Reciprocal regulation of amino acid import and epigenetic state through Lat1 and EZH2

Stephen G Dann^{1,*}, Michael Ryskin², Anthony M Barsotti², Jonathon Golas², Celine Shi², Miriam Miranda², Christine Hosselet², Luanna Lemon², Judy Lucas², Maha Karnoub³, Fang Wang², Jeremy S Myers², Scott J Garza¹, Maximillian T Follettie², Kenneth G Geles², Anke Klippel³, Robert A Rollins² & Valeria R Fantin¹

Abstract

Lat1 (SLC7A5) is an amino acid transporter often required for tumor cell import of essential amino acids (AA) including Methionine (Met). Met is the obligate precursor of S-adenosylmethionine (SAM), the methyl donor utilized by all methyltransferases including the polycomb repressor complex (PRC2)-specific EZH2. Cell populations sorted for surface Lat1 exhibit activated EZH2, enrichment for Met-cycle intermediates, and aggressive tumor growth in mice. In agreement, EZH2 and Lat1 expression are co-regulated in models of cancer cell differentiation and co-expression is observed at the invasive front of human lung tumors. EZH2 knockdown or small-molecule inhibition leads to de-repression of RXR α resulting in reduced Lat1 expression. Our results describe a Lat1-EZH2 positive feedback loop illustrated by AA depletion or Lat1 knockdown resulting in SAM reduction and concomitant reduction in EZH2 activity. shRNA-mediated knockdown of Lat1 results in tumor growth inhibition and points to Lat1 as a potential therapeutic target.

Keywords cancer metabolism; methionine cycle; S-adenosylmethionine; SLC7A5

Subject Categories Cancer; Chromatin, Epigenetics, Genomics & Functional Genomics; Metabolism

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Introduction

It is widely recognized that malignant cells exhibit unique metabolic profiles consistent with elevated anabolic demands as well as adaptation to elevated cellular stress and changes in the microenvironment. Some of these metabolic traits have been exploited by oncologists for both diagnostic and therapeutic intervention. For instance, the high glucose avidity of tumors provides the basis of

2-deoxy-2-(18F)-D-glucose-based positron emission tomography imaging. Another example exists in the elevated rate of nucleotide biosynthesis rendering malignant cells vulnerable to drugs like pemetrexed and 5-fluorouracil, which target enzymes in the purine and pyrimidine synthesis pathways.

Over the past decade, cancer sequencing efforts have uncovered a number of genetic alterations in metabolic enzymes. Loss-of-function mutations in succinate dehydrogenase (SDHA) and fumarate hydratase (FH) result in accumulation of their substrates succinate and fumarate, respectively. In addition, neomorphic gain of function mutations in IDH1 and IDH2 leads to accumulation of 2-hydroxyglutarate (2-HG) (Dang *et al*, 2009; Gross *et al*, 2010; Xiao *et al*, 2012). In these circumstances, the accumulating metabolite acts as a competitive inhibitor of α -ketoglutarate (α -KG)-dependent enzymes including dioxygenases that affect histone and DNA methylation. Metabolite-mediated inactivation of histone or DNA demethylases reprograms the cancer epigenome, which underscores the fundamental link between cellular metabolism and epigenetics (Sasaki *et al*, 2012; Xiao *et al*, 2012).

While a number of studies have focused on the characterization of the mechanisms modulating the activity of epigenetic demethylases, less is known about how metabolism regulates the epigenetic enzymes involved in methyltransferase reactions in cancer cells. All cellular methyltransferases depend on the methyl donor S-adenosylmethionine (SAM) for activity. The catalytic subunits of the methionine adenosyltransferase (MAT1A & 2A) complexes form SAM by combining a molecule of ATP and Met (Lu & Mato, 2012). The amino acid transporter SLC7A5 (herein referred to as Lat1) has been previously characterized by its substrate selectivity for the branched chain amino acids and bulky amino acids including Met (Mastroberardino *et al*, 1998). This transporter functions at the cell surface in covalent complex with its obligate chaperone SLC3A2 (herein referred to as CD98). In a number of tumor types, Lat1 expression predicts poorer overall survival and a less-differentiated state (Ring *et al*, 2006; Kaira *et al*, 2008; Yanagisawa *et al*, 2012). Further, previous reports describe Lat1 upregulation in response to activation of Myc (Gao *et al*, 2009).

¹ Pfizer Oncology Research Unit, San Diego, CA, USA

² Pfizer Oncology Research Unit, Pearl River, NY, USA

³ Celgene, Summit, NJ, USA

*Corresponding author. Tel: +1 845 602 2370; Fax: +1 845 602 5557; E-mail: stephen.dann@pfizer.com

Here, we describe a novel role for Lat1 as an indirect regulator of a cell's epigenetic state by restricting the availability of *Met*, and thereby reducing the available pool of SAM. Reduction in the cellular SAM pool results in a loss of several histone methyl marks including the EZH2-specific histone H3, lysine 27 trimethylation (3meH3K27), known to play a fundamental role in regulating the expression of genes involved in differentiation (Lee *et al*, 2006). We also describe a feedback loop where EZH2 activity regulates the expression of Lat1 through PRC2-dependent repression of retinoid receptor α (RXR α). Our data suggest that interfering with the activity of amino acid transport may impact the activity of epigenetic regulators and in turn affect transcriptional programs under their control.

Results

Cell populations enriched for Lat1/CD98 surface complexes exhibit higher *Met*-cycle metabolite levels

We sought to metabolically profile heterogeneous lung cancer cell subpopulations as dictated by cell surface expression of the Lat1/CD98 transporter. We took advantage of the well-characterized antibodies specific for CD98, and fluorescent-activated cell sorting was performed on NCI-H1299 and NCI-H520 cancer cell lines to enrich for Lat1 complexes (Fig 1A and B). NCI-H1299 cells enriched for CD98/Lat1 display a more aggressive and invasive phenotype when grown on top of matrigel compared to CD98^{lo} and also form more aggressive tumors in immune-compromised mice (Fig 1C and D). While this is in agreement with previous reports where the CD98/Lat1 amino acid transporter was linked to stem-like properties of cancer cells (Martens-de Kemp *et al*, 2013), it should be noted that both CD98^{hi} and CD98^{lo} cells were able to form tumors with the same penetrance (9/10 mice). In light of these data, we interpret the enrichment or expression of CD98/Lat1 as a measure on a spectrum of cellular differentiation as opposed to a stem-cell state.

Using a targeted metabolomics analysis approach, we phenotyped CD98^{hi/lo} NCI-H1299 and NCI-H520 cells and found significant elevation of several *Met*-cycle intermediates including SAM, S-adenosylhomocysteine (SAH), methylthioadenosine, adenosine, and cystathionine in CD98^{hi} cells (Fig 1E). In this analysis, *Met* itself does not appear to change; however, our sample collection process requires that cells are kept in serum-free media prior to metabolite extraction, which dilutes the cell autonomous read-out for essential amino acids. To overcome this technical limitation, we took advantage of ion exchange chromatography to specifically assay

intracellular *Met* concentrations from CD98 sorted cells. Using this methodology, we find that CD98^{hi} NCI-H1299 cells contain higher concentrations of free *Met*, a known Lat1 substrate (Fig 1F).

CD98^{hi} NCI-H1299 and NCI-H520 cells exhibit an enrichment of 3meH3K27, the EZH2-specific methyl mark associated with stem-cell-like, less-differentiated features in cancer (Fig 1A and B; Iliopoulos *et al*, 2010). In addition, sorted CD98^{hi} NCI-H520 cells propagated in culture after sorting display persistent enrichment of both EZH2 and 3meH3K27 (Fig 1F). While CD98^{hi} sorted NCI-H1299 cells were not enriched for total EZH2, we did observe enrichment of phosphorylated EZH2 at T487 (Fig 1A). This phosphorylated form of EZH2 is indicative of higher activity as previously observed using a recombinant phospho-mimetic EZH2 mutant at this site (Fig 1A; Kaneko *et al*, 2010). In addition to a lack of detectable EZH2 pT487, CD98^{lo} NCI-H1299 cells express lower levels of MAT2A, the catalytic subunit of the MATII complex which may contribute to the observed reduction in histone methylation, *Met* and SAM in these cells (Fig 1A).

Given the enrichment for markers indicative of a less-differentiated state in CD98^{hi} cells, we analyzed indicators of epithelial to mesenchymal transition (EMT), a cellular process previously shown to correlate with cancer cell stemness (Supplementary Fig S1A). While we did not see changes in the expression of E-cadherin, Slug, or Snail between CD98^{lo} and CD98^{hi} NCI-H1299 cells (Supplementary Fig S1A), we did find accumulation of actin stress fibers in the CD98^{hi} fraction (Supplementary Fig S1B). The stress fiber phenotype is indicative of cytoskeletal remodeling that could account for the more aggressive growth of CD98^{hi} cells *in vivo*.

Lat1 and EZH2 expression are reflective of differentiation state

The metabolic, epigenetic, and *in vitro/in vivo* growth phenotypes observed in cells sorted on the basis of cell surface CD98/Lat1 suggest that expression of this complex may be linked to differentiation state. Moreover, expressions of Lat1 and EZH2 are part of a previously identified gene signature that describes an undifferentiated tumor state (Rhodes *et al*, 2004). We sought to evaluate whether any correlation exists between the expression of these genes in *in vitro* models where differentiation can be induced (i.e. spheroid growth & pro-differentiation culture media) or suppressed (i.e. retinoic acid restriction & SV40 large-T antigen expression).

Cancer cell lines can be differentiated through adaptation to 3-dimensional (3D) culture conditions as spheroids (Sutherland, 1988). We find that multiple non-small cell lung cancer (NSCLC) cell lines will adapt to spheroid growth when grown on non-adherent plates in the presence of 2% extracellular matrix (ECM). We

Figure 1. Characterization of lung cancer cells enriched for high CD98/Lat1 complex.

- A NCI-H1299 lung cancer cells sorted for high and low surface CD98 were lysed and analyzed by Western blot with the indicated antibodies.
- B NCI-H520 lung cancer cells sorted for high and low surface CD98 were replated for the indicated time course, lysed, and analyzed by Western blot with the indicated antibodies.
- C NCI-H1299 lung cancer cells sorted for high and low surface CD98 were plated on a matrigel matrix and photographed at 10 $\times$ magnification (scale bar, 100 μ m).
- D NCI-H1299 cells sorted for high and low CD98 expression were implanted sub-cutaneously into Nod-Scid mice (10,000 cells/mouse) and monitored for tumor growth (Note: these data have been censored to remove data from one mouse in each cohort where no tumor formed over the course of the experiment; each data point then represents the mean tumor size from cohorts of 9 mice. Error bars represent SEM, *two-way ANOVA $P < 0.05$ as a function of tumor growth over time).
- E NCI-H1299 and NCI-H520 lung cancer cells sorted for high and low surface CD98 were analyzed by positive-ion LC-ESI mass spectrometry for the metabolites indicated (Bar graphs represent mean values of triplicate experiments, error bars correspond to standard deviation).
- F NCI-H1299 cells sorted for high CD98 surface expression have higher cellular free *Met* concentrations than CD98 low cells (*Student's *t*-test $P < 0.05$; Data is presented as mean value of triplicate experiments, error bars represent SEM).

compared expression in NCI-H1299 cells grown on plastic and as spheroids and found various changes reflective of a more differentiated state (Fig 2A and Supplementary Fig S1C). For instance, the Integrin/FAK pathway was largely downregulated upon spheroid growth and markers of epithelial to mesenchymal transition (EMT) were consistent with epithelial characteristics (Supplementary

Figure 1.

Figure 2. Lat1 and EZH2 expression are reflective of differentiation state.

- A NCI-H1299 lung cancer cells were grown as pulmospheres (left micrograph at 10× magnification), lysed, and compared to lysates from cells grown in traditional plastic cell culture by Western blot with the antibodies indicated.
- B The patient-derived TUM622 cell line was grown as pulmospheres (left micrograph at 10× magnification), lysed, and compared to lysates from cells grown in traditional 2D cell culture by Western blot with the antibodies indicated in the presence and absence of retinoic acid (RA).
- C TUM622 cells were cultured in CnT-23 airway epithelia differentiation media and assayed by Western blot with the indicated antibodies.
- D NCI-H1299 cells undergoing cell cycle block by nocodazole treatment and subsequent release (T0) were lysed and analyzed by Western blot with the indicated antibodies (V denotes vehicle-treated cycling cells; time points taken were 2, 6, and 24 h post-release).
- E Primary human bronchial epithelial cells (NHBE) before and after immortalization with SV40 large-T antigen (left micrograph at 10× magnification) were lysed and analyzed by Western blot with the antibodies indicated. Scale bars, 100 μm.

Fig S1C). Loss of Lat1, CD98, and EZH2 was also observed upon adaptation to spheroid culture (Fig 2A). We next assayed the expression level of the same proteins in the primary NSCLC cell line TUM622 when grown in adherent and spheroid conditions (Damelin *et al*, 2011). Again, we observed a loss of Integrin/FAK pathway signaling, a shift to a more epithelial phenotype and concomitant loss of EZH2, Lat1, and CD98 (Fig 2B and Supplementary Fig S1D).

TUM622 cells grow in defined media, which allowed us to evaluate the effect of retinoic acid (RA), a well-characterized pro-differentiation factor, on the expression of Lat1 and EZH2. We found that upon removal of RA from culture media, Lat1 and CD98 expressions, but not EZH2, were elevated. In the spheroid cultures, Lat1 and CD98 levels were rescued to levels observed in adherently growing cells (Fig 2B). Further, when TUM622 cells are grown in media purposed for propagation of differentiated airway epithelial cells, a reduction in both Lat1 and EZH2 as well as migration of CD98 to a lower molecular weight species is observed over time (Fig 2C). In this setting, expressions of Lat1 and EZH2 are inversely correlated with Involucrin, a known marker of terminal differentiation in squamous epithelial cells (Lee *et al*, 1996).

To rule out differences that may occur due purely to changes in cell cycle, we evaluated the changes in Lat1 and EZH2 expression upon nocodazole block and release in NCI-H1299 cells. No difference in Lat1 levels was observed during 24-h cell cycle progression. On the other hand, EZH2, Cyclin B1, and Cyclin D1 levels did vary over time consistent with previous reports (Fig 2D; Wei *et al*, 2011). Further, growth of TUM622 cells in the absence of RA had no significant effect on cell proliferation over time as assayed by viable cell counts (Supplementary Fig S1E).

In an effort to develop an independent model to explore the relationship between CD98, Lat1, and EZH2 in the transition from the differentiated to undifferentiated state, we immortalized normal human bronchioalveolar cells (NHBE) with lentiviral-delivered SV40 large-T antigen (Fig 2E). In this experimental setting, we again see predictable response in the Integrin/FAK pathway and EMT markers as well as robust elevation of Lat1, CD98, and EZH2 expression (Supplementary Fig S1F and Fig 2E). Of particular note, we observed a correlation of MAT2A expression with Lat1 and EZH2 reminiscent of CD98^{hi} sorted cells suggesting that the *Met*-cycle is upregulated in less-differentiated states (Fig 2B and E).

Lat1 expression is regulated by EZH2 through de-repression of RXR α

Given the correlative link between EZH2 and Lat1 as well as the clear effect of RA on CD98/Lat1 expression described above, we

sought to determine the impact of EZH2 loss on Lat1 expression and *Met* levels. To that end, we constitutively knocked down EZH2 in NCI-H1299 cells. Western blot analysis confirmed the loss of EZH2 and 3meH3K27, and downregulation of CD98/Lat1 (Fig 3A). The observed reduction in Lat1 expression reflects a functional loss as

Figure 3.

cellular levels of *Met* are reduced in cells with EZH2 knockdown (Fig 3B). Furthermore, treatment of NCI-H1299 cells with the EZH2 inhibitor GSK126 resulted in a reduction in Lat1 expression (Fig 3C). The data provide pharmacological confirmation of the connection between EZH2 activity and Lat1 expression.

In order to determine whether Lat1 expression is directly regulated by EZH2 activity, we performed chromatin immunoprecipitation (ChIP) with antibodies against EZH2, SUZ12, 3meH3K27, and RNA Polymerase II (as a positive control of active transcription). We did not observe enrichment of PRC2 complex members or 3meH3K27 at the Lat1 or CD98 promoter indicating that expression of Lat1 and CD98 are regulated by PRC2 via an indirect mechanism (Fig 3D). To ensure our ChIP experiments were properly assaying PRC2 promoter occupancy, we used CCND2 as a positive control PRC2 target gene (Supplementary Fig S2A).

The link between EZH2 and RA-induced differentiation has been previously reported (Epping *et al*, 2005), and the effect of RA on CD98/Lat1 expression appeared to be downstream of EZH2 (Fig 2B). Therefore, we also investigated whether PRC2 directly regulated retinoic acid receptor and retinoid X receptor genes. ChIP experiments revealed significant enrichment of EZH2, SUZ12, and 3meH3K27 at the promoters of RAR β and RXR α , but not RAR α and RXR β in H1299 cells (Fig 3E). In addition, shRNA-mediated knockdown of EZH2 resulted in selective upregulation of RAR β and RXR α , confirming that these genes are directly repressed by the PRC2 complex in these cells (Fig 3G and Supplementary Fig S2B).

Based on these results, we postulated that the EZH2-mediated effect on Lat1 regulation was through direct regulation of either RAR β or RXR α . To test this hypothesis, we used siRNA to deplete either RAR β or RXR α in cells where constitutive knockdown of EZH2 resulted in loss of Lat1 expression. We found that inhibition of RXR α , but not RAR β , rescued Lat1 mRNA and protein to the levels observed in non-targeting control cells (Fig 3F and G and Supplementary Fig S2B). Further, RXR α siRNA alone was sufficient to elevate Lat1 expression at both the mRNA and protein level reminiscent of the RA dropout experiment described above (Fig 3F and G). To confirm that this was, in fact, PRC2 complex dependent and not EZH2 autonomous activity, we repeated the RXR α siRNA experiment during SUZ12 shRNA knockdown and observed similar effects on Lat1 mRNA expression (Supplementary Fig S2C). Finally, we confirm that RXR α binds the Lat1, CD98, and MAT2A promoter, while RAR β does not contribute any promoter binding even to the positive control ABCA1 gene in these cells (Supplementary Fig S2D and E). Taken

together, these data revealed a role for RXR α as a negative regulator of Lat1 and suggest that the PRC2 complex potentiates Lat1 expression by directly repressing RXR α gene expression.

Lat1 and EZH2 co-segregate in NSCLC clinical samples as a function of tumor stage

To understand the clinical relevance of the preclinical observations previously described, we examined EZH2 and Lat1 expression across gene expression data sets obtained from samples of patients with lung cancer. Using both proprietary (GeneLogic) and external (Oncomine) mRNA expression databases, we found that both EZH2 and Lat1 are upregulated in NSCLC tumor samples (both adenocarcinoma and squamous cell carcinoma subtypes), while the related gene homologues EZH1 and SLC7A7 (i.e. γ -Lat1) are down regulated (Fig 4A and B, Supplementary Fig S3A and B). We developed IHC methodology to assay for EZH2 and Lat1 expression in tissue microarrays of samples of patients with NSCLC of various stages. Statistical analysis of staining patterns shows a significant overlap of Lat1 with EZH2 expression (Fisher's exact test $P = 1.98E-04$), and expression of each also significantly correlates with increasing stage of tumor (Fisher's exact test $P = 4.45E-08$ and $1.78E-07$, respectively; representative images are shown in Fig 4B). Co-staining individual samples for EZH2 and Lat1 in lung squamous cell carcinoma reveal that these proteins are co-expressed in the same cell populations. Specifically, we observed robust staining for both EZH2 and Lat1 within the least differentiated, most rapidly proliferating, tumor cells adjacent to the stroma (Fig 4C).

Loss of Lat1 by shRNA knockdown limits *Met*-cycle metabolites and reduction in H3K27 methylation

Based on the observed interplay between metabolic and epigenetic effectors, we reasoned that epigenetic state could be modulated simply by restricting the cellular availability of amino acids (AA). Given the ability of Lat1 to transport *Met* substrate, we sought to evaluate its functional role in terms of cellular *Met* availability and growth. We find that shRNA-mediated knockdown of Lat1 in NCI-H1299 lung cancer cells results in a significant reduction in *Met* (Fig 5A) as well as total SAM concentrations (Fig 5B). Based on these data, we next interrogated the effect of Lat1 knockdown on the EZH2 substrate H3K27 and observed a reduction in H3K27

Figure 3. Repression of RXR α by EZH2 induces Lat1 expression.

- A Constitutive knockdown of EZH2 by lentivirally delivered shRNA in NCI-H1299 lung cancer cells was assayed by Western blot for the antibodies indicated (N denotes non-targeting shRNA, A and B denote unique EZH2 shRNA targeting oligonucleotides).
- B Cellular *Met* concentrations were measured in cells constitutively expressing shRNA targeting EZH2 (*Student's *t*-test $P < 0.05$ relative to non-targeting shRNA; bar graphs represent the mean of triplicate experiments, error bars represent SEM).
- C NCI-H1299 cells were treated with the EZH2 inhibitor GSK126, lysed, and assayed by Western blot with the antibodies indicated.
- D Chromatin immunoprecipitation of Actin B, SLC7A5 (Lat1), and SLC3A2 (CD98) promoter elements from NCI-H1299 cells were assayed for occupancy by EZH2, Suz12, 3meH3K27, and RNA Polymerase II using quantitative PCR (Untreated denotes control immunoprecipitations with no DNA; bar graphs represent the mean of triplicate experiments, error bars represent SEM).
- E Chromatin immunoprecipitation of RAR α , RAR β , RXR α , and RXR β promoter elements from NCI-H1299 cells were assayed for occupancy by EZH2, Suz12, 3meH3K27, and RNA Polymerase II using quantitative PCR (Untreated denotes control immunoprecipitations with no DNA; bar graphs represent the mean value of experiments carried out in triplicate, error bars represent SEM).
- F, G Protein expression during RXR α siRNA and EZH2 shRNA knockdown was assayed (F) by Western blot with the antibodies indicated and (G) by real-time PCR with the probes indicated (bar graphs represent the mean of triplicate experiments, error bars represent SEM).

Figure 4. Lat1 and EZH2 are co-expressed in clinical samples from patients with lung cancer.

- A** Normal/tumor matched-pair samples from lung squamous cell carcinoma patients profiled from GeneLogic database for SLC7A5 (Lat1), EZH2, SLC7A7 (y+Lat1), and EZH1.
- B** Lat1 and EZH2 immunohistochemical staining of a moderately differentiated lung carcinoma patient with heaviest staining at the tumor/stroma interface, little to no staining in the islands of differentiated tissue (micrographs taken at 10 $\times$ magnification on the left, corresponding blow-up of the marked inset area on the right). Analyses of Lat1 and EZH2 in 38 matched-pair normal/tumor lung samples showed staining exclusively in the tumor epithelium as compared to normal lung tissue (Fisher's exact test $P = 4.45\text{E}-08$ and $1.78\text{E}-07$, respectively) and co-staining with proportional intensity in the same samples (Fisher's exact test $P = 1.98\text{E}-04$).
- C** Co-localization of Lat1 (membrane, pink) and EZH2 (nuclear, brown) in a representative patient sample of lung squamous cell carcinoma showing heaviest staining at the tumor/stroma interface (micrographs taken at 20 $\times$ magnification; black line added manually to distinguish squamous-like differentiated cells from proliferating invasive front).

di- and tri-methylation (Fig 5C). Importantly, we find a number of histone methyl marks are reduced upon knockdown of Lat1 including substrates for MLL1 (H3K4) and MMSET (H4K20), suggesting that SAM substrate channeling from Lat1-specific *Met* entry is not limited to EZH2 activity (Supplementary Fig S4A). In order to substantiate a model of *Met* restriction on the cellular SAM pool and epigenetic state, we used shRNA to knockdown MAT2A and interfere directly with the *Met*-cycle. Due to the critical and non-redundant role of MAT2A in multiple cellular processes, it was essential to employ a doxycycline-inducible

shRNA system to complete these experiments. We find that depletion of MAT2A phenocopies loss of Lat1, leading to a comparable reduction in 3meH3K27 (Fig 5D). Based on the shRNA experiments described above, it follows that NCI-H1299 cells depleted of AAs will respond with reduced 3meH3K27 (Fig 5E). Using an unrelated cell line (HEK-293), we find that 3meH3K27 reaches a minimal level at 4 h post-depletion thereby showing that amino acid dependency on 3meH3K27 is not limited to the lung cancer models used in these studies (Fig 5F). Moreover, restriction of *Met* alone is sufficient to reduce 3meH3K27, whereas restriction

of a control amino acid (cysteine) has no effect on epigenetic state (Fig 5G).

Lat1 depletion interferes with NCI-H1299 tumorigenicity

Finally, we investigated the contribution of Lat1 to cancer cell growth using shRNA knockdown in NCI-H1299 cells. Although knockdown of Lat1 did not affect cell growth on plastic, the same hairpins impaired NCI-H1299 cell growth when plated on top of extracellular matrix reminiscent of the CD98^{hi} sorted cells (Fig 5G). We also find that shRNA-mediated loss of Lat1 results in impaired invasion through matrigel-coated Boyden chambers, suggesting that at the invasive tumor front, Lat1 may facilitate the expansion of tumor boundaries into the surrounding tissue (Supplementary Fig S4B). Ultimately, we sought to determine whether loss of Lat1 would result in significant tumor growth inhibition *in vivo*. We observed NCI-H1299 tumor growth inhibition consequent to Lat1 shRNA-mediated knockdown (Fig 5I). Likewise, induction of MAT2A shRNA in the same tumor model also manifested robust tumor growth inhibition strengthening the model that Lat1 and MAT2A operate within the same metabolic/epigenetic axis (Supplementary Fig S4C).

Discussion

Malignant cells rewire their transcriptional, signaling, and metabolic networks to suit the biosynthetic demands of unchecked proliferation. It is apparent that these networks are interconnected and highly responsive to changes in one another. For instance, mutations in metabolic genes such as IDH1, IDH2, SDH, and FH lead to alterations in metabolites that interfere with the activity of dioxygenases, including histone demethylases, by virtue of competition with α -KG. As a consequence, the broad epigenetic imbalance results in transcriptional and signaling deregulation (Xiao *et al*, 2012). Fluctuations in a number of metabolites have been shown to affect a wide range of epigenetic regulators. For example, in addition to its substrate function within the Krebs's cycle and as an anabolic building block, Acetyl-CoA levels also directly impact histone acetylation (reviewed in Kaelin & McKnight, 2013). In yeast, Acetyl-CoA concentrations in the cell dictate the activity of histone

acetyltransferases such as GCN5 leading to broad dynamic changes in transcriptionally active chromatin (Cai *et al*, 2011). Additionally, in mammalian cells, histone acetylation requires the conversion of citrate into acetyl-CoA by ATP-citrate lyase (Wellen *et al*, 2009). Further, removal of histone acetylation marks can be mediated by members of the sirtuin family, which are dependent on another metabolite, nicotinamide adenine dinucleotide (NAD⁺) as a cofactor for the deacetylation reaction (Kaelin & McKnight, 2013).

Here, we describe a previously unknown and reciprocal regulation between extracellular *Met* uptake and epigenetic state (modeled in Fig 6). Our data suggest that Lat1 is critical for transport of the essential amino acid *Met* and maintenance of the steady state cellular SAM concentration. Reduction in SAM levels correlates with loss of Lat1 as well as the histone methylation mark catalyzed by EZH2, 3meH3K27. In accordance with these observations, expressions of EZH2 and Lat1 are tightly correlated in cell culture models of differentiation. Furthermore, expression of both proteins is high and coincident in clinical samples of lung cancer compared to adjacent normal tissue and positively correlates with tumor stage. Our study also shows that an enrichment of the CD98/Lat1 complex specifies a cancer cell population inherently more aggressive *in vitro*, reflected in their organization of actin stress fibers and growth on top of matrigel and *in vivo* reflected by their accelerated tumor growth kinetics. The CD98/Lat1^{hi} cell characteristics are correlated with high EZH2 activity. In agreement, CD98/Lat1^{hi} cells have elevated *Met*-cycle intermediates indicative of their requirement for SAM in modulating EZH2 epigenetic effects.

In a reciprocal feedback relationship, we also identify Lat1 as a downstream biomarker of EZH2 activity. Our first indication of this relationship came from observations in spheroid cell cultures, where Lat1 and EZH2 levels drop but only Lat1 decreased in a RA-dependent manner. Loss of PRC2 activity by either shRNA- or small-molecule-mediated inhibition also reduced Lat1 levels in cells, and this effect is abrogated in the absence of RXR α . We postulate that RA binds RXR α resulting in activation and DNA binding of this nuclear hormone receptor. That RXR α can influence the induction, and in this case, suppression of target genes upon activation by RA is likely through distinct binding partners at genetic loci associated with differentiation or progenitor phenotypes, respectively.

These data can be explained mechanistically by the observation that EZH2, SUZ12, and 3meH3K27 (markers of transcriptional

Figure 5. Lat1 expression dictates availability of methionine for *Met*-cycle activity, epigenetic state, and tumor growth.

- A Cellular *Met* was measured in NCI-H1299 cells after shRNA-mediated Lat1 knockdown (*Student's *t*-test $P < 0.05$ relative to non-targeting control).
- B Cellular S-adenosylmethionine (SAM) concentration was measured using the Mediamics Bridge-It assay from NCI-H1299 cells after lentivirally mediated delivery of constitutive stable shRNA molecules targeting Lat1 (*Student's *t*-test $P < 0.05$ relative to non-targeting control; bar graphs represent the mean of triplicate experiments, error bars represent the SEM).
- C Lysates from NCI-H1299 cells were analyzed by Western blot with the antibodies indicated after lentivirally mediated delivery of constitutive stable shRNA molecules targeting Lat1.
- D NCI-H1299 cells stably expressing doxycycline-inducible shRNA oligonucleotides that target MAT2A were induced with 250 ng/ml doxycycline (Dox), lysed and analyzed by Western blot with the antibodies indicated.
- E NCI-H1299 cells depleted of amino acids in 10% dialyzed serum for 6 h were lysed and analyzed by Western blot with the antibodies indicated.
- F HEK293 cells deprived of amino acids in media containing dialyzed serum for 2, 4, and 6 h were lysed and analyzed by Western blot with the antibodies indicated.
- G NCI-H1299 cells depleted of cysteine (-C) or methionine (-M) in 10% dialyzed serum were lysed and analyzed by Western blot with the antibodies indicated.
- H Growth of NCI-H1299 cells on matrigel matrix was assayed by phase contrast microscopy upon shRNA-mediated Lat1 knockdown (photographed at 10 $\times$ magnification, scale bar indicates 100 μ m).
- I NCI-H1299 sub-cutaneous xenografts harboring constitutive stable shRNA targeting Lat1 (100,000 cells/mouse) were monitored for tumor growth as compared to non-targeting shRNA (error bars represent SEM, *two-way ANOVA $P < 0.05$ as a function of Lat1 knockdown effect on tumor growth over time). Data points represent the mean value of tumor size from 15 mice in each cohort.

repression) but not RNA Polymerase II (indicative of active transcription) were found at the RXR α promoter. Of particular note, we recognize a disconnect where EZH2 knockdown results in a modest

reduction in Lat1 mRNA but a robust reduction in Lat1 protein. This suggests that EZH2 plays a role in Lat1/CD98 protein stability perhaps through RXR α -independent regulation of degradation

Figure 5.

Figure 6. Illustrative model of a Lat1/EZH2 feedback loop active in lung cancer.

The Lat1/CD98 transporter complex acts as an entry point for Met which, along with ATP, is used as substrate by MAT2A to form SAM. SAM acts as the methyl donor for histone methyltransferase reactions such as the di- and tri-methylation of H3K27 performed by EZH2 in the PRC2 complex. PRC2 activity directly suppresses the transcription and subsequent translation of RXR α , which is a RA-dependent negative regulator of Lat1 transcription. Thus, high EZH2/Lat1 activity is self-reinforcing by ultimately alleviating the inhibitory activity of RXR α on Lat1 expression (Closed arrow points denote positive, activation signals whereas closed circle points denote negative, inhibitory signals).

pathways. Development of small-molecule candidates targeting EZH2 and their utility in the clinic will ultimately determine whether Lat1 expression can act as a surrogate biomarker for inhibitor response.

Finally, we present data that underscore the critical role of Lat1 in supporting tumor growth. The anti-tumor effect mediated by loss of Lat1 was only evident after day 7 when most tumors reached an average size of 100 mm³. One potential explanation for this observation is that a threshold tumor size may exist at which malignant cells become more dependent on Met uptake. We hypothesize that as tumors grow and increase their Met demand for protein and SAM synthesis, they become more dependent on Lat1. Interfering with this transporter compromises the ability of tumor cells to replenish the Met pool resulting in metabolic crises. It should be noted that K_m of EZH2 for SAM has been reported *in vitro* at approximately 1.2 μ M, while the K_i for SAH is approximately 7.5 μ M (Richon *et al*, 2011). Given the likely higher affinity for SAM, we would predict higher EZH2 activity in a SAM-enriched cell. The ratio of SAM:SAH also plays a role in allosteric regulation of EZH2 and other methyltransferases in a cellular context and remains a caveat when interpreting our results.

Corroborating these data is the finding that MAT2A knockdown also results in robust tumor growth inhibition in the same model.

Interestingly, while MAT2A expression does not appear to be affected by loss of EZH2, its promoter is bound by RXR α suggesting some EZH2-independent but RXR α -dependent regulation of this metabolic enzyme. In order to exploit SAM metabolism as a therapeutic strategy in oncology, it will be of utmost importance in future studies to fully evaluate the contribution of various methionine endpoints (i.e. translation, epigenetics, SAM-dependent biosynthesis, etc.).

Materials and Methods

Cell lines, spheroid, and 3D cell culture assays

HEK293, NCI-H1299, and NCI-H520 cell lines were purchased from ATCC and grown in cell culture as per ATCC recommendations. Derivation of TUM622 cells was previously described and grown as per author's recommendation. Primary Normal Human Bronchioalveolar Epithelial (NHBE) cells were purchased from Lonza and subcultured as per the manufacturer's recommendation.

Experiments involving NCI-H1299 or TUM622 cells grown as pulmospheres were as follows. 10,000 cells were plated in full media containing 2% extracellular matrix (Matrigel) on a low

binding 6-well plate. Spheroid morphology and growth was monitored over the course of 10–15 days and photographed as explained below.

Experiments involving NCI-H1299 cells grown on top of matrigel were as follows. 300 μ l of ice-cold 80% matrigel in full media was plated into a 24-well cell culture dish and allowed to harden for 2 h at 37°C. NCI-H1299 cells were treated with siRNA or sorted for CD98 as described, and 100,000 cells were seeded in 1 ml of full media onto the matrigel plate. Cell morphology and growth was monitored over the course of 10–15 days and photographed as explained below.

Experiments involving cellular invasion assays were performed using the Cultrex BME Cell Invasion assay as per the manufacturer's suggested protocol (Trevigen).

siRNA mediated knockdown

Transient knockdown experiments were carried out as per the manufacturer's recommendation. Briefly, Silencer Select siRNA oligonucleotides (Life Technologies) targeting *SLC7A5* or *RXRA* were transfected with Lipofectamine™ RNAiMAX. NCI-H1299 cells were seeded 100,000 cells/well in a 6-well plate 24 h prior to transfection. Oligonucleotide and transfection reagent were complexed according to the manufacturer's instructions and delivered to cells at a final concentration of 5 nM. siRNA oligonucleotide sequences used in this study are found in Supplementary Table S1.

Fluorescent-activated cell sorting

Adherent cells were trypsinized and washed with PBS prior to incubation with mouse monoclonal anti-CD98 (Supplementary Table S2) antibody for 1 h at 4°C. Excess primary antibody was washed off in a 1% FBS/PBS solution three times followed by further incubation with PE-tagged secondary antibody for a further 30 min at 4°C. Cells were washed again with 1% FBS/PBS to remove excess secondary antibody, passed through a cell strainer and run on a FACSARIA III (BD Biosciences) to collect the top and bottom 5% of cells expressing surface CD98 into full media.

NCI-H1299 subcutaneous Xenografts

NCI-H1299 cells sorted for CD98^{hi} or CD98^{lo} sub-populations (1×10^4 cells) were injected subcutaneously into the right flank of female Nod-Scid mice. Groups of 10 mice received subcutaneous inoculation of CD98^{hi} or CD98^{lo} NCI-H1299 cells. NCI-H1299 stable cells (1×10^5) expressing stable Lat1, EZH2, or non-targeting shRNAs were injected subcutaneously into the right flank of female Nod-Scid mice. Groups of 15 mice received subcutaneous inoculation of pooled Lat1, EZH2, or non-targeting shRNAs expressing NCI-H1299 cells. All animals were completely dissected and photographically documented. *In vivo* tumor growth was measured weekly using standard caliper measurements and statistically analyzed using repeated-measures ANOVA.

Amino acid HPLC, SAM assay, and metabolomic LC-MS

Amino acid analysis was completed on a Biochrom 30+ amino acid analyzer as per manufacturer's instructions. Briefly, cells were

grown on a 6-well plate, rinsed with ice-cold PBS, and scraped into 60 μ l of ice-cold PBS. The scraped cell suspension was disrupted in water bath sonicator, and cellular debris removed by 4°C, 10 min centrifugation at 21,000 g. Fifteen microliters of the resulting cellular lysate was used as injection volume to analyze and compare to amino acid standards.

Cellular SAM was assayed using the Bridge-It S-adenosylmethionine cellular assay kit (Mediomics) as per the manufacturer's instructions. Briefly, cells grown in a 6-well plate were rinsed with ice-cold PBS and then lysed in 150 μ l ice-cold 0.2% perchloric acid and 0.08% β -mercaptoethanol. Ten microliters of the resulting lysate was combined with assay reagents, incubated for 60 min at room temperature, transferred to a non-binding black bottom 96-well plate, and assayed on a Victor³V plate reader (PerkinElmer) with 495 nm excitation and 665 nm emission settings.

Metabolomic LC-MS was performed on cells grown on a 6-well plate 24 h after FACS sorting for CD98^{hi} and CD98^{lo} populations. Cell pellets were rinsed briefly with ice-cold PBS and extracted with 200 μ l cold MeCN/MeOH/H₂O 40/40/20 for 10 min, followed by extraction with 300 μ l cold water for 5 min. Ten microliters of 2 μ M iso-ATP and 5-fluoro-2-methylpyridine (injection standards) was added to the pooled metabolites, and the resulting solutions were centrifuged at $15,000 \times g$ at 4°C for 15 min. Thirty microliters of the extracts were loaded onto an Scherzo SM-C18 150 $\times$ 2.0 mm column (Imtakt USA) at a flow rate of 0.3 ml/min and column oven temperature of 30°C. The mobile phases used for metabolite separations were water containing 0.1% formic acid (mobile phase A) and acetonitrile containing 0.1% formic acid (mobile phase B). The following gradient was used: 0–6 min: 0% B, 6–16 min 0–50% B, 16–18 min 50–100% B, 18–20 min 100% B, 20–20.5 min 100–0% B, and 20.5–30 min 0% B. The column effluent was delivered directly to an API 4000 mass spectrometer (AB SCIEX) with a heated turbo electrospray ionization source operated in multiple reaction monitoring (MRM) mode.

Gene expression analysis by qRT-PCR

One-Step quantitative RT-PCR was performed on 100 ng of total RNA from NCI-H1299 cells using Superscript III Platinum One-Step qRT-PCR System (Invitrogen) according to the manufacturer's protocol. Gene expressions of interest were measured using TaqMan Gene Expression Assays (Applied Biosciences) on the CFX96 Real-Time System (BioRad). Results were represented as fold change using the $\Delta\Delta C_t$ method, following normalization to RPN1. TaqMan primer/probe sets used in this study are found in Supplementary Table S1.

Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR)

ChIP-qPCR was carried out following the protocol outlined in De Abreu *et al* (2010). Briefly, NCI-H1299 cells were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by adding lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated, and the DNA sheared to an average length of 300–500 bp. Genomic DNA was prepared by treating chromatin with RNase, proteinase K and heated for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended, and the resulting DNA was quantified on a NanoDrop

spectrophotometer. The total chromatin yield was determined by extrapolation to the original chromatin volume.

Purified chromatin (20–30 µg) was precleared with protein G agarose beads (Invitrogen). Genomic DNA regions of interest were immunoprecipitated using the antibodies indicated (Supplementary Table S2). After incubation at 4°C overnight, protein G agarose beads were used to isolate the immune complexes followed by washes, elution from the beads with SDS buffer, and RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65°C, and ChIP DNA was purified by phenol–chloroform extraction and ethanol precipitation.

qPCRs were carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using Input DNA. The sequences of ChIP-qPCR primers are listed in Supplementary Table S1.

Stable cell line engineering

All lentiviral-delivered constitutive shRNAs were purchased from Sigma as high titer virus, and all shRNA sequences are found in Supplementary Table S1. Stable NCI-H1299 cells were created by exposing cells at 50% confluency in a 6-well plate to pseudotyped lentiviral particles at an MOI of one for a 24-h period in the presence of 8 µg/ml polybrene. After 24 h, media was replaced with full media and cells were grown to confluence and then passaged into T-175 culture flasks with puromycin selection (100 µg/ml) until resistant cell colonies formed and antibiotic induced cell death ceased. Several hundred resistant colonies were pooled and expanded to form the stable cell lines employed herein.

SV40 large-T antigen was delivered to NHBE as pseudotyped lentiviral particles at an MOI of one (Capital Biosciences). NHBE with large-T antigen were passaged 20 times beyond the point of telomeric crisis occurring in NHBE without large-T antigen.

Lentiviral-delivered inducible MAT2A shRNAs were purchased as DNA constructs from Collecta, Inc. Lentivirus were packaged as per manufacturer's instructions, and stable cell lines were generated as described above.

Immunoblotting, chromatin immunoprecipitation, and real-time PCR

Standard Western blotting techniques were used for all immunoblotting experiments described. Cell lysates were generated from 2D and spheroid growth conditions using RIPA buffer containing 40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 5% glycerol, 1% NP40, 1% Na deoxycholate, 0.1% SDS, protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktails I and III (Sigma). Antibodies used in this study are found in Supplementary Table S2.

Microscopy and immunohistochemistry

Micrographs of live cells grown in 2D and spheroid cell culture were taken using phase contrast settings on a Zeiss AxioScope at the indicated magnifications.

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections using standard protocols and developed

via DAB detection and horseradish peroxidase-conjugated secondary antibody. Tissue microarrays with both normal and tumor tissue were procured from US Biomax. Slides were counterstained using standard hematoxylin staining protocol.

Supplementary information for this article is available online:

<http://emboj.embopress.org>

Author contributions

SGD completed and designed experiments and/or data collection included in all Figures. MR completed the majority of experiments presented in Fig 3. AMB completed the experiment presented in Fig 3C. JG developed and applied immunohistochemistry assays presented in Fig 4. CS completed live cell sorting experiments presented in Fig 1. MM completed the matrigel growth assay presented in Fig 5H. CH, LL, JL and SJG completed the *in vivo* assays presented in Fig 1D, 5I, and Supplementary Fig S4C. MK completed the statistical analysis from immunohistochemical staining of lung cancer tissue microarrays. FW and JSM completed the metabolomic profiling and bioinformatic analysis presented in Fig 1E. KGG completed the experiment presented in Fig 2C. MTF contributed the GeneLogic mRNA expression profiling data presented in Fig 4A and Supplementary Fig S3. AK, RAR and VRF contributed experimental design and data interpretation essential in completing the manuscript.

Conflict of interest

All listed authors are or were full-time employees of Pfizer, Inc. with no relationships that they believe could be construed as resulting in an actual, potential, or perceived conflict of interest with regard to the manuscript submitted for review.

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