. S8g) is sufficient to rescue maximal respiration but not basal respiration. This suggested a role of the CALCRL-BCL2 axis in maintaining some aspects of mitochondrial function in response to AraC. This was not related to energy production, as neither mitochondrial ATP production nor ECAR were affected (Fig. S8i, j). Finally, BCL2 rescue almost entirely inhibited basal apoptosis induced by the

measurements revealed a modest but significant reduction in basal OCR, whereas maximal respiration was conserved, indicating that mitochondria remain functional (Fig. S8b). We also consistently observed a significant decrease in mitochondrial ATP production by shCALCRL (Fig. S8c). We and other groups have previously shown that chemoresistant cells have elevated oxidative metabolism and that targeting mitochondria in combination with conventional chemotherapy may represent an innovative therapeutic approach in AML12,23,27. Since depletion of CALCRL modestly decreased OCR and more greatly decreased mitochondrial ATP in AML cells (Fig. S8b, c), we assessed cellular energetic status associated with AraC. Knockdown of CALCRL significantly abrogated the AraC-induced increase in basal respiration and maximal respiration (Fig. S8d). Moreover, we observed a decrease in mitochondrial ATP production in response to AraC upon CALCRL silencing (Fig. S8e), whereas glycolysis (e.g., ECAR) was not affected (Fig. S8f).

Fig. 5 Depletion of CALCRL sensitizes AML cells to chemotherapy through induction of DNA damages. a Four days after transduction, MOLM-14 or OCI-AML3 cells were treated with AraC or idarubicin for 48 h. Then cell viability was assessed by MTS assay and values were normalized to untreated condition (n = 3 independent experiments). Curve fit to calculate IC50 was determined by log (inhibitor) vs response (three parameters). b Graph shows the percentage of AnnV+/7-AAD+ cells. Four days after transduction, cells were treated with chemotherapeutic agents for 48 h before flow cytometry analysis (n = 4 independent experiments, unpaired t-test). c Colonies in methylcellulose were counted 1 week after transfection of primary AML samples with shCTR or shCALCRL 5 nM AraC (n = 7 primary AML samples, paired t-test). d Detection of double strand breaks by alkaline comet assays in MOLM-14 cells untreated or treated 24 h with AraC (1 µM). Representative pictures of nuclei in the same experiment. e Quantification of alkaline comet tail moments for one representative experiment (135–413 nuclei were analyzed for each treatment, unpaired t-test) out of four. Data are mean ± s.e.m.
Chemotherapy reduced both percentage of human cells (Fig. 5a) and levels of secreted ADM (Fig. 5b) in the bone marrow of mice. The correlation between leukemia burden and cell injection: MOLM-14 expressing indicated inducible shRNAs were injected into the tail vein of NSG mice. Ten days later, when disease was established, mice were treated with 30 mg/kg/d AraC for 5 days. On day 18, a group of mice was kept to follow survival (d). The other group was treated with AraC or idarubicin (h) treated mice were transduced with indicated shRNA. After 4 days cells were treated with AraC or idarubicin ex vivo for 48 h. Cell viability was assessed by MTS assay and values were normalized to untreated condition (n = 1 in quadruplicate). Curve fit to calculate IC50 was determined by log (inhibitor) vs response (three parameters). f Western blotting for CALCRL, RAD51, CHK1, BCL2, and β-ACTIN. Cellular extracts were collected 2 days after mice sacrifice. g, h Human cells from vehicle (g) or AraC (h) treated mice were transduced with indicated shRNA. After 4 days cells were treated with AraC or idarubicin for 48 h and cell viability was assessed by MTS assay (n = 1 in quadruplicate). Curve fit to calculate IC50 was determined by log (inhibitor) vs response (three parameters) test. Data are mean ± s.e.m.

Next, we aimed at determining the role of CALCRL in the LSC function maintenance of the RIC population. Leukemic cells from patients collected at diagnosis were injected into NSG mice, and after engraftment and treatment with AraC, human viable AML cells constituting MRD were collected and transfected with siCTR or siCALCRL before LDA transplantations into secondary recipients (Fig. 7g). A significant reduction in LSC frequency was observed in the samples depleted from CALCRL compared to the controls in the two primary AML samples tested (Fig. 7h, i).

We next examined cell surface expression of CALCRL in patients before and after intensive chemotherapy (Fig. 7j). Treatment decreased the percentage of blasts in the bone marrow (Fig. 7k), accompanied with a significant enrichment in CALCRL- positive cells (Fig. 7l). Moreover, we observed a continuous enrichment in CALCRL+ blasts following chemotherapy (12.9% at diagnosis, 32.8% at day 35, 81% at relapse; Fig. 7m). We further performed an ex vivo assay on a relapse sample followed by LDA in NSG mice (Fig. 7n). We demonstrated that CALCRL depletion significantly reduced LSC frequency, highlighting a critical role for CALCRL in the maintenance of the clone present at relapse (Fig. 7o, p). Recently Shlush et al. proposed an elegant model of relapses with two situations: in the first one called “relapse origin-primitive” (ROP), relapse originated from rare LSC clones only detectable in HSPC or after xenotransplantation. In the second model, called “relapse origin-committed” (ROC), the relapse clone
arose from immunophenotypically committed leukemia cells in which bulk cells harbored a stemness transcriptional profile. We analyzed this transcriptomic database and found that at the time of diagnosis, CALCRL expression was higher in blasts with ROC than with ROP phenotype, in accordance with the expression of CALCRL in cells harboring stem cell features (Fig. S9C).

Interestingly, CALCRL was strongly increased at relapse in ROP patients, which correlated with the emergence of a clone with stem cell properties at this stage of the disease (Fig. S9d). These observations supported the hypothesis of the preexistence of a relapse-relevant LSC population, rare (ROP) or abundant (ROC), expressing high levels of CALCRL.
Altogether, these results strongly support the conclusion that CALCR preserves LSC function after chemotherapy, thus representing an attractive therapeutic target to eradicate the clone at the origin of relapse.

Discussion

LSC-selective therapies represent an unmet need in AML due to high plasticity and heterogeneity not only of the phenotype\textsuperscript{2,14,15} but also for the drug sensitivity\textsuperscript{12,13} of the LSC population. However, fundamental studies focusing on intrinsic properties of LSCs such as their resistance to chemotherapy are crucially needed for the development of better and more specific therapies in AML.

Our study provides key insights of LSC biology and drug resistance and identifies the ADM receptor CALCR as a master regulator of RICs. Our work first shows that CALCR gene is overexpressed in the leukemic compartment compared to normal hematopoietic tissue based on Eppert’s study that functionally characterizes LSCs. CALCR could be specifically upregulated by LSC-related transcription factors such as HIF1\textalpha or ATF4\textsuperscript{41,42}. Indeed, both ADM and CALCR possess the consensus hypoxia-response element (HRE) in the 5\textprime-flanking region and are HIF1\textalpha-regulated genes\textsuperscript{43}. Recently, it has been demonstrated that the integrated stress response and the transcription factor ATF4 is involved in AML cell proliferation and is uniquely active in HSCs and LSCs\textsuperscript{42,44}. Interestingly, maintenance of murine HSCs under proliferative stress but not steady-state conditions is dependent on CALCR signaling\textsuperscript{45}. Accordingly, CALCR might support leukemic hematopoiesis and overcome stress induced by the high proliferation rate of AML cells. As opposed to ADM, CGRP is slightly expressed in BM from AML patient biopsies at diagnosis and its expression does not have a prognostic value in AML. Under inflammatory conditions to regulate immune responses or post-chemotherapeutic stress responses, continuous CGRP secretion is increased in normal hematopoietic cells or relapse-initiated cells as paracrine signal\textsuperscript{35,46}. In this context, CGRP is one of the components of the CALCR effects, while the ADM-CALCR is the major driver of these RICs in an autocrine-dependent manner.

Our findings clearly show that targeting CALCR expression impacts clonogenic capacities, cell cycle progression, and genes related to DNA repair and genomic stability. Cancer stem cells and LSCs are predominantly quiescent, thereby spared from chemotherapy. However, recent studies suggested that LSCs may also display a more active cycling phenotype\textsuperscript{47,48}. C-type lectin CD93 is expressed on a subset of actively cycling, non-quiescent AML cells enriched for LSC activity\textsuperscript{17}. Recently, Pei et al.\textsuperscript{46} showed that targeting the AMPK-FIS1 axis disrupted mitophagy and induced cellular cycle arrest in AML, leading to the depletion of LSC potential in primary AML. These results are consistent with the existence of different subpopulations of LSCs that differ in proliferative state. Moreover, FIS1 depletion induces the down-regulation of several genes (e.g., CCND2, CDC25A, PLK1, CENPO, AURKB) and of the E2F1 gene signature that were also identified after CALCR\textsuperscript{45} knockout. Recently, it has been proposed that E2F1 plays a pivotal role in regulating the CML stem/ progenitor cells proliferation and survival status\textsuperscript{32}. Several signaling pathways, for instance MAPKs, CDK/cyclin, or PI3K/ AKT, have been described to be stimulated by ADM/CALCR axis and may control pRB/E2F1 complex activity\textsuperscript{47,48}. Other signaling mediators activated in LSCs such as c-Myc and CEBP\alpha regulate E2F1 transcription and allow the interaction of the E2F1 protein with the E2F gene promoters to activate genes essential for DNA replication at G1/S, cell proliferation, and survival in AML\textsuperscript{49–51}. Therefore, our analysis of cellular signaling downstream of CALCR uncovers new pathways crucial for the maintenance and the chemoresistance of LSCs.

The characterization of RICs, which are detected at low threshold at diagnosis and strongly contributes to chemotherapy resistance, is necessary to develop new therapies with the aim of reducing AML relapses. Boyd and colleagues have proposed the existence of a transient state of LSCs during the immediate and acute response to AraC that is responsible for disease regrowth foregrowing the recovery of LSC pool\textsuperscript{53}. In this attractive model and in the dynamic period of post-chemotherapy MRD, CALCR-positive AML cells belong to this leukemia-regenerating cell subpopulation and CALCR is essential for the preservation of the LSC potential of primary chemoresistant AML cells. It would be interesting to determine whether chemotherapy rather primarily spares CALCR-positive cells and/or induces an adaptive response that increases the expression of CALCR.

In summary, while two distinct chemo-sensitive and chemoresistant LSC subpopulations coexist in AML, our study further defines this therapy-resistant LSC subpopulation responsible for relapse in patients and PDX as RICs. Our data also identify CALCR as an AML actor of RICs. CALCR is involved in chemoresistance mechanisms, and its depletion sensitizes AML cells to chemotherapy in vitro and in vivo. Finally, our results pinpoint CALCR as a new and promising candidate therapeutic target for eradicating the LSC subpopulation that initiated relapse in AML.

Methods

Human studies. De-identified primary AML patient specimens are from Toulouse University Hospital (TUH), Toulouse, France. Frozen samples of patients diagnosed with AML were obtained from TUH after signing a written informed consent for research use in accordance with the Declaration of Helsinki, and stored at the HIMIP collection (BB-0033-00060). According to the French law, HIMIP biobank collection has been declared to the Ministry of Higher Education and Research (DC 2008-307, collection 1) and obtained a transfer agreement for research applications (AC 2008-129) after approval by our institutional review board and ethics committee (Comité de Protection des Personnes Sud-Ouest et Outremer II). Clinical and biological annotations of the samples have been declared to the CNIL (Comité National Informatique et Libertés, i.e., Data processing and
The cut-off was increased to >0.5% for AML#3 because the expression was measured only based on hCD45+ mCD45.1-. Limiting dilution analysis was performed using L-calc software. List of antibodies used in this work: Anti-CALCRL (Jean-Emmanuel Sarry lab), Anti-mCD45.1 PERCP/CY5.5 (BD, Cat# 560580), Anti-hCD45 APC (BD, Cat# 555485), Anti-hCD34 AF700 (BD, Cat# 561440), Anti-hCD38 PECY7 (BD, Cat# 335825), Anti-hCD33 PE (BD, Cat# 555450), and Anti-hCD34 BV421 (BD, Cat# 562890).

**Immunohistochemistry.** Expression protein was investigated by immunoreactivity scoring on tissue microarrays containing pre-therapeutic bone marrow samples from intensively treated AML patients. Studies on the tissue microarray have been approved by the institutional review board of the University of Münster. Detailed information on the AML tissue microarray cohort and CALCRL expression has been published previously (Angenendt et al. 2009). The tissue microarrays were stained using the anti-ADM (Abcam, Cat# ab69117) antibody as described previously (Angenendt et al. 2009). Brieﬂy, following deparafﬁnization and heat-induced epitope unmasking, 4 μm tissue sections were incubated with the primary antibodies, followed by suitable secondary and tertiary antibodies (Dako). Immunoreactions were visualized with a monocular APAAP-complex and a fuchsin-based substrate-chromogen system (Dako). Counterstaining was performed with Mayer’s haemalum (Merck). Two investigators who were blinded towards patient characteristics and outcome independently assessed intensity of staining (1 = no weak, 2 = moderate, 3 = strong staining intensity) and percentage of positive cells at each intensity level. Subsequently, H-scores were calculated as described previously [H-score = 1 × (percentage of blasts positive at 1) + 2 × (percentage of blasts positive at 2) + 3 × (percentage of blasts positive at 3)] (Angenendt et al. 2009). There was a good inter-investigator agreement (r = 0.91 for ADM, p < 0.0001). Samples from 179 AML patients were evaluable for CALCRL and ADM. Images were taken using a Nikon Eclipse 50i microscope equipped with a Nikon DX-MV2.

**Western blot analysis.** Proteins were resolved using 4–12% polyacrylamide gel electrophoresis Bis-Tris gels (Life Technology, Carlsbad, CA) and electro-transferred to nitrocellulose membranes. After blocking in Tris-buffered saline (TBS) 0.1%, Tween 20%, and 5% bovine serum albumin, membranes were immunostained overnight with appropriate primary antibodies followed by incubation with secondary antibodies conjugated to HRP. Immuno-reactive bands were visualized by enhanced chemiluminescence (ECL; Supersignal West Pico; Thermo Fisher Scientiﬁc) with a Syngene camera. Quantification of chemiluminescent signals was done with the GeneTools software v4.3.80 from Syngene. List of antibodies used in this work was: anti-CASPASE-3 (CST, Cat#9662; 1/1000), Anti-ACTIN (Millipore, Cat# MAB1501; 1/10,000), anti-CALCRL (Elabscience, Cat# ESAP13421; 1/1000), anti-RAD51 (Abcam, Cat# ab133534; 1/1000), anti-BCL2 (CST, Cat# 2872; 1/100), anti-E2F1 (C-20)(Santa Cruz, Cat# sc-193; 1/1000), anti-CHKI (Santa Cruz, Cat# sc-8408; 1/1000), anti-RAMP1 (3B9) (Santa Cruz, Cat# sc-293438; 1/1000), anti-RAMP2 (B-5) (Santa Cruz, Cat# sc-356240; 1/1000), anti-RAMP3 (G-1) (Santa Cruz, Cat# sc-365313; 1/1000), anti-ADM (Thermo Fisher Scientiﬁc, Cat# PAS-24927; 1/1000), anti-CGRP (Abcam, Cat# ab47027; 1/1000), anti-PARP (Thermo Fisher Scientiﬁc, Cat# 44-698C; 1/1000), and anti-alpha/beta-Tubulin (CST, Cat# 2148; 1/1000).

**Cell death assay.** After treatment, 5 × 105 cells were washed with PBS and resuspended in 200 μl of Annexin V-binding buffer (BD, Cat# 556420). Two microliters of Annexin-V-FITC (BD, Cat# 556454) and 7-amo-actinomycin D (7-AAD; Sigma Aldrich) were added for 15 min at room temperature in the dark. All samples were analyzed using LSFortessa or CytoFLEX flow cytometer.

**Cell cycle analysis.** Cells were harvested, washed with PBS, and fixed in ice-cold 70% ethanol at −20 °C. Cells were then permeabilized with 1× PBS containing 0.25% Triton X-100, resuspended in 1× PBS containing 10 μg/ml propidium iodide and 1 μg/ml RNAse, and incubated for 30 min at 37 °C. Data were collected on a CytoFLEX flow cytometer.

**Clonogenic assay.** Primary cells from AML patients were thawed and resuspended in 100 μl Nucleofector Kit V (Axuma, Cologne, Germany). Then, cells were nucleofected according to the manufacturer’s instructions (program U-001 Axuma, Cologne, Germany) with 200 nM siRNA scrambled (ON-TARGETplus Non-targeting siRNA #2, Dharmacon) or anti-CALCRL (SMARTpool ON-TARGETplus CALCR siRNA, Dharmacon). Cells were adjusted to 1 × 106 cells/ml final concentration in H4230 methylcellulose medium (STEMCELL Technologies) supplemented with 10% 5637-CL as a stimulant and then plated in 35-mm plates in duplicate and incubated to grow for 7 days in a humidified CO2 incubator (5% CO2, 37 °C). At day 7, the leukemia colonies (>5 cells) were scored.

**Plasmid cloning, shRNA, lentiviral production, and leukaemic cell transduction.** shRNA sequences were constructed into pLKO.TET-ON or bought cloned into pLKO vectors. Each construct (6 μg) was co-transfected using lipofectamine 2000 (20 μl) in 10 cm dish with psPax2 (4 μg, provides packaging proteins) and pMD2.G (2 μg).
provides VSV-g envelope protein) plasmids into 293T cells to produce lentiviral particles. Twenty-four hours after cell transfection, medium was removed and 10 ml opti-MEM with 3% Pen/Strep was added. At about 72 h post-transfection, 293t culture supernatants containing lentiviral particles were harvested, filtered, aliquoted, and stored at −80 °C in a freezer for future use. On the day of transduction, cells were infected by mixing 2 × 10^6 cells in 2 ml of freshly thawed lentivirus and Polybrene (Sigma-Aldrich, Cat#157689) at a final concentration of 8 μg/ml. At 3 days post infection, transduced cells were collected, one million cells were sorted. List of plasmids used in this study: pCDH-Puro-BiL (Cheng et al.134, Addgene plasmid #46971), Tet-pLKO-puro (Wiederschain et al.35, Addgene plasmid #21915), CALCRL MISSION shRNA (shCALCRL#1, Sigma-Aldrich, Cat# TRCN0000053679; shCALCRL#2, Sigma-Aldrich, Cat# TRCN0000056793; shCALCRL#3, Sigma-Aldrich, Cat# TRCN0000036959). List of shRNA sequences: shCALCRL#1 FW CTTATCTCCTGTTGCAATTA, shE2F1 FW GCCGATGAGCTTCACTGAA, shRAMP2 FW CCCTCCTCCGACAAGAGA, and shRAMP3 FW GAGCTTAGACCTCTGTTGA.

**IC50 experiments.** The day before experiment, cells were adjusted to 3 × 10^5 cells/ml final concentration and plated in a 96-well plate (final volume: 100 μl). To measure half-maximal inhibitory concentration (IC50), increased concentrations of AraC or idarubicin were added to the media. After 2 days, 20 µl/ml of MTS solution (Promega) was added for 2 h and then absorbance was recorded at 490 nm with a 96-well plate reader. The doses that decrease cell viability to 50% (IC50) were analyzed using nonlinear regression (inhibitor) vs (response parameters) with GraphPad Prism (v6 and v8) software.

**Measurement of oxygen consumption in AML cultured cells using Seahorse assay.** All XF assays were performed using the XF24 Extracellular Flux Analyser (Seahorse Bioscience, North Billerica, MA). The day before the assay, the sensor cartridge was placed into the calibration buffer medium supplied by Seahorse Biosciences to hydrate overnight. Wells of Seahorse XF24 microplates were coated with 50 µl of Cell-Tak (Corning; Cat#354240) at a concentration of 22.4 µg/ml (final concentration). Then, Cell-Tak coated Seahorse microplates were rinsed with distilled water and AML cells were plated at a density of 10^3 cells/well with XF base minimal DMEM media containing 11 mM glucose, 1 mM pyruvate, and 2 mM glutamine. Then, 180 µl of XF base minimal DMEM medium was placed into each well and the microplate was centrifuged at 80 g for 5 min. After 1 h incubation at 37°C in CO2-free atmosphere, basal oxygen consumption rate (OCR, as a mitochondrial respiration indicator), and extracellular acidification rate (ECAR, as a glycolysis indicator) were performed using the Seahorse XF24, and analyzed using Wave software (version 2.6.1).

**Alkaline comet assays.** Alkaline comet assays were performed with OxiSelect Comet Assay Kit and according to the manufacturer’s instructions (Cell Biolabs Inc.). Electrophoresis was performed at 4 °C in alkaline condition at 20 V during 30 min. Slides were visualized by using a Zeiss microscope (Zeiss). Electrophoresis was performed at 4 °C in alkaline condition at 20 V during 30 min. Comet tail moments were measured with ImageJ software (version 1.52).

**RNA microarray and bioinformatics analyses.** For primary AML samples, human CD45+ CD34+ were isolated using cell sorter cytometer from engrafted BM mice (for 3 primary AML specimens) treated with PBS or with AraC. RNA from AML cells was extracted using Trizol (Invitrogen) or RNeasy (Qiagen). For BM mice (for 3 primary AML specimens) treated with PBS or with AraC. RNA purity was monitored with NanoDrop 1ND-1000 spectro-photometer and RNA quality was assessed through Agilent 2100 Bioanalyzer with RNA 6000 Nano assay kit. No RNA degradation or contamination were detected (RIN > 9). Of the total RNA, 100 ng was analyzed on Affymetrix GeneChip© Whole Transcript (WT) Expression Arrays P/N 703174 Rev. 2.

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Author contributions

Competing interests

The authors declare no competing interests.

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