Université de Montréal

Identification of the molecular pathways mediating the anti-AML activity of statins

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Mémoire présenté
en vue de l'obtention du grade de de Maître ès sciences (M.Sc.)
en Biologie Moléculaire
option Générale

Résumé

La leucémie myéloïde aiguë (LMA) est fréquente chez l'adulte. Malgré un taux de survie très faible des patients après 5 ans (environ 27%), le traitement de la LMA a peu changé au cours des quarante dernières années. Par le biais d'un criblage de viabilité à haut débit réalisé dans le but d'identifier de meilleures molécules anti-LMA, nous avons mis en évidence la capacité des statines (inhibiteurs de l'hydroxy-methyl-glutary-CoA reductase (HMGCR)) à cibler de manière différentielle les différents sous-types de LMA. De manière intéressante, les échantillons de LMA appartenant à des patients classés «bon prognostic», sont 10 fois plus sensibles aux statines que les spécimens provenant de patients présentant un pronostic défavorable. Cet effet discriminatoire est perdu avec l'analogue de statine A405, synthétisé par notre équipe et qui n'a pas d'activité anti-HMGCR, suggérant que l'inhibition d'HMGCR est essentielle pour l'effet discriminatoire des statines. Les statines sont des médicaments hypocholestérolémiants et sont largement connues pour leur action inhibitrice sur l'HMGCR, une enzyme limitante de la voie de synthèse du mévalonate. En plus d'être d'excellents inhibiteurs de la production de cholestérol, les statines sont également connues pour leurs effets pléiotropiques sur d'autres branches de la voie du mévalonate. Ces branches jouent des rôles importants dans diverses fonctions cellulaires telles que la transduction du signal, la synthèse des protéines et la régulation du cytosquelette. Dans cette étude, nous avons émis l'hypothèse selon laquelle l'effet anti-LMA différentiel des statines est modulé par une ou plusieurs sous-branche(s) de la voie du mévalonate. Comme preuve de principe, nous avons confirmé que la réduction de l'activité d'HMGCR, médiée par shARN dans la lignée cellulaire OCI-AML5, conduit à une augmentation significative de la sensibilité aux statines mais pas à l'analogue A405. Nous avons également démontré que la perte complète du récepteur HMGCR, obtenue par CRISPR, est létale et que la supplémentation en mévalonate rétablit à la fois la viabilité cellulaire et la sensibilité aux statines. Pour disséquer davantage l'activité anti-LMA des statines, nous avons utilisé la technique d'ARN interférence pour cibler chacune des sous-branches en aval de la production de mévalonate et étudier la conséquence de ceci sur la viabilité cellulaire ainsi que sur la sensibilité aux statines. Notre étude a montré que la suppression individuelle des enzymes des sous-branches n'affecte pas significativement la sensibilité des cellules OCI-AML-5 aux statines. Fait in-

téressant, nous avons observé que la réduction de l'enzyme au point de ramification de la voie, la farnésyl diphosphate synthase (FDPS), est létale dans les cellules OCI-AML-5. De manière surprenante, nous avons également observé que la réduction de FDPS provoque une réponse biphasique aux statines. Alors qu'une concentration élevée d'atorvastatine aggrave le phénotype causé par la réduction du FDPS, une faible concentration conduit à un sauvetage presque complet de ce phénotype. Ces résultats ont permis de mettre en évidence un nouveau mécanisme de résistance aux statines dans la LMA. Dans l'ensemble, cette étude souligne l'importance de comprendre le contexte métabolique de la LMA avant traitement, afin de prévenir la résistance au médicaments.

Mots-clés : Leucémie myéloïde aigüe, Statines, chimiogénomique, métabolisme du cancer, identification de cibles, résistance thérapeutique.

Abstract

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults. The overall 5-year survival is as low as ~27%; however treatment for AML has only recently evolved with marginal impact on this low survival. In a large viability screen performed to identify better anti-AML molecules, we identified statins (3-Hydroxy-3-Methyl-glutary-CoA reductase (HMGCR) inhibitors) for their ability to differentially target AML specimens. Interestingly, AML specimens belonging to good outcome AML patients were 10 times more sensitive to statins compared to specimens belonging to adverse outcome patients. This discriminatory effect is lost with statin analog A405, synthesized by our team, which lacks anti-HMGCR activity, suggesting that HMGCR inhibition is essential for statin's discriminatory effect. Statins are cholesterol-lowering drugs and are widely known for their inhibitory action on HMGCR, a rate-limiting enzyme in the mevalonate pathway. In addition to being excellent inhibitors of cholesterol production, statins are also known for their independent pleiotropic effects attributed by other products of the mevalonate pathway. These branches play important roles in various cellular functions such as signal transduction, protein synthesis and cytoskeletal regulation. In this study, we hypothesized that statin's anti-AML differential effect is modulated by sub-branch(es) of the mevalonate pathway. As proof of principle, we confirmed that shRNA-mediated reduction of HMGCR activity in OCI-AML-5 cell line led to a significant increase of sensitivity to statins but not to its analog A405. We further demonstrated that complete loss of HMGCR using CRISPR technology was lethal and that mevalonate supplementation rescued both cell viability and sensitivity to statins. To further dissect the anti-AML activity of statins, we used RNAi technology to target each of the downstream sub-branches of the mevalonate pathway and investigated the consequences on cell viability and statin sensitivity. Our study showed that knocking down enzymes of single sub-branches did not affect significantly OCI-AML-5 cells sensitivity to statins. Interestingly, we observed that reduction of branch point enzyme, farnesyl diphosphate synthase (FDPS) was lethal in OCI-AML-5 cells. Unexpectedly, we also observed that FDPS knockdown caused a biphasic response to statin. While high concentrations of atorvastatin aggravates the phenotype caused by FDPS reduction, low concentrations led to a near complete rescue of this phenotype. These results uncovered a novel unsuspected statin resistance mechanism in AML.

Overall, this study highlights the importance of understanding the metabolic background of AML prior to drug treatment, in order to prevent drug resistance.

Keywords: Acute myeloid leukemia, statins, chemo-genomics, cancer metabolism, target identification, therapy resistance

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Liste des abréviations

allo-HSCT: Allogenic HSCT

AML: Acute myeloid leukemia

AMPK: AMP-activated protein kinase

CBF: Core-binding factor

CBF-AML: Core binding factor acute myeloid leukemia

CN-AML: Cytogenetically normal AML

CoQ10: Coenzyme Q10

COX: Cytochrome oxidase

CR: Complete Remission

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DMAPP: Dimethylallyl diphosphate

DMSO: Dimethyl sulfoxide

Dox: Doxycycline

ELN: European LeukemiaNet

ETC: Electron transport chain

FAB: French-American-British

FDPS: Farnesyl diphosphate synthase

FH: Fumarate hydratase

FNTA: Farnesyl tranferase α subunit

FNTB: Farnesyl tranferase β subunit

FPP: Farnesyl diphosphate

FT: Farnesyl transferase

GGPS1: Geranylgeranyl diphosphate synthase 1

GGT-I: geranylgeranyl transferase I

GGT-II: Geranylgeranyl transferase II

GPCR: G-protein-coupled receptors

HiDAC: High-dose cytarabine

HIF-1α: Hypoxia-inducible factor-1α

HMGCR: Hydroxy-methyl-glutaryl-CoA reductase

HSC: Hematopoietic stem cell

HSCT: Hematopoietic stem cell transplantation

HSCT: Hematopoietic stem cell transplantation

ICMT: Isoprenylcysteine Carboxyl Methyltransferase

IDH: Isocitrate dehydrogenase

inv(16) : inv(16)/t(16;16);CBFB-MYH11

IPP: Isopentenyl diphosphate

LDL: Low density lipoprotein receptor

LSC: Leukemic stem cells

MDS: Myelodysplastic syndrome

MM: Multiple myeloma

OS: Overall survival

PGGT1B: Geranylgeranyl transferase type I β subunit

qRT-PCR: Quantitative real-time PCR

REP: Rab escort protein

ROS: Reactive oxygen species

SDH: Succinate dehydrogenase

sgRNA: small guide RNA

shRNA: short hairpin RNA

SRE: Sterol regulatory elements

SREBP: Sterol regulatory element binding proteins

TKI: Tyrosine kinase inhibitor

TRIT1: tRNA isopentenyltransferase 1

WHO: World Health Organization

Remerciements

First and foremost, I would like to offer my profoundest appreciation and gratitude to my professor, Dr. Guy Sauvageau who gave me the opportunity to be a part of his lab. You have always supported me, I truly appreciate the valuable time you took out for me in spite your busy schedule. Thank you for your encouragement and valuable advice. I am very fortunate to be a part of your laboratory; this journey has been a very knowledgeable one.

I would like to thank Dr. Marie-Eve Bordeleau and Dr. Jana Krosl for supporting me as mentors during my graduate journey in the lab. I sincerely thank them for your help during the project and for the scientific training I received. Your scientific advice and support during my masters has definitely helped me evolve as a scientist.

I express my heartfelt gratitude to Dr. Jalila Chagraoui who helped me in this project, taught me several techniques and gave me a strong foundation in this field. Thank you for your constant encouragement and support, my graduate training would be incomplete if not for your guidance. Your advice always helped me troubleshoot, think outside the box and find a way out of the many problems I encountered. I sincerely want to thank Isabel and Nadine for their tremendous help and support in helping me carry out my RNAi screen successfully. I truly appreciate the valuable time and effort you put in for me. I would also like to thank Dr. Bernhard Lehnertz, Dr. Celine Moison, Dr. Richard Bisaillon, Tara MacRae, Laura Simon, Simon Girard and Muriel Draoui for their valuable advice and suggestions that often helped me take correct decisions at critical situations. I express my gratitude to Dr. Anne Marinier and her team for synthesizing compounds that were critical in my project. I would also like to thank Jean Duchaine, Raphaelle Lambert, Daniele Gagne, Gael Dulude and Annie Gosselin and their core facility platforms. I am truly indebted to the facilities at the IRIC. I am also very grateful to the monetary support I received from the Cole foundation and University of Montreal.

Finally, I would like to extend my gratitude to my friends Maria Sanchez Osuna, Roger Palou, Swati Singh, Saranya Sankar, Unain Ansari, Melanie Criqui and my family who always supported me in my endeavors during my time at IRIC.

Chapter 1- Introduction

1.1 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is the second-most common hematological disorder diagnosed in adults. AML is characterized by infiltration of immature myeloid progenitors in the bone marrow and peripheral blood [1]. These highly proliferative blasts, often poorly or abnormally differentiated lead to the impairment of the normal functions of the blood. AML is clinically recognised as a heterogeneous disorder. Several lines of evidence have shown that AML occurs as a multi-step transformation process and initiates from mutations in hematopoietic stem cell (HSC) giving rise to leukemic stem cells (LSC), present at the apex of the neoplastic hierarchy [2]. Unfortunately for AML patients, treatment has not advanced much in the past four decades and overall 5-year survival remains as low as 27% [3].

1.1.1 Classification of AML

Two principal systems have been established to define AML sub-types: the French-American-British (FAB) system and the World Health Organization (WHO) system [4].

1.1.1.1 FAB classification system

The FAB system is the oldest system of AML classification and dates back to 1976. It uses morphological and cytochemical features to classify AML and defines eight classes that ranges from M0 (myeloblasts) to M7 (megakaryocytes) (Table 1) [5]. FAB system classifies AML sub-types based on the degree of maturity and resemblance of leukemic blasts with normal myeloid progenitor cells [6]. The cut-off for AML diagnosis was set at >30% blasts in the bone marrow; anything below this cut-off was considered to be myelodysplastic syndrome (MDS). The prevalent advantage of this system was the ease of its use; however, it also came with several limitations. Some of the major shortcomings lied in the lack of inclusion of clinical and cytogenetic data and the lack of reproducibility arising from inter-observer variations [7].

FAB Subtype	Name
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

Table 1 : The FAB Classification System

1.1.1.2 WHO classification system

In 2001, the WHO system of classification was established to improve AML classification and articulate the new advances made in AML diagnosis. Unlike the FAB system, WHO classification takes into account genetic alterations, immunophenotypic as well as clinical features of AML [8]. This was updated in 2008 [9] followed by further revision in 2016 [10]. The current WHO classification of AML consists of four major entities; these include AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related AML, and AML not otherwise specified (Table 2). The WHO classification additionally lowered the threshold for diagnosis to 20% blasts in the blood or bone marrow. One of the limitations of the WHO system is that it does not take into account the prognostic relevance of certain mutations in genes such as FLT3-ITD mutation.

AML with recurrent genetic abnormalities

- AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
- APL with PML-RARA
- AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
- AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
- AML with t(6;9)(p23;q34.1);DEK-NUP214
- AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
- AML with BCR-ABL1
- AML with mutated NPM1
- AML with biallelic mutations of CEBPA
- AML with mutated RUNX1

AML with myelodysplasia-related changes

- AML with history of myelodysplastic syndromes (MDS)
- Morphological evidence of dysplasia in ≥ 2 myeloid cell lineages
- AML with myelodysplastic related cytogenetic abnormalities including monosomy 5/monosomy 7 and deletion 5q or 7q

Therapy related AML

- AML occurring due to prior treatment of chemotherapy such as alkylating agent or radiation therapy

AML not otherwise specified

- AML with minimal differentiation
- AML without maturation
- AML with maturation
- Acute myelomonocytic leukemia
- Acute monoblastic/monocytic leukemia
- Pure erythroid leukemia
- Acute megakaryoblastic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis

Table 2 : WHO classification (adapted from [11])

1.1.2 Prognostic factors in AML

Prognosis in AML depends on several factors including AML sub-type, cytogenetic anomalies and mutations as well as age and medical history of patients. Based on these, prognostic factors can be divided into two main categories referred to as patient-related and AML-related factors [12]. Patient-related factors include age, performance status and organ function [13]. Patient age at diagnosis is the major patient-related prognostic factor. Elderly patients over 60 years of age are associated with poorer outcomes. Among leukemia-related factors, cytogenetic anomalies and gene mutations are central in predicting treatment outcomes of AML patients. For instance, AML with translocations in core-binding factor (CBF) transcription factor complex such as translocation t(8;21) and inversion (16) are generally associated with a favorable treatment outcome [14]. On the contrary, if AML patients with CBF anomalies have co-existing mutations in the KIT gene, they are associated with an adverse risk [14].

The European LeukemiaNet (ELN) system was introduced in 2010 in order to develop a standardized approach to stratify AML patients based on their prognosis [16]. The ELN system added prognostic significance to the pre-existing AML classification. According to this system, risk categories are classified into four groups, namely favorable, intermediate-I, intermediate-II, and adverse sub-groups (Table 3). One of the milestones of this system was the sub-division of the cytogenetically normal AML (CN-AML) into two groups: favorable and intermediate-I subgroups based on mutational status of NPM1, CEBPA, and FLT3 genes. Since its advent, numerous studies have improved the ELN classification by additionally cumulating other genetic mutations to the ELN groups [17, 18].

Sub-group	Cytogenetic/genetic anomalies
Favorable	• t(8;21)(q22;q22); RUNX1-RUNX1T1 • inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 • Mutated NPM1 without FLT3-ITD (normal karyotype) • Mutated CEBPA (normal karyotype)
Intermediate-I	• Mutated NPM1 and FLT3-ITD (normal karyotype) • Wild-type NPM1 and FLT3-ITD (normal karyotype) • Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate-II	• t(9;11)(p22;q23); MLLT3-MLL • Any cytogenetic anomaly not classified as favorable or adverse
Adverse	• inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 • t(6;9)(p23;q34); DEK-NUP214 • t(v;11)(v;q23); MLL rearranged • Monosomy 5 or del(5q); monosomy 7; abnormal 17p; • Complex karyotype (≥ 3 chromosomal abnormalities)

Table 3 : ELN classification (adapted from [15])

1.1.3 Standard AML therapy

Although several advances in research have improved classification of AML, treatment for AML patients has not improved significantly. Standard treatment for AML patients consists of two components, referred to as remission (induction) therapy and post-remission therapy. AML treatment begins with induction therapy with the aim to completely eradicate morphologically visible leukemia in the blood and bone marrow and achieve complete remission (CR) in AML patients.

Induction therapy generally consists of a "7+3" regimen, irrespective of mutational background and cytogenetic anomalies. This includes continuous intravenous infusion of cytarabine for seven consecutive days followed by anthracycline administration (such as daunorubicin or idarubicin) for the next three days. Both these drugs are responsible for inhibition of DNA synthesis. Cytarabine works as an anti-metabolite and incorporates in the DNA, while anthracyclines are DNA intercalating agents and inhibit topoisomerase II enzyme. Depending on the age of the patients, strength of the therapy varies. Younger AML patients are given more intense chemotherapy than older patients (> 60 years) [19].

Post-remission therapy aims at eradicating any residual leukemic cells that persist after the induction therapy. This generally consists of three types: intensive chemotherapy (consolidation), maintenance therapy and high dose chemotherapy (conditioning) followed by hematopoietic stem cell transplantation (HSCT) [12]. For post-remission therapy, the cytogenetic risk factors of AML patients are taken into consideration for therapy selection. Patients associated with favorable cytogenetics are generally administered with repetitive cycles of high-dose cytarabine (HiDAC) chemotherapy for 3 – 5 days. For patients with intermediate or adverse risk AML, HSCT- either allogenic or autogenic- is usually the recommended choice of therapy [20]. The major obstacle in allogenic HSCT (allo-HSCT) is the risk of graft-versus-host disease due to incompatibility between the donor and the recipient that ultimately leads to the rejection of donor's transplanted hematopoietic stem cells. However, allo-HSCT has the advantage over autologous HSCT of the phenomenon called graft-versus-leukemia effect which refers to the ability of the donor's immune system to eliminate host's leukemic cells. For older AML patients,

HSCT is rarely offered and therapy is usually less intensive and consists of one or two cycles of high dose chemotherapy [20, 21].

1.2 High-throughput screening approach to identify better anti-AML molecules

As mentioned earlier, AML is a very heterogeneous disease and standard treatment for AML remains the same for the past three to four decades. In the search for better therapeutics targeted to specific AML sub-groups, we performed a large viability screen with a library of approximately 5,000 compounds that included chromatin modifiers, kinase inhibitors, off-patent drugs, molecules targeting G-protein coupled receptors (GPCRs) and some compounds proprietary to IRIC. A major hindrance in the identification of good anti-AML molecules is due to the occurrence of AML cells quickly differentiating in culture. This makes current assay conditions inefficient in correctly recapitulating *in vivo* drug responses of AML. To circumvent this limitation, we used novel culture conditions established in the laboratory using the compounds UM729 and SR1 that inhibit differentiation of AML cells and allow their maintenance and expansion *ex vivo* [22]. The screen comprised of 20 primary human AML specimens that were selected to represent the genetic heterogeneity of AML. Drugs that achieved >50% proliferation inhibition of AML cells compared to DMSO treated controls were considered as 'hits'.

1.2.1 Identification of statin's discriminatory effect in AML

One group of hit compounds were statins, HMGCR (hydroxy-methyl-glutaryl-CoA reductase) inhibitors, which had the ability to discriminate between AML specimens. This was evident from the unique association between the *in vitro* statin sensitivity of patient derived specimens and the patient's clinical outcome. Specimens derived from AML patients with a good overall survival (OS> 3 years) were more sensitive to statins compared to specimens derived from patients with a poor overall survival (OS <1 year) (Figure 1, a and b). We further validated this finding in a second screen with specimens that belonged to AML patients with either successful treatment outcome (OS > 3 years with standard chemotherapy and no HSCT)

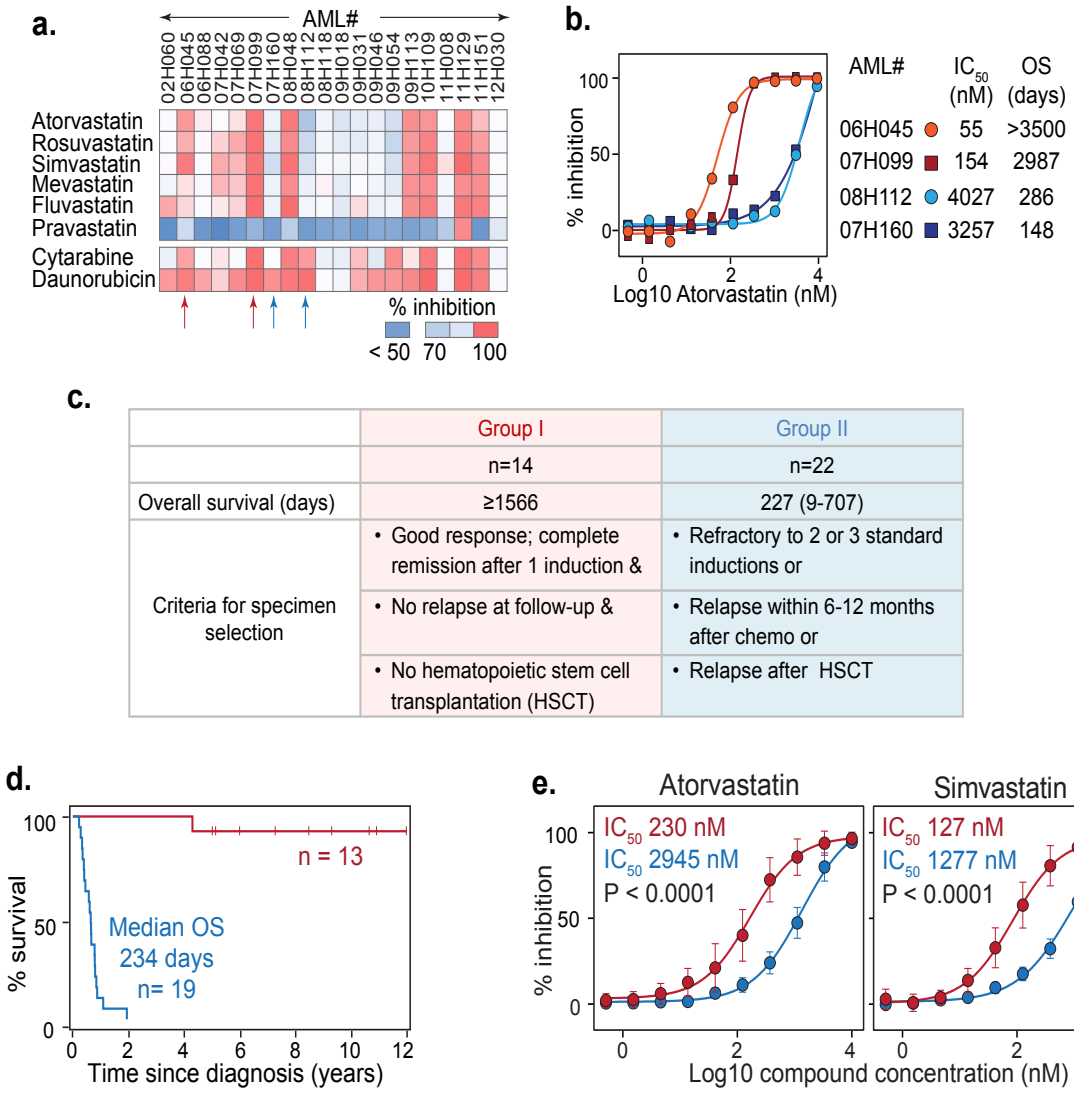


Figure 1. Identification of statin's discriminatory effect in AML. (a) Primary chemical screen with 20 de novo AML specimens identifies variability in statin responses depending on patient's clinical outcome. Red and blue arrows represent good and poor overall survival of AML patients respectively and (b) dose response assays of these patient derived AML specimens to atorvastatin. (c) Selection criteria of clinical characteristics of specimens in screen II (d) Kaplan-meier curves depicting survival of AML patients in screen II. (e) Dose response assays of atorvastatin and simvastatin of AML specimens in screen II. (Work done by Jana Krosl (unpublished data) in the Sauvageau laboratory)

or that were refractory to treatment (OS < 1 year, induction chemotherapy-resistant that relapsed irrespective of HSCT) (Figure 1, c and d). We observed a significant 10-fold difference between the IC_{50} values of the two groups of specimens (Figure 1e), further confirming the results observed in the initial screen.

1.2.2 HMGCR inhibition is key to statin's discriminatory effect

Statins work by blocking the activity of the HMGCR enzyme. To identify if statin's discriminatory effect is indeed mediated via HMGCR inhibition, we questioned if statin analogs that lack HMGCR inhibitory activity would retain statin's discriminatory effect. In collaboration with Anne Marinier's team at IRIC, we performed structure-activity relationship studies to identify analogs of statins that lack anti-HMGCR activity. We identified UM135405 (hereafter, A405) an analog of Atorvastatin that completely lost the ability to inhibit HMGCR, as measured by *in vitro* assays (Figure 2, a and b). We found that A405 completely lost the discriminatory effect observed with the statins (Figure 2c) thus suggesting that HMGCR inhibition is essential for statin's discriminatory effect in AML. Further, we observed that even with the loss of HMGCR inhibition, A405 retained some anti-AML activity; this suggested the possibility that a 'second' target of the statins exists.

1.2.3 CBF-AML - Most statin-sensitive AML sub-group

In order to improve our understanding of statin's anti-leukemic effect, we sought to identify if specific sub-groups, mutations or genetic anomalies could be associated with *ex-vivo* statin sensitivity. To this end, we tested statins in a third screen with more than 200 *de novo* AML specimens that were sequenced and characterized with clinical annotations, enabling a complete mutational and cytogenetic landscape of AML patient specimens. The screen uncovered that specimens belonging to the CBF-AML sub-group were the most statin sensitive among other AML specimens (Figure 3a). CBF-AML is associated with chromosomal anomalies with either translocation t(8;21) or inversion inv(16)/t(16;16) (inv (16)) that lead to the disruption of the core-binding transcription factor essential for the transcription of genes crucial during hematopoiesis. We additionally observed that AML with mutations in IDH2 (Isocitrate dehydrogenase

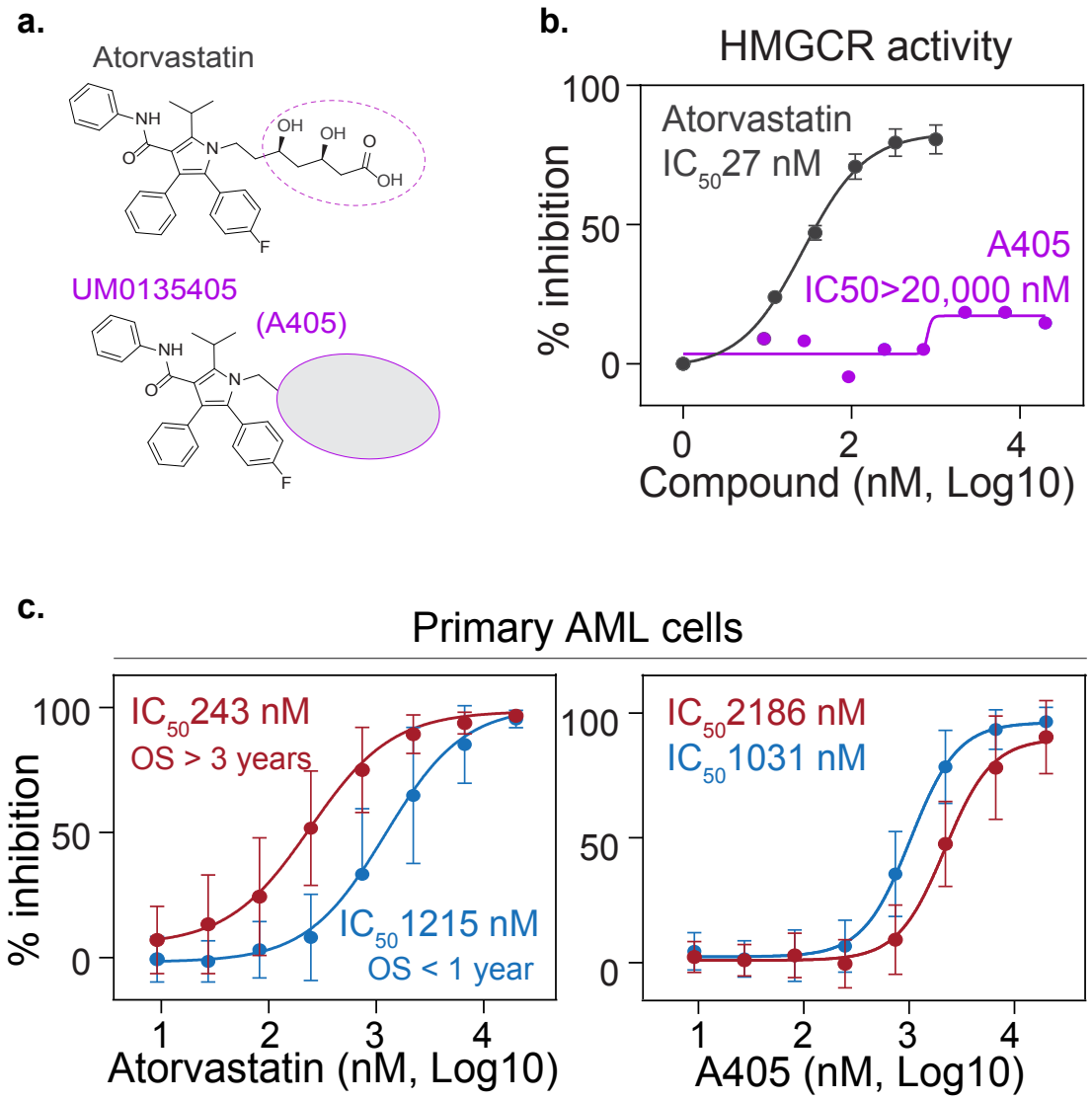


Figure 2. HMGCR inhibition is key to statin's discriminatory effect. (a) Chemical structures of atorvastatin and its A405 analog and (b) HMGCR inhibition activity of compounds measured with cell-free HMGCR activity assay kit. (c) Dose response assays of atorvastatin (left) and A405 (right) on primary AML specimens. Discriminatory effect is lost in the case of A405. (Work done by Jana Krosl (unpublished data) in the Sauvageau laboratory in collaboration with Anne Marinier's team)

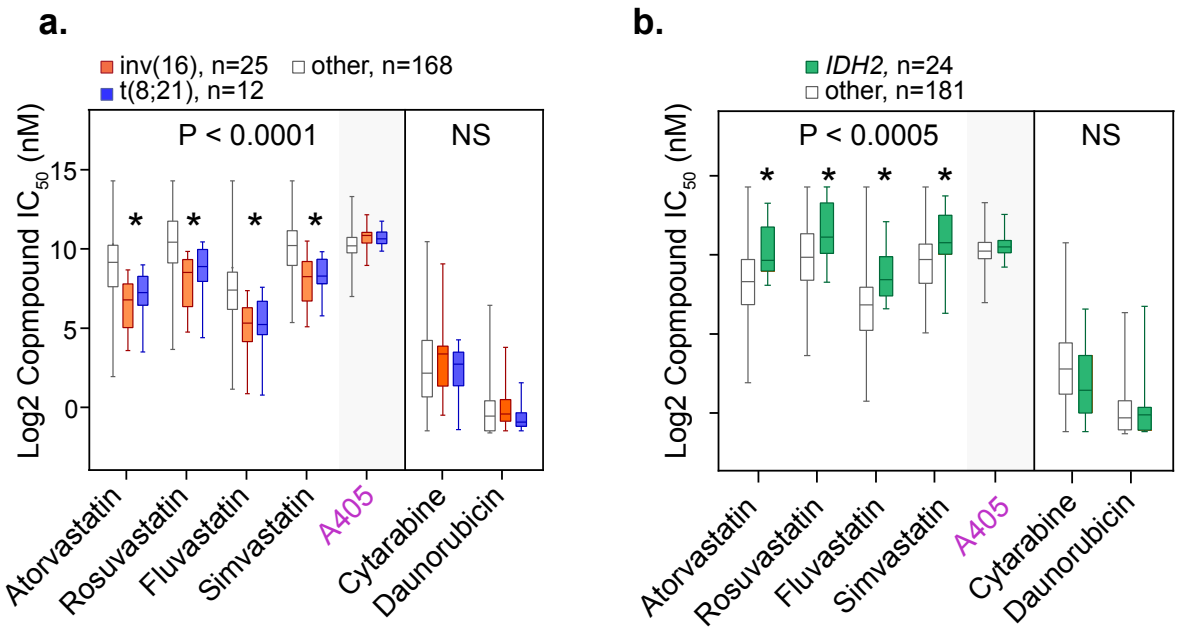


Figure 3. Identification of statin-sensitive and resistant AML sub-groups. IC50 values derived from the large chemical screen with 200 de novo AML specimens against statins identifies (a) CBF AML sub-group, defined by translocation t(8;21) or inversion inv(16)/t(16;16) (inv (16)) anomalies, are most statin sensitive and (b) AML with mutations in IDH gene are most statin resistant compared to other AML sub-groups. No significant changes in IC50 values are observed with either standard chemotherapeutic agents (cytarabine and daunorubicin) or A405. (Work done by Jana Krosl (unpublished data) in the Sauvageau laboratory)

isoform 2) gene were the most statin-resistant AML sub-group (Figure 3b).

1.3 Statins: Cholesterol lowering blockbuster drug

Statins are cholesterol-lowering drugs that were identified from fungal origin in the late 1900s and are widely prescribed for the treatment and prevention of cardiovascular and coronary heart diseases. Interestingly, the beneficial effects of statins are not restricted only to the cholesterol pathway. Statins have been implicated for their pleiotropic effects on other mechanisms and signalling pathways through their impact on the mevalonate pathway.

1.3.1 Pharmacology of Statins

1.3.1.1 Mechanism of action

Statins work by inhibiting hydroxylmethyl glutaryl coenzyme A (HMG-CoA) reductase enzyme and thus, inhibits the formation of mevalonate, the precursor of cholesterol [23]. HMG-CoA reductase (HMGCR) catalyses the reduction of HMG-CoA to mevalonate and is considered the rate-limiting enzyme of the mevalonate pathway and subsequent cholesterol biosynthesis pathway [24].

The crystal structure of the catalytic portion of the HMGCR provided a mechanistic insight on statin-mediated HMGCR inhibition [25]. Statins bind and occupy a part of the binding pocket for the HMG-CoA, thus inhibiting the access of HMG-CoA to the enzyme's active site. Structural studies revealed that statins competitively inhibit HMGCR for HMG-CoA but not its co-enzyme NADPH. Pharmacological studies demonstrated that statins had the ability to bind to HMGCR with an affinity of approximately 3 orders of magnitude higher than its endogenous substrate, HMG-CoA [26]. The affinity of the enzyme for the inhibitor is in the nanomolar range, while that of the HMG-CoA is in the high micromolar range [27]. In other words, statins act as competitive antagonists of the HMGCR enzyme and bind to the enzyme with a much higher affinity than its original substrate. Thus, statins were found to be remarkably powerful drugs to lower blood cholesterol levels via their inhibitory action on the HMGCR.

1.3.1.2 Chemical structure and functional properties

The structure of statins is similar to the natural substrate of HMGCR, thus bestowing them the ability to bind to the enzyme with very high affinity. Chemically, statin structure is constituted of two distinct components covalently linked to each other: a pharmacophore, a moiety shared by all statins, and a hydrophobic ring moiety.

The pharmacophore of statins is a modified HMG-CoA-like moiety (3,5- dihydroxyglutaric acid) which structurally resembles the HMGCR substrate HMG-CoA and its intermediate transition state, mevaldyl-CoA [25, 28]. The statin pharmacophore is responsible for the binding to the active site of the HMGCR enzyme, essential for the binding of HMG-CoA substrate. This makes statins capable of inhibiting HMGCR in a competitive, reversible and dose-dependent manner [29]. In statins such as lovastatin and simvastatin, the HMG-CoA like moiety is present as an inactive lactone form, which is hydrolysed *in vivo* to expose the active open acid form of the pharmacophore [30]. Additionally, the HMGCR enzyme is stereo-selective and 3R,5R stereochemistry of statins allows them to be efficient inhibitors.

The latter component of the statins, the hydrophobic rigid ring moiety, is essential for the forming binding interactions with the HMGCR enzyme. These interactions aid in improving the proximity of the inhibitor with the enzyme and reduce the competition of the enzyme's natural HMG-CoA substrate. Different statins have different ring moieties. For instance, partially reduced naphthalene ring is present as a substituent in statins such as lovastatin, simvastatin and pravastatin, while atorvastatin and cerivastatin contain a pyrole and pyridine group respectively [23]. Differences in the chemical structures of the ring moiety across statins not only have an impact on statin's mode of binding but also governs the distinct molecular and clinical properties of the statins. Moreover, the substituents of the ring structures affects the solubility of the statins and consequently several of their pharmacokinetic characteristics, including drug absorption, distribution, metabolism and excretion [31].

1.3.2 Types of statins

Depending on their origin, statins are classified into two types: type I and type II. Type

I statins consist of naturally occurring statins that were first discovered as secondary metabolites in fungi. Endo *et al.*, the pioneers in the discovery of statins, identified the world's first fungal-derived statin Mevastatin, isolated from *Penicillium citrinum* [32]. Other naturally occurring statins belonging to type I class include simvastatin, lovastatin and pravastatin and share structural homology to Mevastatin. The structural similarity arises from their common hydroxy-hexahydro naphthalene ring system, that contains different substituent side chains attached to it [33]. Type II statins, on the other hand, contain synthetically derived statins. The structures of type II statins are very different from natural statins but contain the statin pharmacophore, HMG CoA-like moiety essential for the HMGCR inhibition (Figure 4). These include cerivastatin, atorvastatin, fluvastatin, rosuvastatin as well as pitavastatin and have differences in their bioavailability, half-lives, metabolism, excretion and other pharmacokinetic properties [34].

Statins are classified based on their solubility properties and hydrophilicity. The common pharmacophore of statins inherently imparts a 'hydrophilic' nature to the statins due to its strong polar hydroxyl, carboxylate component. The ring moiety further contributes to statin's overall hydrophilic or lipophilic characteristics. Among lipophilic statins are atorvastatin, fluvastatin, cerivastatin and simvastatin, while rosuvastatin and pravastatin are hydrophilic statins. These solubility characteristics of statins have been found to play a major role not only on cholesterol inhibition effects but also its pleiotropic effects. Several clinical studies have been performed to explore the differences between the lipophilic and hydrophobic statins and assess the benefits of both [35-37]. Hydrophilic statins are more hepato-selective because they specifically require active transport to enter into the liver, which is the major site for cholesterol synthesis and thus claimed to have lesser toxic effects. On the other hand, lipophilic statins, by the virtue of their hydrophobic nature, can enter through passive diffusion across cell membranes of both hepatic as well as non-hepatic tissue. Some studies demonstrated using *in vitro* experiments that lipophilic statins had a higher propensity to cause statin-induced myopathies compared to hydrophilic statins [38].

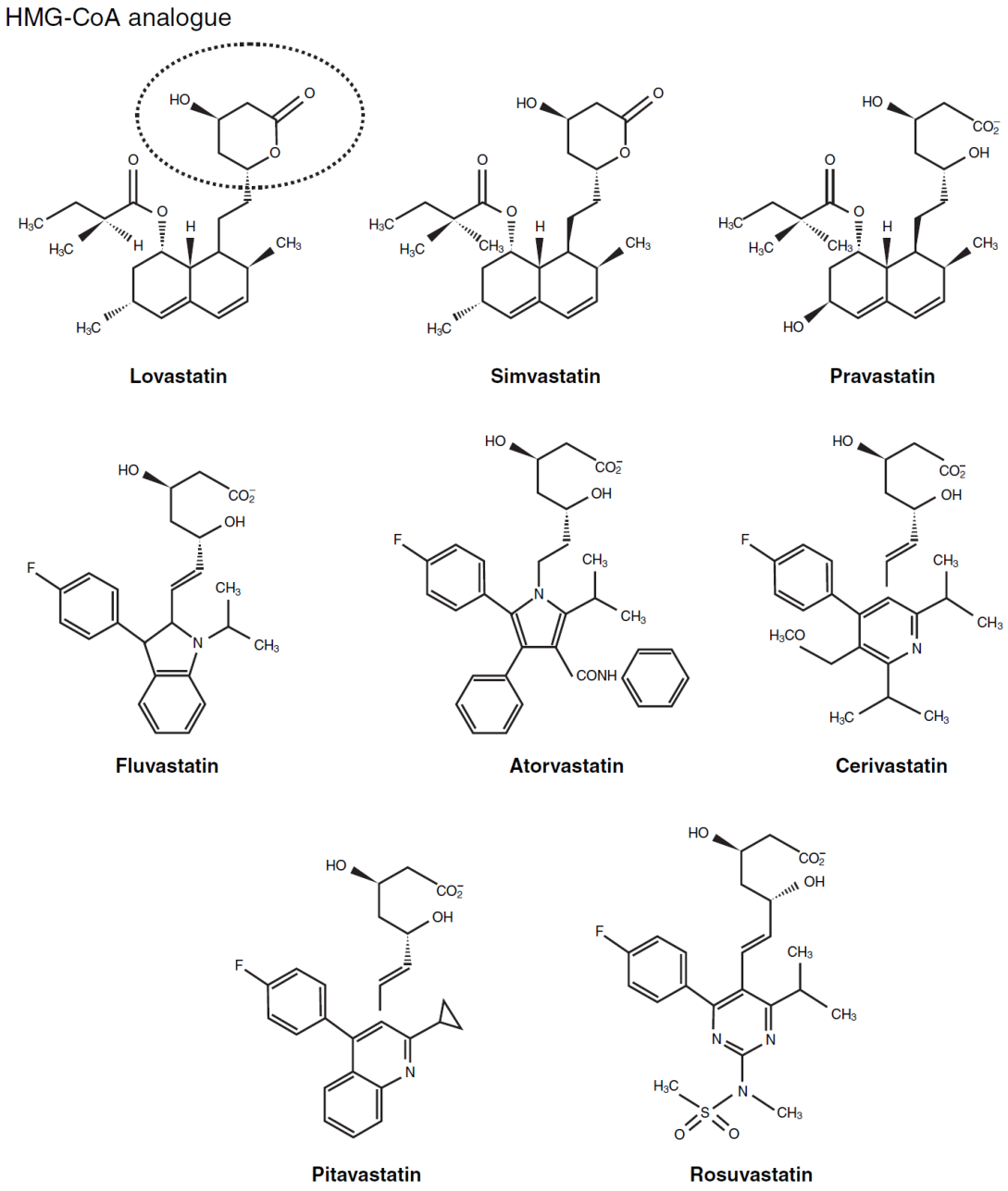


Figure 4. Statin structures. Chemical structures of statins and derivatives. The pharmacophore, HMG-CoA-like moiety (encircled) is shared among statins.

1.4 The Mevalonate Pathway

1.4.1 Cancer metabolism & the mevalonate pathway

Reprogramming of cellular energy metabolism in tumor cells is now recognised as an important hallmark of cancer [39]. Tumor cells have been found to alter their metabolism in order to supply macromolecular precursors and provide energy to support their abnormal growth and survival [40]. Metabolic reprogramming in tumors could occur as a direct consequence of mutations in genes encoding for enzymes involved in metabolism. Examples of these include mutations in genes encoding for enzymes of the Krebs cycle such as isocitrate dehydrogenase (IDH), fumarate hydratase (FH), succinate dehydrogenase (SDH) [41, 42]. Altered metabolic activity could additionally occur as a result of the activation of signalling cascades that promote an increase in the anabolic program. These include mutations in genes encoding for key players in the PI3K-AKT-mTOR that lead to over-activation of the signalling network [43], or due to the altered activity of enzymes in metabolic pathways such as aerobic glycolysis and the mevalonate pathway [44, 45].

The mevalonate pathway is an important biosynthetic pathway for the synthesis of cholesterol and other isoprenoid-derived intermediates that are vital for cellular function and survival. This anabolic pathway is controlled by the activity of HMGCR, which converts HMG-CoA to mevalonate. Several studies have provided evidences demonstrating the mevalonate pathway as being oncogenic. In B-cell chronic lymphocytic leukemia (CLL), mevalonic acid was found to acquire the ability to cause transformation [46]. Work from another study showed that ectopic expression of the HMGCR enzyme was capable of promoting transformation in primary mouse embryonic fibroblasts, suggesting that HMGCR plays a role as an oncogenic metabolite [47]. Data analysis of primary breast cancer patient specimens has also shown that higher expression of genes associated with the mevalonate pathway correlate with poor prognosis and reduced survival [47]. Additionally, *in vivo* studies have further demonstrated that administration of mevalonate to mice with breast cancer xenografts promotes tumor growth [48]. Thus, there are increasing evidences that the mevalonate pathway plays a significant role in cancer, strongly suggesting the therapeutic potential of targeting the mevalonate pathway.

1.4.2 Mevalonate pathway derived branches and their metabolites

The mevalonate pathway was initially identified for its role in cholesterol biosynthesis. Several studies on the fate of mevalonate later proved that products of the mevalonate pathway were steadily incorporated into proteins as well [49, 50]. This led to the identification of isoprenoid moieties and their role in protein post-translational modifications. Isoprenoids are derived from a common 5-carbon compound building block isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Sequential head-to-head or head-to-tail condensations lead to the formation of several complex isoprenoid moieties that are then added onto biological molecules. Thus, the mevalonate pathway is essential for the formation of sterol and non-sterol isoprenoids (Figure 5) and each of these branches are briefly described in the following sections below.

Cholesterol branch. The first committed step in the process of cholesterol biosynthesis is the formation of squalene from farnesyl diphosphate (FPP) catalysed by the squalene synthase enzyme (FDFT). Squalene undergoes cyclization reactions to form lanosterol and after a series of additional reactions, cholesterol is formed.

Cholesterol plays an important role in steroidogenesis as a precursor of steroid hormones, essential for tumor growth and progression, implicating its usefulness as a therapeutic target [51, 52]. Cholesterol is an essential component of the cellular membrane as well as lipid rafts. Thus, cholesterol is required in rapidly proliferating cells for its incorporation into cell membranes. Moreover, lipid rafts play a key role in signal transduction. Lipid raft depletion by cholesterol-depleting drugs leads to increased apoptosis in cancers such as breast cancer and prostate cancer [53]. Studies in AML have proved that upregulation of cholesterol is essential for survival of leukemic cells and inhibition of cholesterol synthesis can sensitize AML cells to therapeutics [54, 55]. Further, leukemic cells, but not normal mononuclear cells, have aberrant feedback repression of cholesterol synthesis which is associated with an increase in cell survival [56].

Protein isoprenylation branch. Isoprenylation is a process involving post-translational

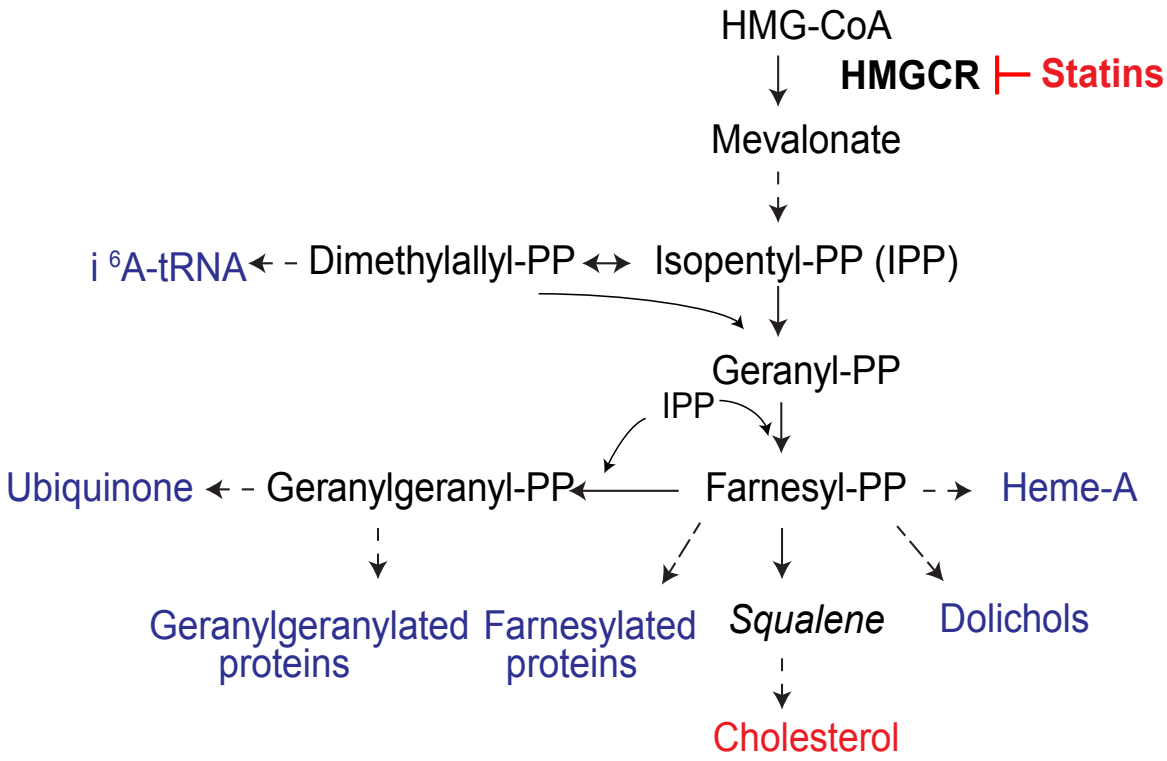


Figure 5. The mevalonate pathway. Schematic representation of the mevalonate pathway and the formation of steroid and non-steroid isoprenoid end products. Inhibition of HMGCR via statins blocks the production of mevalonate, the precursor of downstream sub-branches of the mevalonate pathway that are critical for cell proliferation and survival.

lipid modification of proteins by covalent attachment of 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid groups to conserved cysteine residues at the C-terminus of proteins. This enhances the overall lipophilicity of proteins and facilitates interactions with cell membranes. Thus, prenylation enables proteins to perform functions such as signal transduction and vesicular trafficking.

There are three types of prenyltransferase enzymes involved in post-translational modification of proteins: farnesyl transferase (FT), geranylgeranyl transferase I (GGT-I) and geranylgeranyl transferase II (GGT-II). FT transfers a farnesyl moiety onto the cysteine of proteins with C-terminal CaaX motif (C- cysteine, a – any aliphatic amino acid, and X - usually methionine/glutamine/serine/alanine/cysteine). GGT-I transfers the geranylgeranyl moiety onto the cysteine of a closely identical CaaX motif (X -either leucine/isoleucine). The two enzymes share a common farnesyl tranferase α subunit (FNTA) and distinct catalytic subunits, farnesyl tranferase β subunit (FNTB) and geranylgeranyl transferase type I β subunit (PGGT1B) in FT and GGT- I respectively, that determine the specificity of the prenyl groups to be transferred. On the contrary, GGT-II enzyme transfers two geranylgeranyl moieties onto cysteine residues of a rather distinct CXC or CC motifs. The enzyme requires the presence of rab escort protein (REP) for the addition of the prenyl groups on the protein [57]. The main targets of GGT-II enzyme are Rab proteins, which are essential for vesicular trafficking. Hence, GGT-II is also referred to as Rab geranylgeranyl transferase [58, 59]. Some of the prenylated proteins modified by FT and GGT-1 include nuclear surface membrane proteins (lamins) and GTP-binding proteins (Ras, Rho, Rac, Rap, etc.). Ras proteins play an important role in proliferation, differentiation and survival, and mutations in these proteins (H-Ras, K-Ras and N-Ras) lead to aberrant signaling and tumor initiation. [60, 61]. Thus, inhibition of prenylation has been shown to alter Ras functionality and thus suggested as a therapeutic target. Isoprenylated proteins often undergo additional processing steps involving proteolysis and methyl-esterification by isoprenylcysteine carboxyl methyltransferase (ICMT) that further increase hydrophobic interactions.

Genetic screens performed in the past have also revealed the importance of the inhibition of the prenylation branch in driving statin's anti-tumor activity. Linda Penn's group demon-

strated with genome-wide dropout RNAi screen that knocking down of genes encoding Geranylgeranyl diphosphate synthase 1 (GGPS1) enzyme (upstream to the geranylgeranylation and the ubiquinone branch) enhanced statin-induced apoptosis in lung cancer [62]. The screen also revealed that knocking down genes upstream to the mevalonate pathway and essential for the regulation of the mevalonate pathway, such as HMG-CoA synthase (HMGCS1) and sterol regulatory element binding transcription factor 2 (SREBP2) enhanced sensitivity to statins. Another study from Todd Golub's group used an *in vivo* RNAi pooled screen to target the enzymes in the mevalonate pathway in AML and showed that enzymes involved in the farnesylation and geranylgeranylation such as FNTA and ICMT genes were crucial for driving leukemic activity in LSCs [63].

Dolichol branch. Dolichol is a metabolite derived from isoprenoid molecules essential for synthesis of glycoproteins. Phosphorylated dolichols are also known to be components of cellular membranes to increase membrane fluidity [64]. Some studies suggest that repression of dolichol synthesis slows cancer cell proliferation due to a decrease in the expression of glycosylated IGF-I receptor that is essential for transition to the S phase of the cell cycle [65]. In AML, a study showed that reduction of dolichol pools led to loss of the glycosylated tyrosine kinase receptor FLT3 leading to mislocalization and altered signalling of the receptor, thus suggesting statins could overcome tyrosine kinase inhibitor (TKI) resistance in FLT3/ITD AML [66].

Ubiquinone branch. Ubiquinone or Coenzyme Q10 (CoQ10) is synthesized via condensation reactions using geranylgeranyl pyrophosphate as a substrate. This is an essential component of complex I of the electron transport chain (ETC) in the mitochondria. The reduced form of ubiquinone, ubiquinol has been well established for its anti-oxidant properties and is known to protect the phospholipids of cell membranes as well as the serum lipoproteins [67]. In liver cancer, studies showed evidence that ubiquinone branch is critical for carcinogenesis and supplementation of ubiquinone could reverse the anti-tumor activity of statins [68]. Contrarily, a study demonstrated that ubiquinone supplementation was not sufficient to rescue the statin's inhibitory effect in AML cells [69].

Heme A branch. Heme A serves as a vital electron carrier of the cytochrome oxidase (COX) in the mitochondrial complex IV: cytochrome C oxidase of the ETC. Synthesis of Heme A begins with the farnesylation by Cox10 enzyme of Heme B (protoheme) into Heme O. Heme O undergoes a few additional modifications to form Heme A [70, 71]. The farnesylation serves as a lipophilic anchor for the positioning of Heme A within the COX complex [72]. Studies in ovarian and endometrial cancer have demonstrated that complex IV inhibition leads to degradation of the hypoxia-inducible factor-1α (HIF-1α) which is well associated with tumor metastasis, angiogenesis and therapy resistance [73, 74]. A study performed in AML with mutant IDH showed that suppression of the cytochrome C oxidase leads to the sensitization of the AML sub-group to BCL-2 inhibitors [75].

i^6A-tRNA branch. This branch involves the post transcriptional modification of tRNA and is driven by the tRNA isopentenyltransferase 1 (TRIT1) enzyme [76]. TRIT1 modifies tRNA by the addition of isopentenyl on the adenosine 37 residue to form N_6-isopentenyladenosine (i^6A) tRNA [77]. In mammalian cells, the only tRNA with such modification is the Selenocysteyl (Sec) tRNA (tRNA ([Ser]Sec)) and is essential for selenoprotein production. The i^6A modification on the tRNA enables the decoding of specific stop codons, UGA as selenocysteines in selenoproteins [78]. Selenoproteins have been well studied for their role in maintaining the cellular redox homeostasis [79]. In leukemia, studies have suggested that selenoproteins have a protective function and play a role in LSC apoptosis [80]. Another study in lung cancer showed that TRIT1 could possibly play a tumor suppressor role as TRIT1 expression negatively correlated with lung carcinogenesis [81].

1.4.3 Regulation of the mevalonate pathway

The fine tuning of the mevalonate pathway is crucial for cells to receive the right supply of metabolites supporting growth without over accumulation of toxic products, including cholesterol [50]. The HMGCR enzyme, the rate-determining enzyme of the pathway, is subject to tight regulation and controlled by multivalent feedback mechanisms at the transcriptional, translational and post-translational level. At the transcriptional level, shortage of cholesterol or steroid isoprenoids activates sterol regulatory element binding proteins (SREBP), that enhances

transcription of genes of the cholesterol synthesis enzymes as well as the low density lipoprotein receptor (LDL) by binding to promoter regions containing sterol regulatory elements (SRE) [82]. Non-steroid derived isoprenoids regulate HMGCR at the translational level by undefined mechanisms [24]. Stability and activity of HMGCR is regulated by post-translational mechanisms. HMGCR enzyme is rapidly degraded by the 26S proteasome through its interaction with gp78, ubiquitin-E3 ligase; mediated again by both mevalonate derived sterols and non-sterols [83]. Finally, activity of HMGCR is regulated by inactivating phosphorylation by AMP-activated protein kinase (AMPK), an enzyme subject to activation by the AMP: ATP ratio in the cell [84]. Therefore, HMGCR is the master regulator of controlling the flux of metabolites in the mevalonate pathway.

1.5 Clinical applications of statins in cancer

Studies have demonstrated that statin usage is associated with 20-28% reduction in overall cancer incidence [1]. One instance of this was described in a study by Farwell *et al.* that revealed that total cancer incidence was 9.4% in statin users with cardiovascular diseases compared to non-statin users with cancer incidence of 13.2% [2]. Likewise, several observational and meta-analysis studies has shown that statin usage was associated with lower cancer risks in cases of prostate, hepatocellular, gastric and esophageal cancers [3-6]. Meta-analyses and the pre-clinical results obtained with statins rationalized the repositioning of statins as anti-cancer agents and statins made their way into clinical trials for cancer patients. In the early 1990s, Lovastatin was the first statin to be approved to enter into a clinical trial for patients with solid tumors. Lovastatin dosage of 25 mg/kg/day was administered to cancer patients for seven consecutive days which resulted in statin plasma concentrations of 0.1 to 3.9 μM [7]. This dosage was 50 times higher than the dose administered to treat hypocholesteremia and the major side effects of statins was the lovastatin-induced myopathy. Although this toxicity could be reversed by the co-administration of ubiquinone to patients, large interpatient variability was reported and there was no correlation between the extent of the inhibitory effect or the plasma drug concentrations with the dosage administered [7]. Several other clinical trials have followed suit and as of now (July 2018), https://www.clinicaltrials.gov/ reports a total of 154 entries for statins

that are or have been part of clinic trials for cancer. These include atorvastatin, lovastatin, simvastatin, fluvastatin, rosuvastatin and pravastatin for several solid tumors as well as hematological cancers, including AML. In AML, statins including atorvastatin, lovastatin and pravastatin were approved to enter clinical trials. In order to assess the safety and efficacy of lovastatin in AML, a clinical trial was initiated and AML patients were administered with 10–20 mg/kg/day for 2 weeks. However, due to high drug-related toxicity issues, the full regimen of the lovastatin could not be completed and the trial had to be terminated [8]. In another single case study, an elderly female with relapsed AML was administered with a reduced lovastatin dose of 2 mg/kg/day for a prolonged period of 54 days. In this case, AML blast counts decreased during the treatment and reduced counts were maintained for three months post-treatment [9]. Other clinical trials of statins for treatment of AML patients are summarised in table 4. Although data from the clinical trials with statins in AML provides some promising results, understanding how statin's mediate their anti-AML effect will be very crucial to decrease the toxicity issues and side-effects and completely benefit patients.

Statin	AML	Study phase	Dosing schedule	Study status	Results: Response rate/ Survival	Reference
Atorvastatin (n=50)	Tumor protein 53 (p53) mutated and wild type AML patients	Phase I	80 mg/day Atorvastatin, 1 - 4 weeks before surgery	Not yet recruiting	None	NCT0356 0882
Lovastatin (n=23)	Refractory or relapsed AML	Phase I/II	0.5 mg/kg/day Lovastatin (days 1-7), at day 14, lovastatin doses given in two fold increments (upto 24 mg/kg/day) 3.0 g/m^2 HiDAC Cytarabine every 12 hours (day 3-7)	Terminated	CR of 53.8% (No statistical analysis provided)	NCT0058 3102
Pravastatin (n=6)	Refractory or relapsed AML	Phase I/II	Pravastatin (days 1-10), Cyclosporine (days 5-9), Etopside (days 5-9), mitoxantrone hydrochloride (days 5-9)	Terminated	No CR achieved	NCT0134 2887

Pravastatin (n=36)	Relapsed AML	Phase II	1280 mg/day Pravastatin (Days 1-8), 1.5g/ m^2 Cytarabine (days 4-6), 12 mg/m^2 Idurubicin (days 4-6)	Completed	Response rate of 75% , Median OS 12 months	[85]
Pravastatin (n=24)	Newly diagnosed AML/MDS	Phase II	1280 mg/day Pravastatin (Days 1-8), 1.5g/ m^2 Cytarabine (days 4-7), 12 mg/m^2 Idurubicin (days 4-6)	Completed	Good CR in 50% patients	NCT01831232 [86]
Pravastatin (n=37)	Acute myeloblastic leukemia	Phase I	40- 1680 mg/day Pravastatin (Days 1-8) 1.5g/ m^2 Cytarabine (days 4-7), 12 mg/m^2 Idurubicin (days 4-6)	Completed	CR of 73%	[87]

Table 4 : Summary of clinical trials involving statins in AML treatment

General rationale and hypothesis

AML is a clinically heterogeneous disease and is characterised by several distinct molecular and cytogenetic anomalies. The heterogeneous nature of AML is thought to be responsible for current therapeutics to perform inefficiently in patients which highlights the requirement for sub-group specific anti-AML therapy. The need for better therapeutics is more urgently required for adverse outcome AML patients, who are often refractory to treatment and relapse after therapy. Identification of drug-targetable networks in both good- and poor-outcome AML is a requisite for the development of efficient sub-group specific anti-AML therapy.

We previously identified statin's ability to discriminate between AML specimens depending on the treatment outcome of patients they originated from. Preliminary investigation in the Sauvageau lab demonstrated that the HMGCR inhibition is essential for statin's differential anti-AML activity. This suggested that the mevalonate pathway is somehow more crucial for the leukemic activity in the good-outcome AML patient samples; thus making them more sensitive to statins compared to the poor-outcome patient samples. As previously described, the mevalonate pathway bifurcates into different biosynthetic sub-branches and play crucial roles in several cellular functions. Moreover, many of these branches have previously been implicated in several different cancers, including AML. **These preliminary evidences led us to hypothesize that statin's HMGCR-dependent anti-AML effect is modulated by sub-branch(es) of the mevalonate pathway.** The goal of the project is to identify components or mechanisms that drive statin sensitivity using genetic and chemical approaches.

Specific Objectives

1) Identify statin sensitive cell lines.

2) Validate that HMGCR is the target for statin induced anti-AML activity.

3) Identify sub-branch(es) of the mevalonate pathway, downstream of HMGCR, contributing to anti-AML activity of statins.

Chapter 2: Results

2.1 Identification of statin sensitive cell lines

In order to identify a suitable model system to approach our hypothesis, we tested a series of cell lines for statin sensitivity. We chose AML cell lines with cytogenetic anomalies of the CBF-AML sub-group such as Kasumi-1 and SKNO-1 (t(8;21)), ME-1 (inv (16)) as well as non-CBF-AML cell line OCI-AML-5 cells. We tested statin sensitivity of cell lines and performed flow-cytometric assessment using a viability dye and a fixed volume of beads in order to measure viable cell counts. Cell viability was assessed as a measure of viable (PI or 7-AAD negative) cells as a percentage of the mock (DMSO) treated cells. Our methodology is advantageous over other assays as it is a direct measure of cell viability rather than an indirect estimation of viable cells obtained by measuring general metabolism or enzyme activity (in assays such as MTT reduction assays or ATP assays). Cell death was assessed by measuring the percentage of PI/7-AAD positive cells as compared to mock (DMSO) treated cells. All experiments were performed in serum-starved conditions in order to avoid compensatory effects of cholesterol or other lipids in the serum. We observed that OCI-AML-5 cells and Kasumi-1 cells were sensitive to statins (Figure 6) and good model cell lines to carry out our objectives. Other cell lines tested, the SKNO-1 cell line had a very long doubling period (~50 hours) and ME-1 cell line had poor viability recovery in DMSO (control condition) and hence, were not continued in the study.

2.2 Validation that HMGCR is the target for statin induced anti-AML activity

2.2.1 shHMGCR cloned in a doxycycline-inducible system

The pharmacological target of statins is known to be HMGCR. As a proof-of-principle experiment, we sought to validate that HMGCR is the target of statin induced anti-AML activity in OCI-AML-5 cells. In order to approach this objective, we wished to knockdown the HMGCR enzyme and to observe changes in statin sensitivity. We expected to observe an increase in sensitivity upon HMGCR knockdown. We designed shRNAs targeting the HMGCR gene in a tet-in-

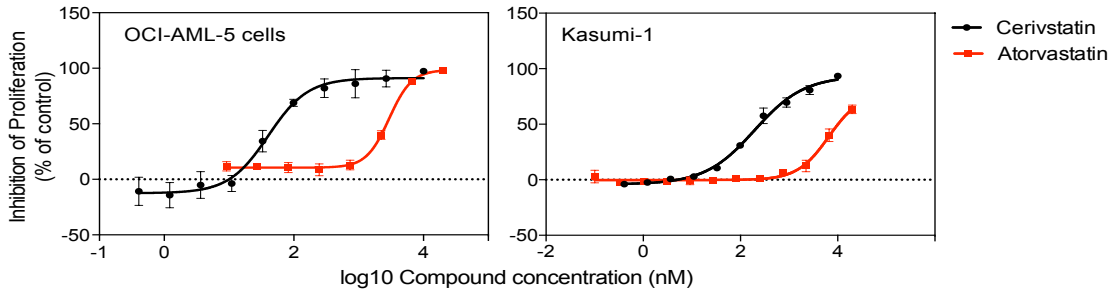


Figure 6. Testing of cell lines with statins. Dose response assays of atorvastatin and cerivastatin with AML cell lines, OCI-AML-5, (left) and Kasumi-1 (right). Vertical axis represents inhibition of proliferation, measured by calculating viable cell counts as a percentage of the DMSO-treated (control) condition. Horizontal axis represents increasing concentrations of compound. (Done in quadreplicates; OCI-AML-5: representative image of n=4 independent experiments, Kasumi-1: n=1 independent experiment)

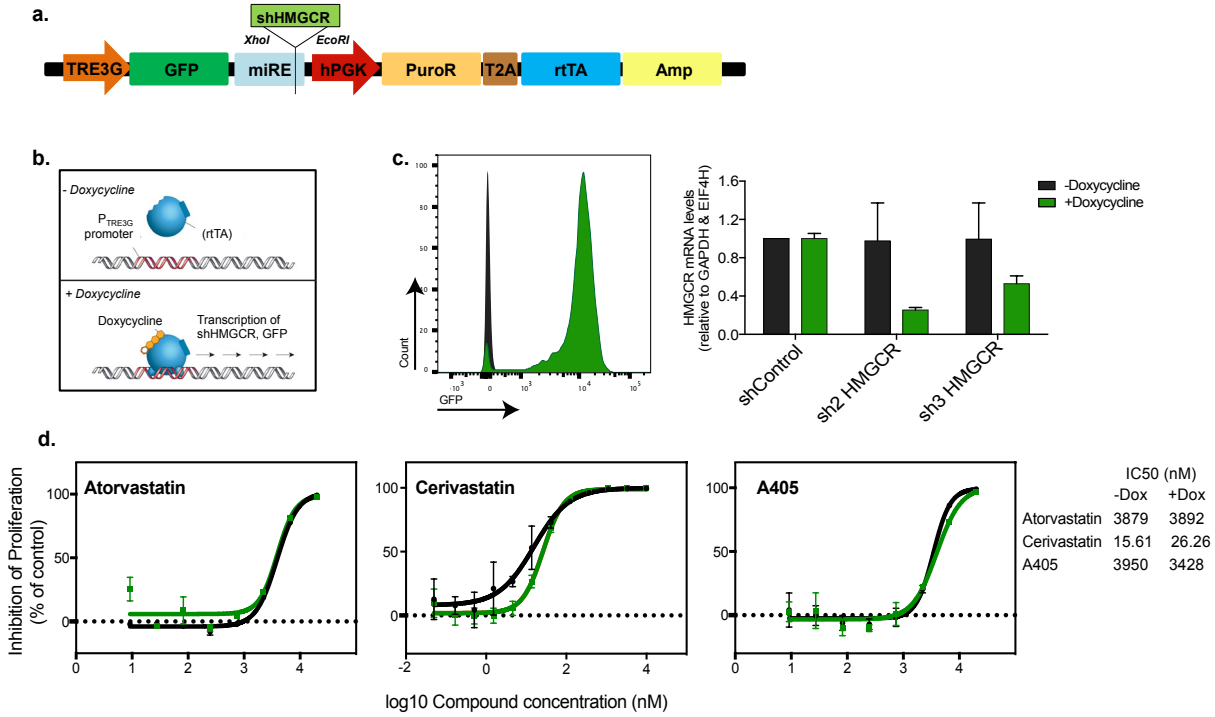


Figure 7. shRNA targeting HMGCR in a doxycycline-induced system. (a) Schematic representation of lentiviral plasmid used to clone shRNA targeting the HMGCR gene. (b) Expression of genes encoding both shRNA and GFP are under the control of doxycycline (adapted from adgene). (c) Representative induction of GFP+ population 24 hours post-doxycycline treatment (left) and assessment of HMGCR knockdown efficiency by qRT-PCR, (Representative of n=2 independent experiments) (right). (d) Assessment of the effect of doxycycline on dose response curves of atorvastatin (left), cerivastatin (middle) and A405 (right) and their respective IC50 values (Done in quadreplicates; Representative image of n=2 independent experiments).

ducible system in order to avoid any lethal effects caused by HMGCR loss. The expression of shRNA and GFP marker was controlled by T3G promoter and presence of doxycycline allowed the binding of the rtTA to T3G promoter and expressed the shRNA and GFP (Figure 7, a and b). The shRNAs were stably integrated via lentiviral transduction. Although the Kasumi-1 cell line was a good genetic model to recapitulate the CBF-AML patient specimens, we observed very poor transduction efficiency. The use of other transduction methods such as spinoculation and retronectin bound virus-mediated infection also failed to achieve good infection efficiency and good viability. We therefore chose to use the other statin-sensitive model OCI-AML-5 cell line which was the most statin sensitive cell line among the ones tested.

We were able to successfully transduce shRNAs expressing viruses into the OCI-AML-5 cell line. We observed an efficient induction of GFP expression within 24 hours post doxycycline treatment, with >90% cell population turning GFP+ upon dox-induction (Figure 7c, left panel). As a prerequisite, we tested whether the presence of doxycycline had an effect on anti-AML activity of both statins and A405 and found no change in sensitivity to the drugs (Figure 7d). Thus, doxycycline did not affect the sensitivity of statins or A405 in OCI-AML-5 cells. Finally, in order to validate our knockdown efficiency, we used quantitative real-time PCR (qRT-PCR) to measure HMGCR knockdown at the mRNA level. We observed up to 80% knockdown of HMGCR mRNA levels compared to the non-targeting control (Figure 7c, right panel).

2.2.2 Reducing HMGCR levels sensitizes OCI-AML-5 cells to statins

In order to validate if HMGCR is the target of statin-mediated cytotoxic activity, we investigated the impact of HMGCR knockdown on statin sensitivity. We induced the shRNA targeting either HMGCR or Renilla luciferase (non-targeting control, NT) and tested the response to statins by monitoring shifts in the IC_{50} values (Figure 8a). This was determined by cell viability assays after four days of drug treatment. We observed that HMGCR silencing rendered the OCI-AML-5 cells more sensitive towards both atorvastatin and cerivastatin and changes in sensitivity corresponded to the level of knockdown; with cells with the most efficient knockdown being the most sensitive (~10 fold increase in sensitivity) (Figure 8b, top and middle panel). To determine if increased sensitivity following HMGCR knockdown was specific to statins, we

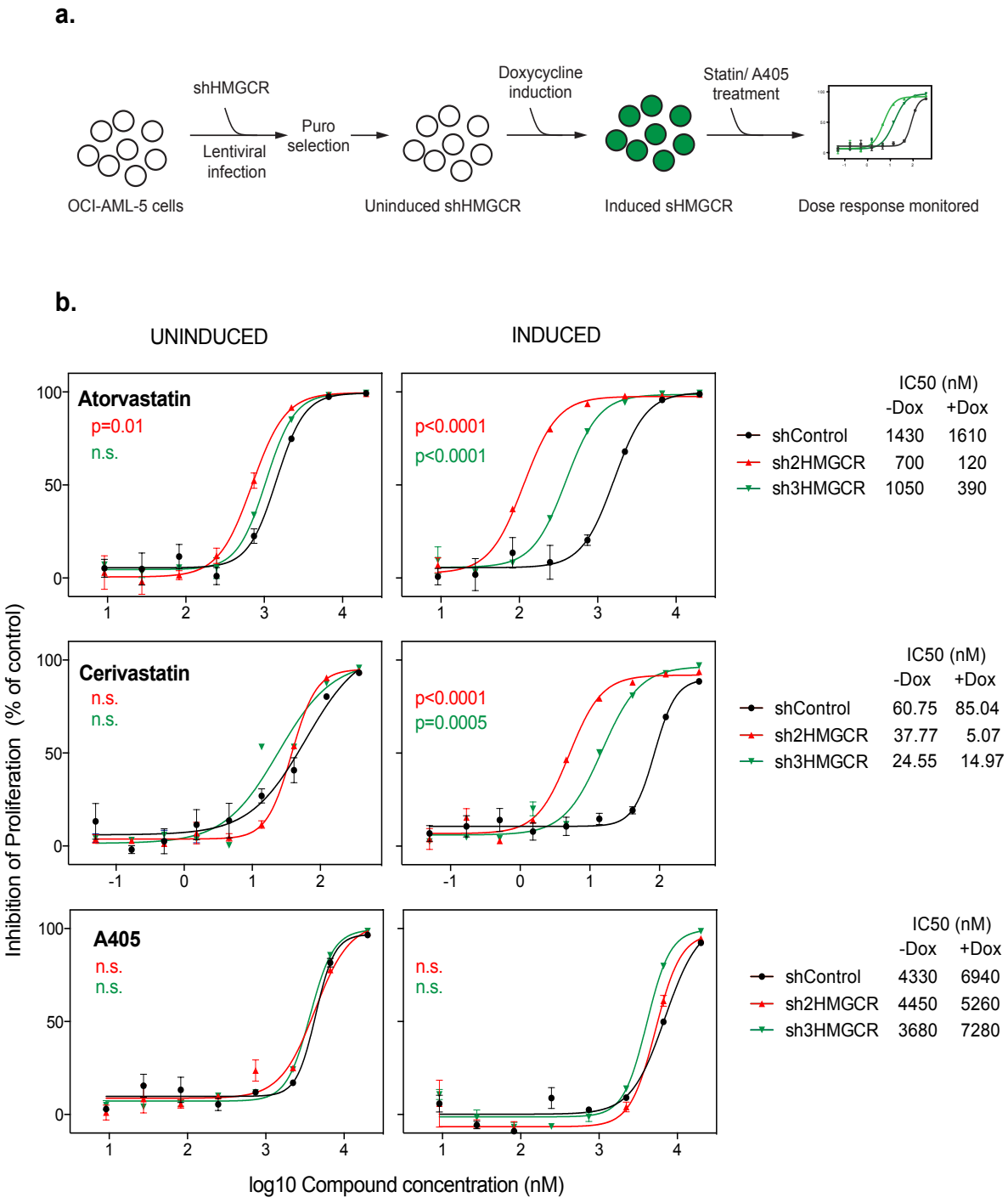


Figure 8. Reducing HMGCR levels sensitizes OCI-AML-5 cells to statins, but not A405 (a) Schema depicting work flow of the experiment. (b) Dose response curves and associated IC50 values of atorvastatin (top), cerivastatin (middle) and negative control-A405 (bottom) with OCI-AML-5 cells expressing shRNA targeting HMGCR or non-targeting control (Luciferase renilla). Two tailed unpaired t-test, n.s. non-significant ($p < 0.05$) (Done in quadreplicates; Representative image of n=3 independent experiments)

tested the response of OCI-AML-5 cells with silenced HMGCR to A405 analog. We used the A405 analog as a negative control as its anti-AML effect is independent of HMGCR inhibition and thus, HMGCR knockdown should not affect sensitivity. As expected, we observed no significant change in sensitivity with A405 irrespective of the knockdown levels (Figure 8b, lower panel). Thus, these results indicated that HMGCR knockdown sensitizes OCI-AML-5 cells to statins, but not to A405.

2.2.3 Mevalonate completely rescues the effect of statins

An intriguing, yet interesting observation made at this point of our study was that during the four days of the assay, we did not observe any decrease in viable cell counts in OCI-AML-5 cells with HMGCR silencing (Figure 9). These preliminary results suggested that HMGCR silencing alone was not sufficient to genetically phenocopy the effect of statins in OCI-AML-5 cells. We reasoned that this observation could be either explained by the possibility of a potential second target of statins being activated in these cells or that remnant HMGCR levels were sufficient to sustain cell survival. Detection of total HMGCR protein levels alone with a western blot would not be sufficient to explain our intriguing observations, but rather total activity levels of HMGCR in the engineered cells would need to be analysed by measuring the conversion of HMG-CoA into mevalonate by liquid chromatography and mass spectrometry [88]. Instead, as an alternative approach, in order to rule out the possibility of the 'additional target', we chose to ask if the downstream product of HMGCR, mevalonate could rescue the cytotoxic effect of the statin. To this end, we treated OCI-AML-5 cells with cerivastatin in a dose dependent manner in the presence or absence of 5mM mevalonic acid for four days and cell viability was determined. We observed that mevalonate was sufficient to completely rescue the effect of the statin (Figure 10).

2.2.4 Complete loss of HMGCR is lethal in OCI-AML-5 cells

We further investigated if complete loss of HMGCR in OCI-AML-5 cells would phenocopy the effect of the statins. To this end, we used CRISPR/Cas9 technology to genetically knock out HMGCR in OCI-AML-5 cells. We designed sgRNA targeting the HMGCR gene and

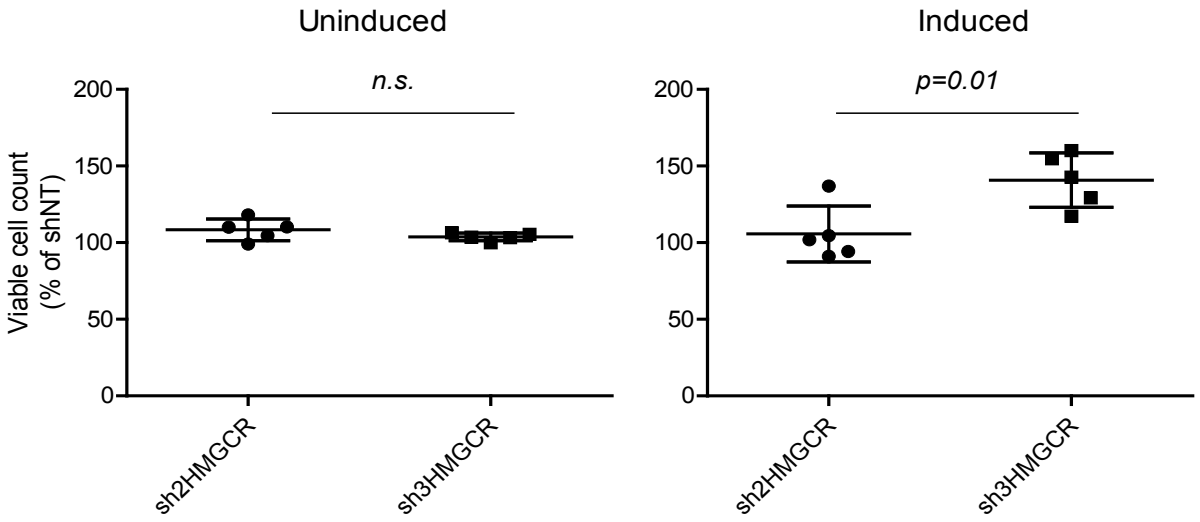


Figure 9. Knocking down HMGCR does not affect viability of OCI-AML-5 cells. Assessment of changes in viability after depletion of HMGCR in doxycycline inducted (right) vs uninduced (left) conditions during assay conditions (four days). Viable cell counts are represented as a percentage of the control cells containing shRNA targeting Luciferase renilla. Two tailed unpaired t-test, n.s. non-significant ($p < 0.05$) (n=5 independent experiments)

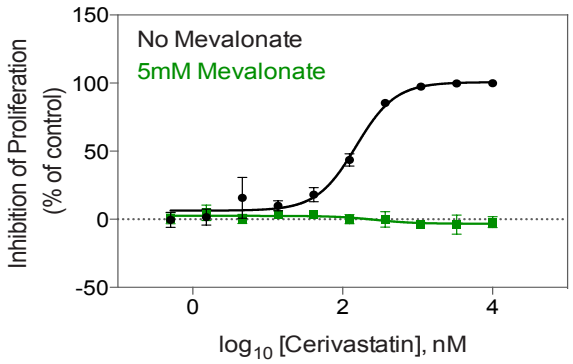


Figure 10. Mevalonate completely rescues the effect of statins. Dose response assays of OCI-AML-5 cells with cerivastatin with and without the supplementation of 5mM mevalonate (curves in green and black respectively). Pilot experiments showed that 5mM was found to be optimal for assay. Inhibition of proliferation, measured by calculating viable cell counts as a percentage of the DMSO or DMSO with mevalonate-treated (control) conditions. (Done in duplicates; Representative of n=3 independent experiments)

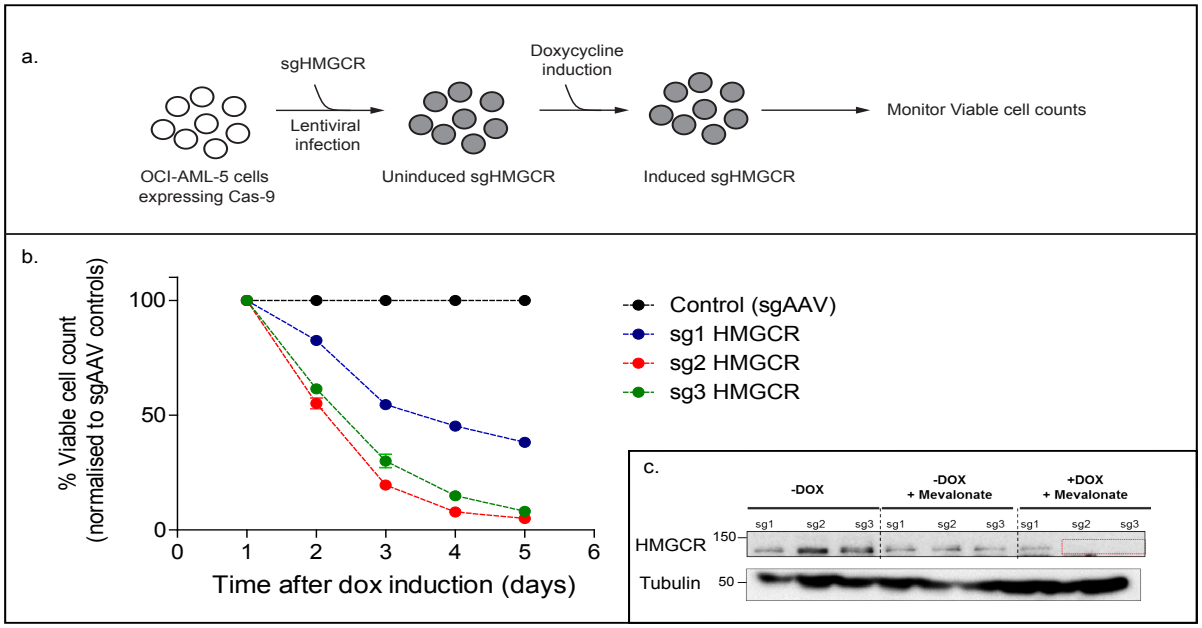


Figure 11. Complete loss of HMGCR is lethal in OCI-AML-5 cells. (a) Schema depicting work flow of the experiment. (b) Assessment of proliferation in OCI-AML-5 cells upon the CRISPR-Cas9 mediated knock-out of HMGCR. Viable cell counts are represented as a percentage of the control cells containing sgRNA targeting AAV. (c) Assessment of the efficiency of HMGCR knockout by western blot analysis. Conditions include doxycycline treated and untreated OCI-AML-5 cells with or without mevalonate. Mevalonate was added to HMGCR knockout condition (+DOX +Mevalonate) for maintaining the viability of the cells and can be compared with HMGCR WT control conditions (-DOX and –DOX +Mevalonate). (Done in duplicates; Representative of n=3 independent experiments)

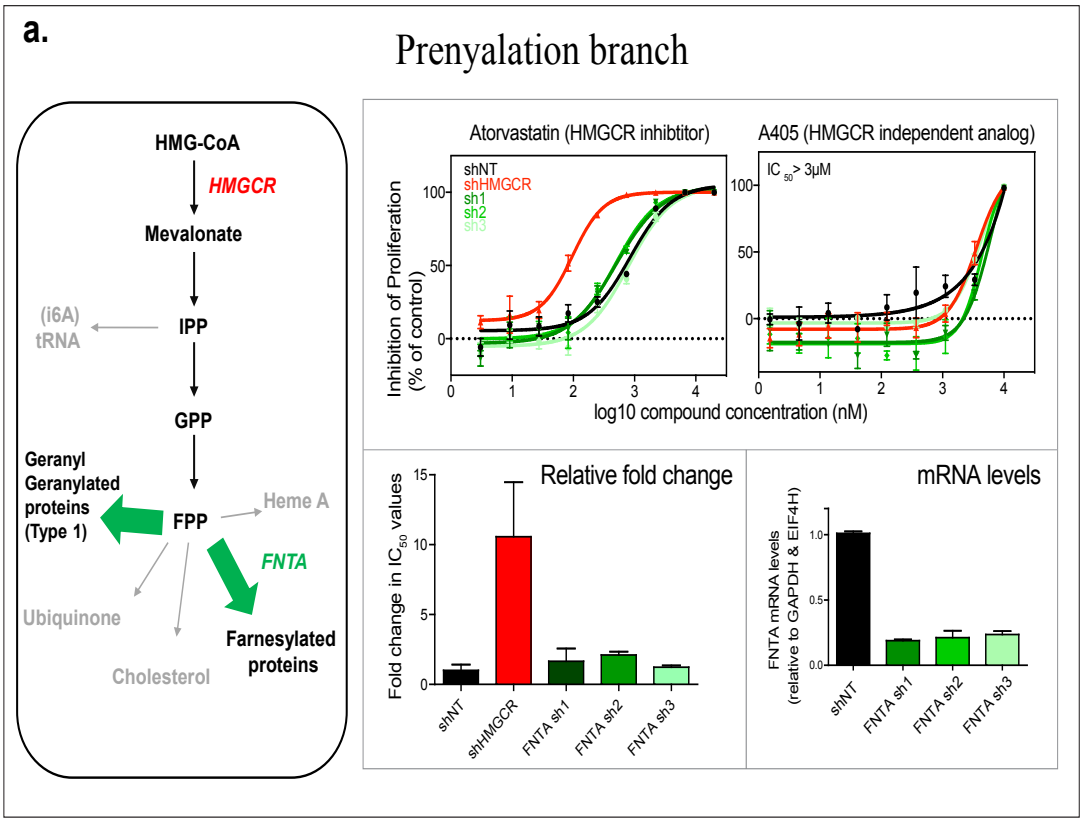
using lentiviruses, infected these into clonal OCI-AML-5 cells expressing an inducible Cas-9 cassette and investigated the effect of loss of HMGCR in OCI-AML-5 cells (Figure 11a). Western blot analysis in OCI-AML-5 cells expressing Cas9 revealed that HMGCR levels were completely depleted by sg2 HMGCR and sg3 HMGCR (Figure 11c), which resulted in rapid cell death (Figure 11b). Thus, complete loss of HMGCR was lethal in OCI-AML-5 cells and knocking out HMGCR genetically phenocopied the effect of statins.

Thus, with both genetic and chemical approaches, we validated that HMGCR is indeed the target of statin-mediated cytotoxic activity in OCI-AML-5 cells and thus, this model system could be exploited to dissect the mevalonate pathway.

2.3 Identification of sub-branch(es) downstream of HMGCR contributing to the anti-AML effect of statins

With our model system validated, we sought out to investigate which of the downstream branch(es) were essential for mediating statin's cytotoxic activity. A majority of the genes coding for enzymes in the mevalonate pathway are essential. Thus, we chose to use inducible RNAi technology to knockdown the enzymes of the different branches of the mevalonate pathway and observe changes in statin sensitivity. OCI-AML-5 cells were transduced with 2-3 shRNAs targeting the key enzymes of the mevalonate pathway. Similar to the previous experiments, A405 was used as a negative control. Knockdown efficiencies were validated at the mRNA level by q-RT-PCR. Changes in sensitivity to statins and A405 upon knockdown of the branches was compared to OCI-AML-5 cells with sh2 HMGCR as a positive control (as we observe a significant shift in IC_{50} values) and the non-targeting shRNA control as a negative control (representative from the screen in Figure 12a).

Enzymes tested in the screen included FDFT (squalene synthase; cholesterol sub-branch) FNTA (common subunit of FT and GGT-I), FNTB (catalytic subunit of FT), PGGT1B (catalytic subunit of GGT-I), RABGGTB (catalytic subunit of GGT-II), ICMT (essential for final methylation post-translation modification for both farnesylated and geranygeranylated proteins), PDSS1 (ubiquinone sub-branch), GGPS1 (common to both ubiquinone and geranylgeranylated


a.
Prenyalation branch
HMG-CoA
HMGCR
Mevalonate
(i6A) tRNA
IPP
GPP
Geranyl Geranylated proteins (Type 1)
FPP
Heme A
FNTA
Ubiquinone
Farnesylated proteins
Cholesterol
Atorvastatin (HMGCR inhibtitor)
A405 (HMGCR independent analog)
shNT
shHMGCR
sh1
sh2
sh3
IC 50 > 3μM
Inhibition of Proliferation (% of control)
log10 compound concentration (nM)
Relative fold change
Fold change in IC50 values
mRNA levels
FNTA mRNA levels (relative to GAPDH & EIF4H)
shNT
shHMGCR
FNTA sh1
FNTA sh2
FNTA sh3

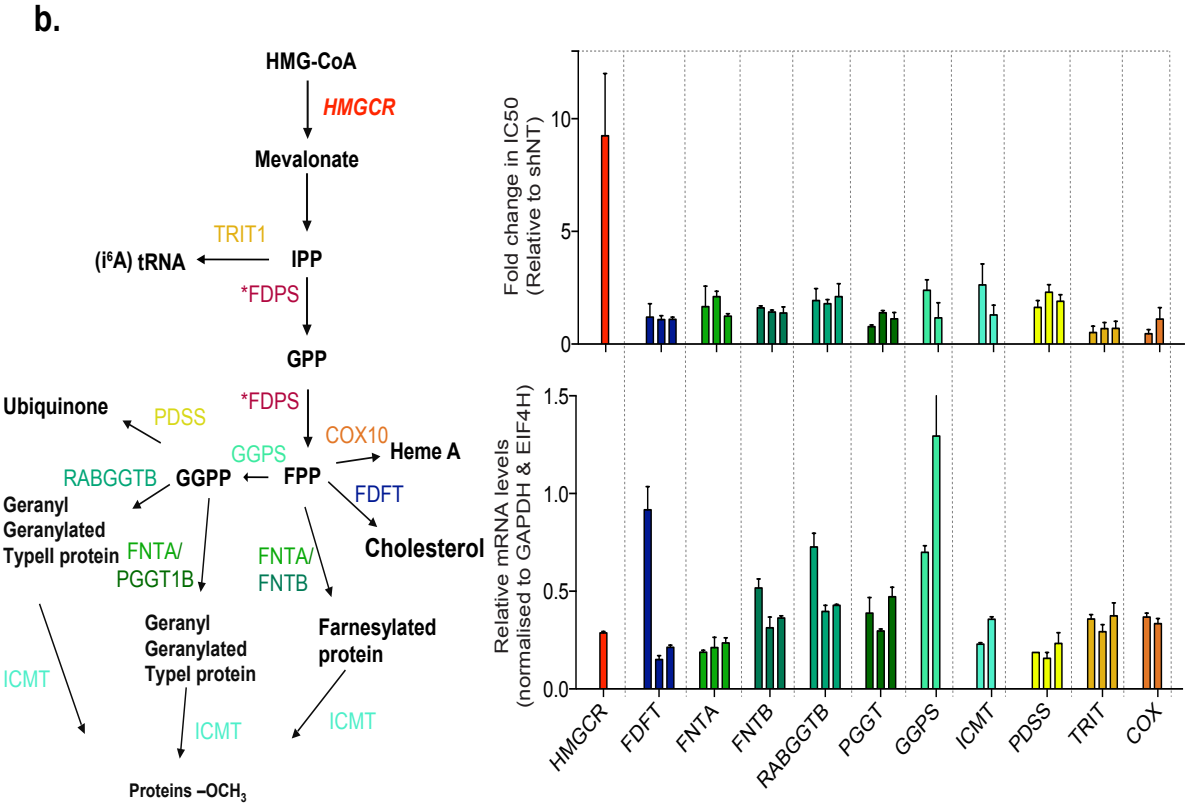

b.
HMG-CoA
HMGCR
Mevalonate
TRIT1
(i6A) tRNA
IPP
*FDPS
GPP
Ubiquinone
PDSS
*FDPS
COX10
Heme A
GGPS
RABGGTB
GGPP
FPP
FDFT
Geranyl Geranylated Typell protein
FNTA/ PGGT1B
FNTA/ FNTB
Cholesterol
Geranyl Geranylated Typel protein
Farnesylated protein
ICMT
ICMT
ICMT
Proteins –OCH3
Fold change in IC50 (Relative to shNT)
Relative mRNA levels (normalised to GAPDH & EIF4H)
HMGCR
FDFT
FNTA
FNTB
RABGGTB
PGGT
GGPS
ICMT
PDSS
TRIT
COX

Figure 12. RNAi screen on the mevalonate pathway. Enzymes in the mevalonate pathway were knocked down with RNAi technology and dose response assays were performed. Representative result from the screen depicted in (a). Dose response curves of OCI-AML-5 cells upon knocking down HMGCR or FNTA after 4 days of treatment with atorvastatin or A405 (top panel). Assessment of FNTA knockdown efficiency by qRT-PCR (bottom right) and fold change in IC50 values of respective dose response curves compared to control cells containing shRNA targeting Luciferase renilla (bottom left). Fold changes calculated by (IC50 value of shRenilla)/(IC50 value of shFNTA) (b) Schematic representation of mevalonate enzymes targeted in the RNAi screen (left panel). Summary of RNAi screen (right panel) describing fold changes in IC50 values of dose response curves compared to control cells containing shRNA targeting Luciferase renilla (top panel) and knock-down efficiencies of shRNA assessed by qRT-PCR (bottom panel). shRNA targeting HMGCR used as a positive control and shows the largest increase in sensitivity and thus, largest fold change in IC50 values. (Done in quadreplicates, n=1 independent experiment). *Results with shRNA targeting FDPS described in Figure 13.

proteins), COX10 (Heme sub-branch), TRIT1 (i^6A t-RNA sub-branch), FDPS (branch-point enzyme) (Figure 12b, left panel). We observed no significant shifts in IC_{50} values by knocking down single branches of the mevalonate pathway upon statin treatment (Figure 12b, and Appendix I). Most of the branches tested had good knockdown levels for targeted enzymes, except for GGPS1 enzyme, which will need to be repeated with better a knockdown. Finally, all the recombinant cells lines tested had IC_{50} values > 3 μM for A405 and we did not observe any changes in sensitivity upon knockdown of the targeted enzymes as expected.

Interestingly, in contrast to HMGCR, knocking down the branch-point enzyme FDPS alone (Figure 13a) led to drastic decrease in the total viable cell counts after 4-days of dox-induction (Figure 13b, left panel). This occurred with concurrent increase in cell death by 50%-60% for all three shRNAs tested (Figure 13b, right panel). Thus, knockdown of the FDPS enzyme was lethal to OCI-AML-5 cells. When the response of OCI-AML-5 cells to statins upon knockdown of FDPS was tested, we observed a biphasic dose response (Figure 13c). At high concentrations of atorvastatin, we observed a lethal effect in cells. However, at a certain window of atorvastatin concentration (1μM – 0.1 μM), we were able to rescue the lethality caused by the FDPS depletion (Figure 13, c and d). At very low atorvastatin concentrations, we lost the 'rescue' effect of statins. This effect was specific to the statin as it was not observed with A405 treatment (Figure 13c).

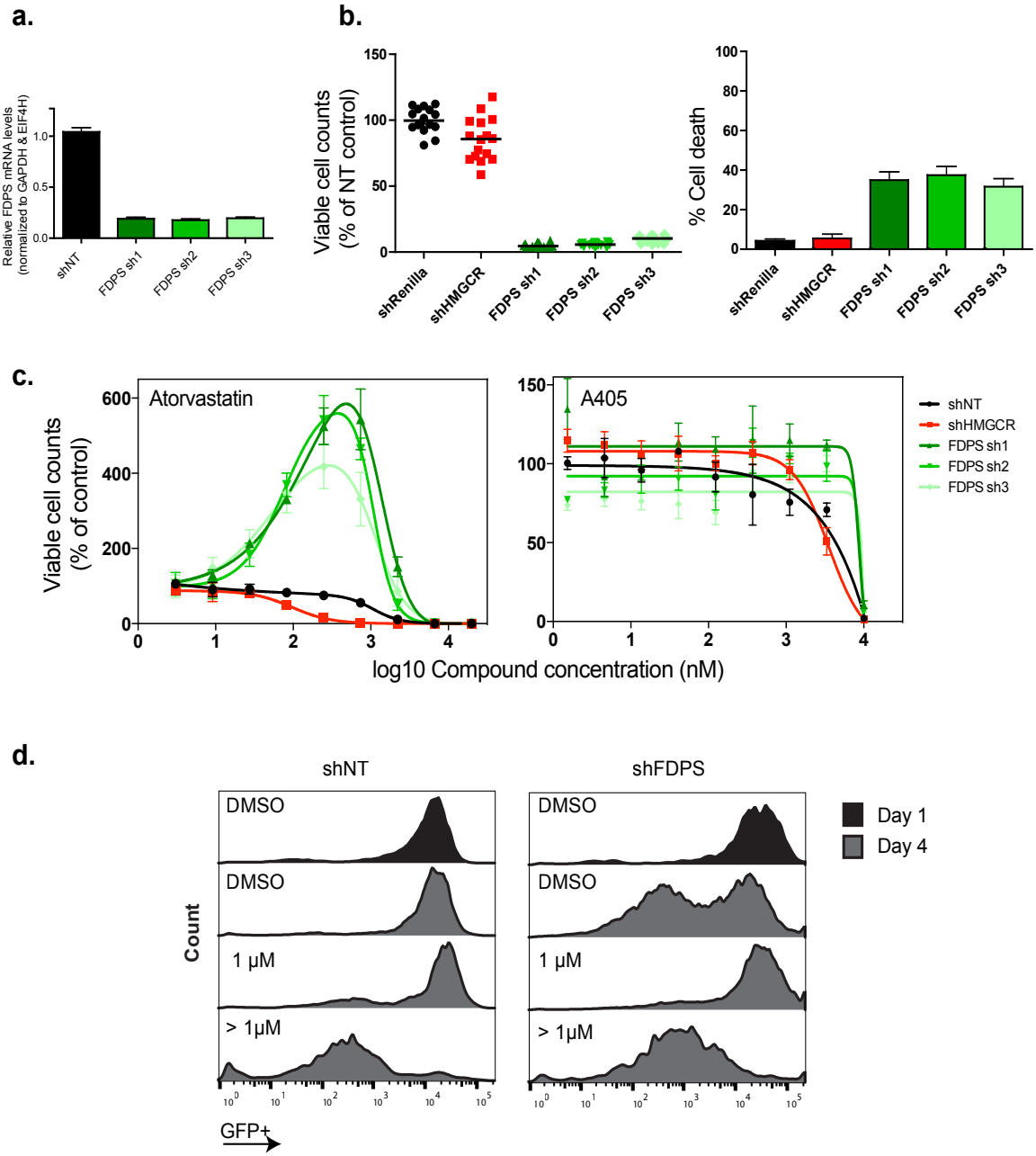


Figure 13. FDPS knockdown lethal in OCI-AML-5 cells, rescuable by statins. (a) Assessment of FDPS knockdown efficiency by qRT-PCR. (b) Assessment of viable cell counts (left) and cell death (right) upon four days of HMGCR or FDPS knockdown in OCI-AML-5 cells. Viable cell counts are represented as a percentage of the control cells containing shRNA targeting Luciferase renilla. (c) Dose response curves of OCI-AML-5 cells upon knocking down HMGCR or FDPS after 4 days of treatment with atorvastatin (left) or A405 (right). Horizontal axis represents viable cell counts as a percentage of cells in control (DMSO treated) conditions. (d) GFP expression measured by flow cytometry in DMSO treated or statin treated conditions at day 1 or day 4 of doxycycline treatment. (Done in quadreplicates, Representative of n=2 independent experiments)

Chapter 3- Methods

3.1 AML cell lines

AML cell lines Kasumi-1 and ME-1 were cultured in RPMI 1640 (Gibco) with 20% heat inactivated serum (Gibco). SKNO-1 and OCI-AML-5 cells were cultured in RPMI 1640 and DMEM (Gibco) respectively with 10% heat inactivated serum and 10 ng/ml GM-CSF.

3.2 Assessment of statin-mediated anti-AML effect

AML cell lines were washed with IMDM media to deplete the presence of serum and transferred in serum-free media which included IMDM (Gibco), 15% BSA-Insulin-transferrin (BIT 9500, Stem cell technologies), and 0.1M β-Mercaptoethanol. In case of OCI-AML-5 cells, 10 ng/ml GM-CSF was supplemented to the media. Cells were treated with Atorvastatin, Cerivastatin or UM0135405 in a dose dependent manner or solvent control (0.1% DMSO) in 384 well plates (Corning 3701). Four days post drug treatment, viable cell counts were determined with a viability dye, 7-AAD or PI and a fixed volume of beads (CountBright Absolute Counting Beads). Experiments were performed with iQue High Throughput Screener (Intellicyt) and subsequent analyses were performed with the iQue software. Dose response analysis and IC_{50} values were determined using Graphpad prism (v6.0).

3.3 RNAi and CRISPR-Cas9

shRNA targeting candidate genes were designed using prediction algorithms described in [89] and cloned into doxycycline inducible vector T3G-GFP-miRE-Pgk-Puro-rtTA. Control vector (shNT) contained shRNA targeting Renilla luciferase. The sgRNAs targeting HMGCR were designed from Sabatani & Lander group [90] and was cloned in plko/tRFP657 lentivirus according to the protocol “Genome-scale CRISPR Knock-Out (GeCKO)” from the Zhang lab (http://genome-engineering.org/gecko/wp-content/uploads/2013/12/lentiCRISPRv2-and-lentiGuide-oligo-cloning-protocol.pdf”). Viruses were transduced in a tetracycline inducible OCI-AML-5 Cas9 cell line.

3.4 Lentiviral production and transduction

HEK293T cells were transfected with 3.33 µg PAX2 packaging plasmid and 1 µg VSV-G envelope plasmid along with 5 µg lentiviral plasmids using 20 µL jetPRIME reagent (Poly-Plus Transfection) according to manufacturer's instructions. Viral supernatant was harvested 48 hours post-transfection and 1 mL soup was added to 300,000 OCI-AML-5 cells in 6 well plates (Sarstedt) along with 3 µg/mL polybrene. Cells were spinoculated by centrifuging them at 32ºC at 1000g for 1.5 hours. Viral soup was then replaced with fresh media (DMEM + 10% Hi-FBS). For shRNA lentiviral infections, 48 hours post infection, cells were washed and media was replaced by 2 µg/mL puromycin containing media. After puromycin selection for 48 hours, cells were washed in order to remove the uninfected dead cells. Infection efficiency, determined by percentage of GFP+ cells, was assessed with flow cytometry using the BD FACS Canto II and always resulted in ≥85% GFP+ cells.

3.4 Real time PCR

Knockdown efficiency was assessed 48 hours post dox-induction. RNA was isolated from cells using TRIzol (ThermoFisher) reagent according to manufacturer`s instructions. cDNA was prepared from the isolated RNA with M-MLV reverse transcriptase (Invitrogen) and random primers (ThermoFisher). Quantitative real time PCR was performed for the candidate shRNA genes using validated Universal ProbeLibrary assays (Roche) on the Viia7 Real time PCR system (Applied Biosystems). Relative mRNA levels were normalized to GAPDH and EIF4H and knockdown efficiency was analysed using Expression Suite software v1.1 (ThermoFisher).

3.5 Western blot analyses

OCI-AML-5 Cas9 cells containing sgRNAs targeting HMGCR were treated with and without doxycycline in the presence or absence of mevalonate, and whole cell extracts were isolated in Laemmli buffer and loaded onto 7.5% SDS-PAGE gels. Proteins were then transferred to activated PVDF membranes, followed by blocking with 5% milk solution prepared in Tris-buffered saline containing 0.1% Tween-20 (TBST) buffer. Membranes were then in-

cubated with 1.3 µg/mL primary rabbit anti-HMGCR antibody (Abcam, ab214018) diluted in TBST containing 5% BSA overnight at 4°C. Membranes were washed thoroughly three times with TBST followed by incubation with secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) in TBST with 5% milk solution for 1h at room temperature. These were washed again three times with TBST and developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore). Images were captured and analysed using ImageQuant LAS4000 (GE Healthcare Life Sciences).

3.6 Screening of mevalonate pathway branches

AML cell lines stably transduced with the shRNAs were washed twice with IMDM media and cells were transferred to in serum-free media containing with 2µg/mL doxycycline for overnight serum starvation. Cells were treated with Atorvastatin or A405 in a dose dependent manner or solvent control (0.1% DMSO) in 384 well plates (Corning 3701) using Echo 555 (Labcyte) machine. Doxycycline was replenished every two days to maintain expression of shRNAs. Four days post drug treatment, viable GFP+ cell counts were determined with a viability dye, 7-AAD and a fixed volume of beads (CountBright Absolute Counting Beads). Experiments were performed with Yeti flow cytometer and subsequent analyses were performed with the Flow Jo v10 software. Dose response analysis and IC_{50} values were determined using Graphpad prism (v6.0).

Chapter 4- Discussion

Heterogeneity of statin responses in AML specimens

Our study adeptly exemplifies how chemo-genomic investigation of AML proved to be key in improvement of AML therapy. We used high-throughput screening approaches to investigate AML responses with the aim to identify small molecules that target specific AML sub-groups. One such chemical screen enabled us to identify the heterogeneity of statin responses in AML. We found this heterogeneity of AML specimens was associated with the clinical outcome of AML patients. Further investigation with larger cohorts of AML specimens enabled us to identify that specific sub-groups of AML, with genetic anomalies in their CBF transcription factor, such as inv(16) and t(8,21), were associated with increased statin sensitivity, while AML with mutations in IDH genes were associated with statin resistance. Thus, our study emphasised the importance of the mutational landscape in AML in predicting responses to AML therapy.

Heterogeneity in statin responses have previously been reported in multiple myeloma (MM) [91]. Clendening *et al.* observed that only a subset of MM cell lines as well as primary MM specimens were sensitive to statins. In this context, they identified that such differences in statin sensitivity were owing to the altered feedback regulation in MM cells; with sensitive MM cells being unable to turn on their feedback upon statin mediated HMGCR inhibition. In our case however, transcriptomic data of primary AML specimens showed that there were no such significant alterations of enzymes involved in HMGCR feedback regulation upon statin treatment among sensitive and resistant cells (Jana Krosl *et al*., unpublished data from our group). Further analyses of activity and protein levels of enzymes involved in feedback mechanisms in the AML specimens would be worth investigating.

The molecular mechanisms mediating differential statin response in cytogenetic AML sub-groups is yet to be elucidated. Several studies have demonstrated that statin impairs glucose uptake and inhibits the glycolysis pathway [92, 93]. Interestingly, tumor cells with mutated IDH activity are known to switch off the glycolytic pathway and are dependent on glutaminolysis to fuel their energy requirements [94]. It is tempting to speculate that in AML with IDH muta-

tions, statins further augment the tumor addiction to glutamine and this could possibly explain the statin resistance. Further, a study exploring glycolytic metabolism demonstrated that AML cells with high glycolytic metabolism were predictive of better overall survival [95]. It would be interesting to investigate the glycolytic metabolic flux in both good outcome AML (such as CBF AML) as well as IDH mutated AML and monitor the effect of statin on rewiring the metabolism in AML cells. Interestingly, there is evidence that IDH1 gene promoter contains SRE elements and that IDH1 gene expression is upregulated by SREBP upon lipid starvation [96]. This raises the possibility that statin-mediated cholesterol depletion in IDH mutated AML cells would increase the mutant IDH gene expression and this might explain the resistance observed with these specimens.

Chemo-genomic dissection of the mevalonate pathway in OCI-AML-5 cells

Preliminary experiments presented here pave the way to further dissection of the mevalonate pathway in AML. As loss of majority of the enzymes of the mevalonate pathway have been shown to be lethal [97], our choice of RNAi technology was advantageous over the CRISPR/Cas-9 system. RNAi system enabled us to leverage AML cells by reducing levels of the pathway enzymes, and observing changes in statin responses. We found that OCI-AML-5 was a good model cell line to investigate the contribution of the branches as they were highly statin sensitive, easily transducible and had a short doubling period. As a pre-requisite, we performed a proof-of-principle experiment in order to determine if OCI-AML-5 cells was indeed a good model in order to investigate the sub-branches of the mevalonate pathway. As expected, we observed that knocking down HMGCR, the target of statins, we were able to increase sensitivity to statins such as atorvastatin and cerivastatin in OCI-AML-5 cells and the degree of sensitivity corresponded to the level of knockdown. Thus, limiting cellular levels of HMGCR enzyme in cells increased sensitivity to statin-mediated inhibition. Further, we observed that this increased sensitivity upon HMGCR depletion was specific to statins and was not observed in the negative control, A405. Thus, with RNAi tools we were able to validate that HMGCR is the target of the statins in OCI-AML-5 cells. These results also suggested that sensitivity of AML cells to statins could be explained by endogenous levels of HMGCR in AML cells and this could possibly

explain the discriminatory effect we observed in AML samples. However, transcriptomic data of AML specimens did not show any significant changes in HMGCR mRNA levels between sensitive and resistant AML specimens (Jana Krosl *et al.*, unpublished data from our group). Assessment of both the activity and the protein levels of HMGCR in the sensitive and resistant AML would be interesting to investigate.

Interestingly, although we observed a considerable increase in statin sensitivity when HMGCR levels are knocked down, we were unable to genetically phenocopy the effect of the statins by knocking down HMGCR alone. Pioneering work of Brown and Goldstein have demonstrated the extent of the tight regulation of HMGCR and that active HMGCR levels as low as 20% are sufficient for mediating normal cellular functions [50]. We speculated that our observations could be explained by this threshold maintained in the cells engineered to express reduced level of HMGCR. We additionally questioned if our observations occurred as a result of other pathways, additional to the mevalonate pathway, promoting leukemic effect in OCI-AML-5 cells. However, upon further investigation, our results showed that exogenous supply of mevalonate could rescue the cytotoxic effects of the statins in OCI-AML-5 cells. Moreover, complete loss of HMGCR was lethal to these cells. Our data confirmed that mevalonate derived products downstream of HMGCR were sufficient to compensate the anti-leukemic effect of the statins and was essential for AML cells survival. Thus, both genetically and chemically we showed that HMGCR is the target of the statins in OCI-AML-5 cells and validated the OCI-AML-5 model system.

With our model system validated, we investigated if inhibition of any of the branches would act in concert with statin's cytotoxic effect. Our preliminary results demonstrated that knocking down enzymes of single sub-branches of the mevalonate pathway was not sufficient to increase sensitivity to statins. Further, simultaneous depletion of both farnesylation and geranylgeranylation (type I) of proteins by knocking down the common sub-unit, FNTA of these enzymes mediating prenylation did not increase statin-sensitivity of OCI-AML-5 cells. Further, simultaneous depletion of all three prenylated proteins by knocking down the common post-translational methylation enzyme, ICMT did not increase statin-sensitivity of OCI-AML-5

cells either. Several groups have demonstrated that statin's anti-tumor effects are mediated via the inhibition of protein prenylation, via farnesyl transferase or geranylgeranyl I transferase [63, 98]. Contrary to this, in our study, we did not observe any significant changes in statin sensitivity upon knocking down these branches. This discrepancy could be explained by the differences in culture conditions used to perform experiments; we used serum-free conditions and depleted cells of serum to prevent the compensation of statin's effect by cholesterol/lipid components present in the serum. Moreover, for all of the branches of the mevalonate pathway, except GGPS1 enzyme, we were successful in obtaining good knockdown levels (as assessed by qRT-PCR). Of note, it will be very important to investigate the protein levels of each enzymes tested as well as their activity to further validate the contribution of these branches in statin's effect, as it is known that this pathway is tightly regulated at both transcriptional and post-translational levels. This would be important to further confirm that single knockdown of branches is not sufficient to increase statin sensitivity.

Additionally, it would be interesting to investigate the impact of complete knockout of pathway enzymes on statin's effect in different cell contexts: sensitive versus resistant cell models. OCI-AML-3, AML-2, HL-60, U937, NB-4, ML-1 are a few AML cell lines that could be investigated for their statin sensitivity/resistance. Comparison of results obtained from both the shRNA and sgRNA screen would be interesting to investigate. Such approaches of performing parallel screens have been found to be more beneficial in identification of drug targets and identifying drug resistance mechanisms [99]. An important consideration to be made is that among the cell lines tested, OCI-AML-5 cells had the shortest doubling time and were the most statin sensitive. Thus, it is likely that these cells have higher cellular requirements for cholesterol or other mevalonate pathway derived metabolites to support their rapid growth. It would be important to investigate if there is a correlation between the growth rates of AML cells and their statin sensitivity and if differences in the growth rates could affect the discriminatory effect of statins. Finally, in order to assess the compensatory mechanisms of any of the sub-branches of the pathway over the other, it would be important to perform combinatorial knockdowns of two or more branches and investigate changes in statin sensitivity. We could additionally use chemical inhibitors to investigate the mevalonate pathway by using commercially available

compounds targeting these branches. These include farnesyl transferase inhibitors (Lonafarnib, FTI-176, FTI-277), geranylgeranyl transferase I inhibitor (GGTI- 2133, GGTI- 286, GGTI-298), cholesterol synthesis inhibitors (zaragozic acid, lapaquistat), and ubiquinone and cholesterol synthesis inhibitor (perillyl alcohol), among others.

Lethality of FDPS knockdown in OCI-AML-5 cells is rescued by low doses of statins

We investigated the role of branch point enzyme FDPS in OCI-AML-5 cells and found that depleting FDPS levels led to a dramatic decrease in viable cell counts accompanied with a substantial increase in cell death. This data suggested that FDPS plays a critical role in the proliferation and survival of leukemic cells. High protein and activity levels of FDPS have been previously associated with high-grade gliomas and FDPS was considered as a candidate oncometabolite as well [100]. Intriguingly, our data showed that lethality of FDPS knockdown could be rescued by treatment with statins. Furthermore, atorvastatin (but not A405) showed a biphasic response in cells engineered to express low level of FDPS. This was unexpected because, as FDPS is downstream to HMGCR, we predicted a similar 'increased statin sensitivity' phenotype as we observed by knocking down HMGCR. Indeed, while high levels of statin expectedly exacerbates the phenotype triggered by lowering FDPS levels in OCI-AML-5 cells, low dose of statin, on the contrary led to a near complete rescue of this phenotype. Importantly, this observation was specific to atorvastatin, as exposure to any doses of A405 analog was unable to rescue the lethal effects of the FDPS knockdown. This led us to speculate how partially inhibiting HMGCR with low concentrations of statin could rescue the cytotoxic effects of the FDPS knockdown.

One possible explanation for these findings lies in the regulatory mechanisms exerted on the feedback system of the pathway. HMGCR is the rate-limiting enzyme of the mevalonate pathway and thus, the mevalonate pathway is tightly controlled via regulation of HMGCR. This occurs at the transcriptional, translational and post-translational level. On the contrary, there are no evidences indicating that FDPS is as robustly regulated as HMGCR. This raises the possibility that regulation of the mevalonate pathway occurs more efficiently via the feedback mech-

anisms of HMGCR rather than FDPS. Moreover, early biochemical studies on the mevalonate pathway have demonstrated that statin inhibition on the HMGCR was used as a tool to amplify the regulatory mechanism on the mevalonate pathway [50]. Based on these findings, we could hypothesize that partial inhibition of HMGCR with low doses of statin leads to the activation of the feedback system and possibly stabilizes the FDPS enzyme (Figure 14a). This hypothesis could be tested by determining the stability of the FDPS using the classical cycloheximide pulse-chase assay with or without statin treatment. Moreover, activity of FDPS enzyme in AML cells could also be determined by measuring the rates of formation of FPP and GPP products from radioactively labelled IPP substrate.

The biphasic response curves of statin in FDPS low context are reminiscent of hormetic dose-response models, wherein a low dose of a compound (or condition) has a stimulatory or beneficial effect while high doses of the compound (or condition) have an inhibitory or toxic effect [101]. Cellular responses to reactive oxygen species (ROS) is often used to exemplify this phenomenon [102-104]. Interestingly, the literature suggests that IPP, the substrate of FDPS plays the role as a powerful antioxidant [105]. This led us to hypothesize that AML cells that have low basal levels of ROS are more susceptible to FDPS depletion. Thus, knocking down FDPS enzyme would increase levels of the upstream substrate, IPP and over accumulation of this antioxidant in the cell would lead to cell death (Figure 14b). On the other hand, partial inhibition of HMGCR with low concentration of statin would decrease the production of IPP, therefore avoiding FDPS-mediated cell death. In order to test this hypothesis, we would have to investigate the basal ROS levels in both statin sensitive and resistant AML cells and IPP: FPP ratio will have to be measured upon statin treatment. Moreover, performing a metabolomics analysis on statin-sensitive versus resistant AML cells would be key in identifying alterations in the flux of the mevalonate pathway.

To summarize, this project demonstrated that both the genetic and metabolic background of AML play key roles in driving statin sensitivity in AML. Moreover, this project highlighted the importance of elucidating the metabolic background in AML to prevent statin resistance in AML patients.

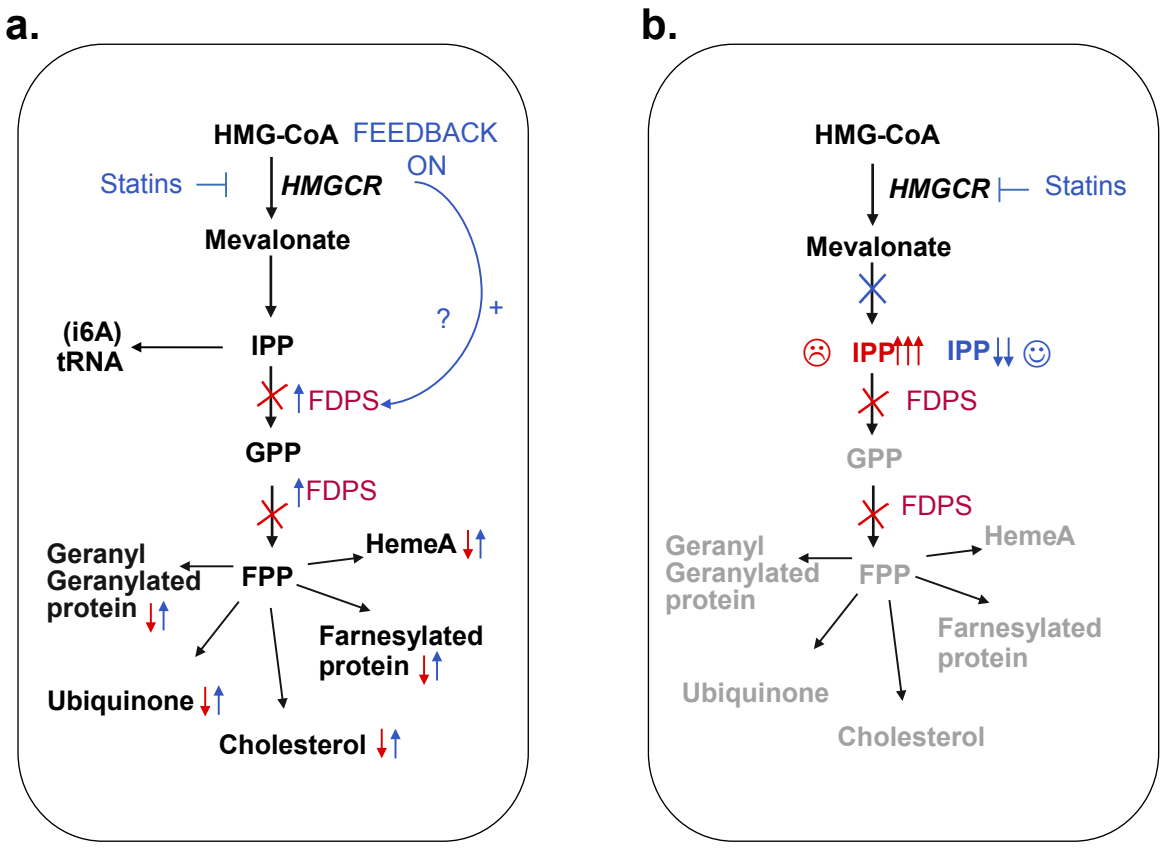


Figure 14. Proposed models explaining rescue mechanisms of cell lethality mediated by loss of FDPS. (a) Feedback mechanism model is based on the hypothesis that FDPS is not as well-regulated as HMGCR. Knocking down FDPS leads to decreased production of downstream metabolites of mevalonate pathway (red arrows) leading to cell lethality. Partial inhibition of HMGCR by low doses of statin (indicated in blue) leads to the activation of the feedback system, and possibly stabilizes the FDPS enzyme leading to formation of downstream metabolites of mevalonate pathway (blue arrows). (b) Model based on the hypothesis that Isopentenyl pyrophosphate (IPP), a potent antioxidant over-accumulates in cells upon FDPS depletion leading to cell lethality (red arrows). Partial inhibition of HMGCR leads to a decrease in the production of IPP (blue arrows), bringing levels of the anti-oxidant back to normal.

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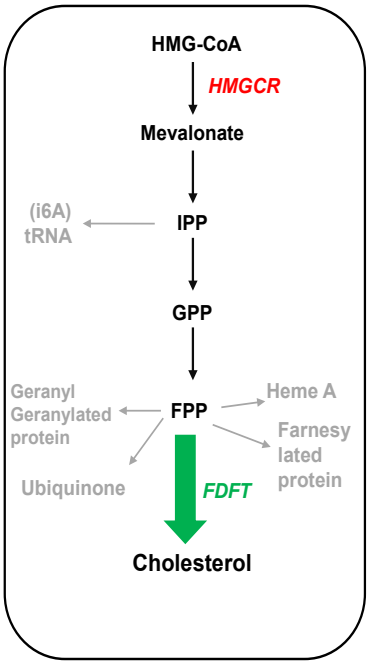
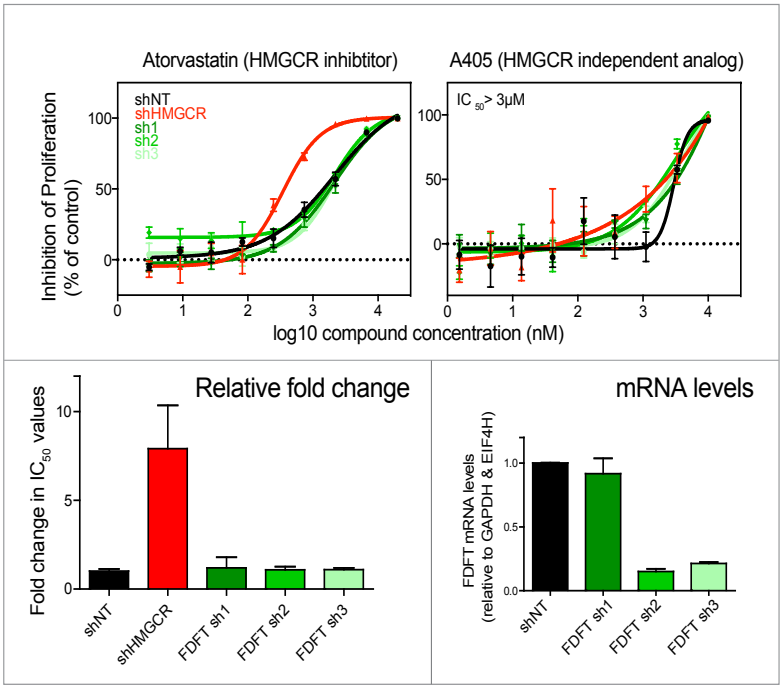
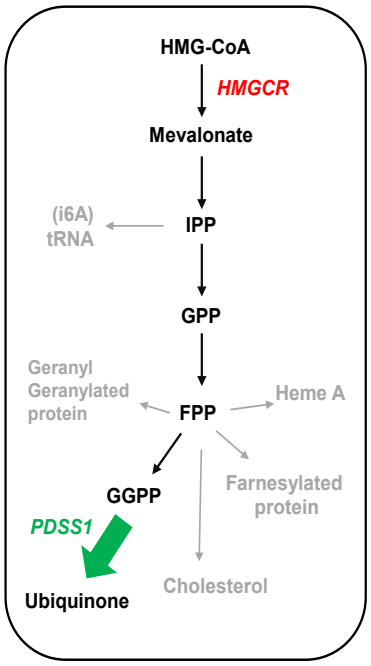
Appendix I- Supplementary figures

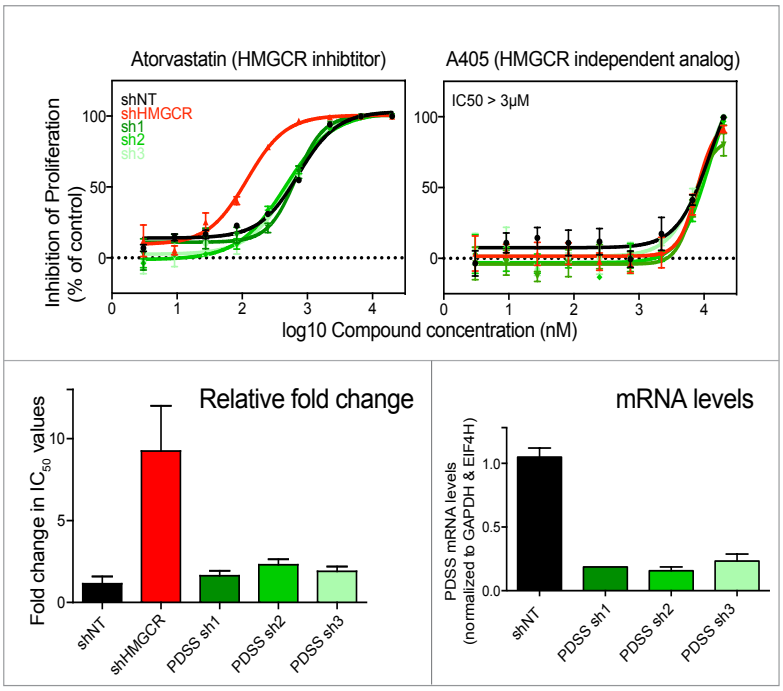
Cholesterol branch



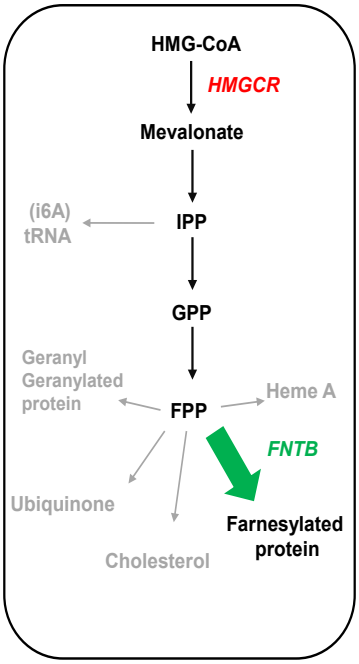


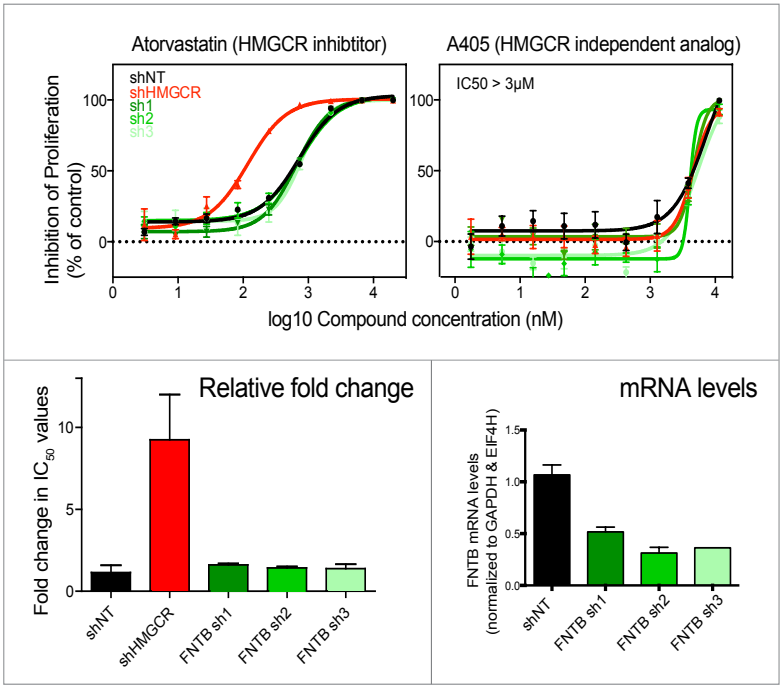
Ubiquinone branch



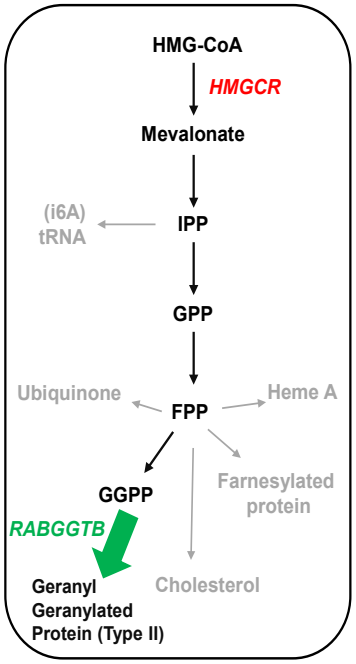


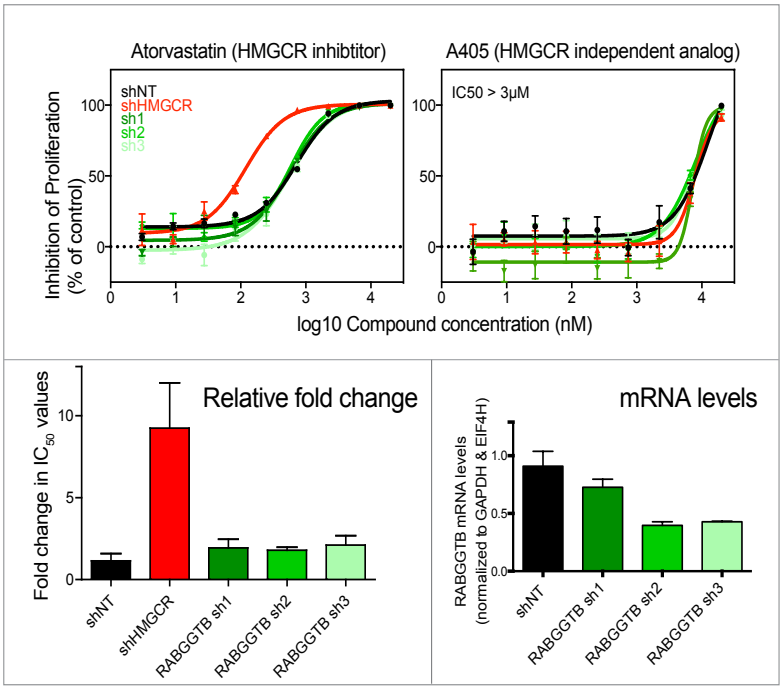
Farnesylation branch



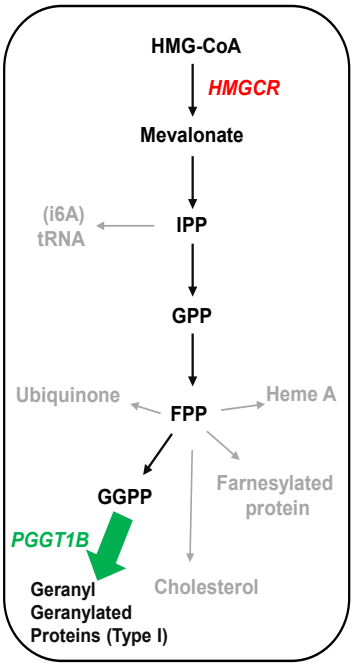


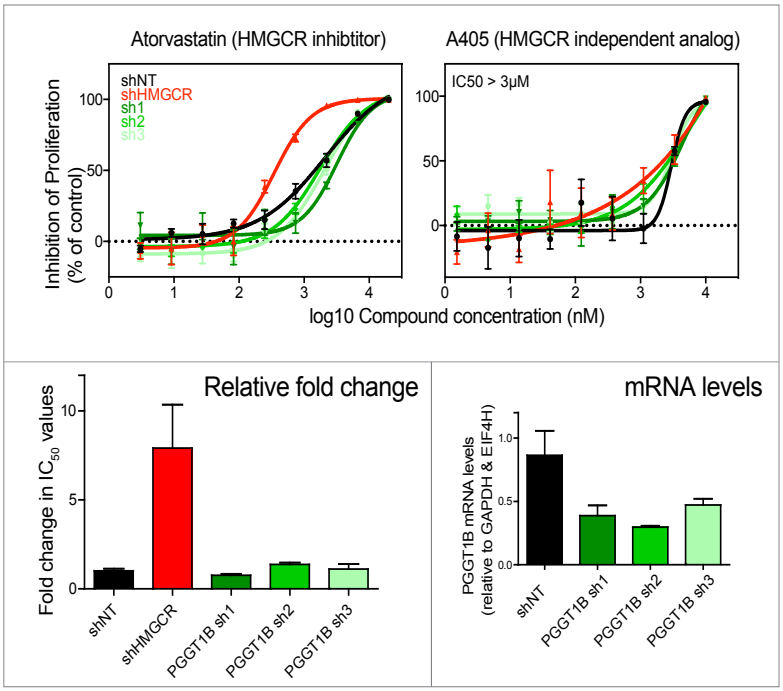
Geranylationgeranylation II branch



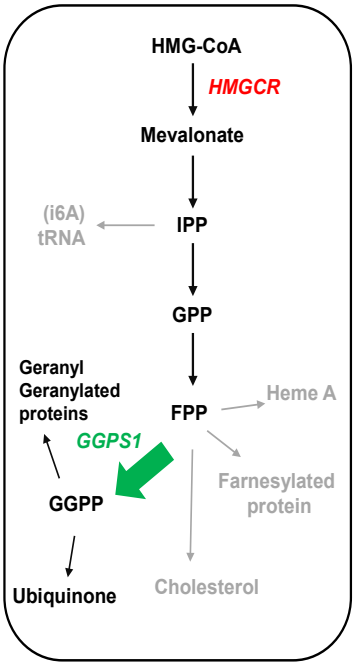


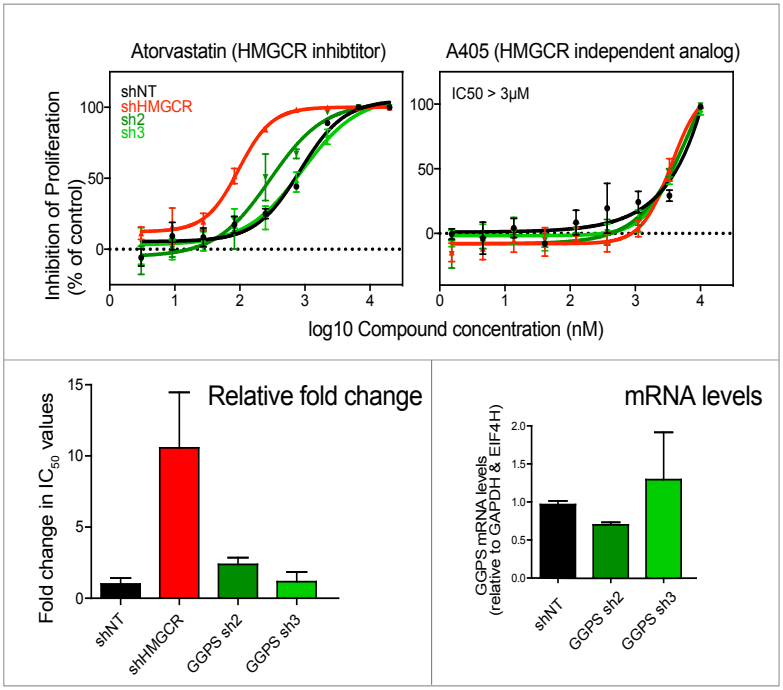
Geranylationgeranylation I branch



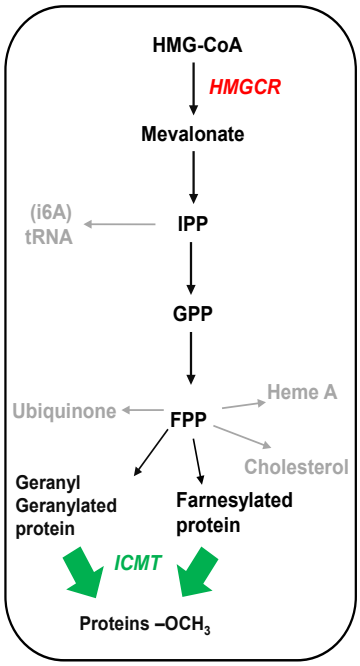


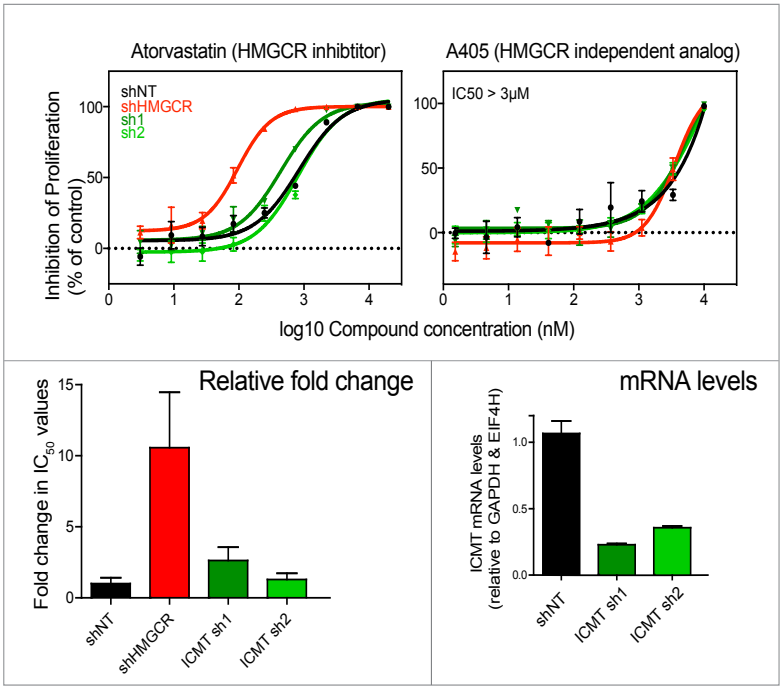
Geranylationgeranylation & Ubiquinone branch



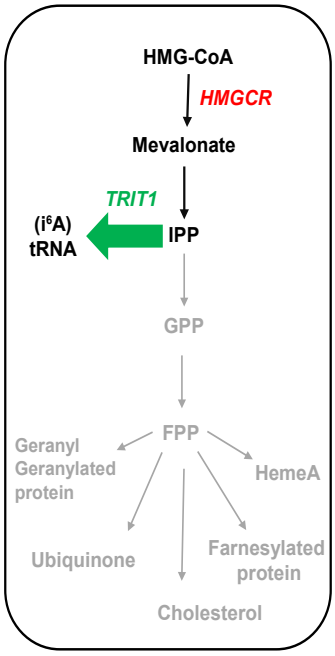


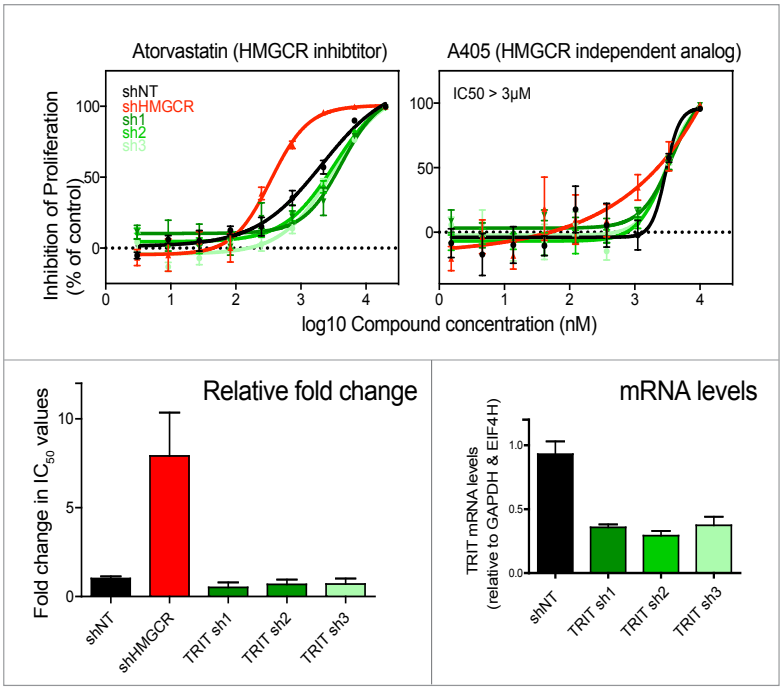
Prenylation branches

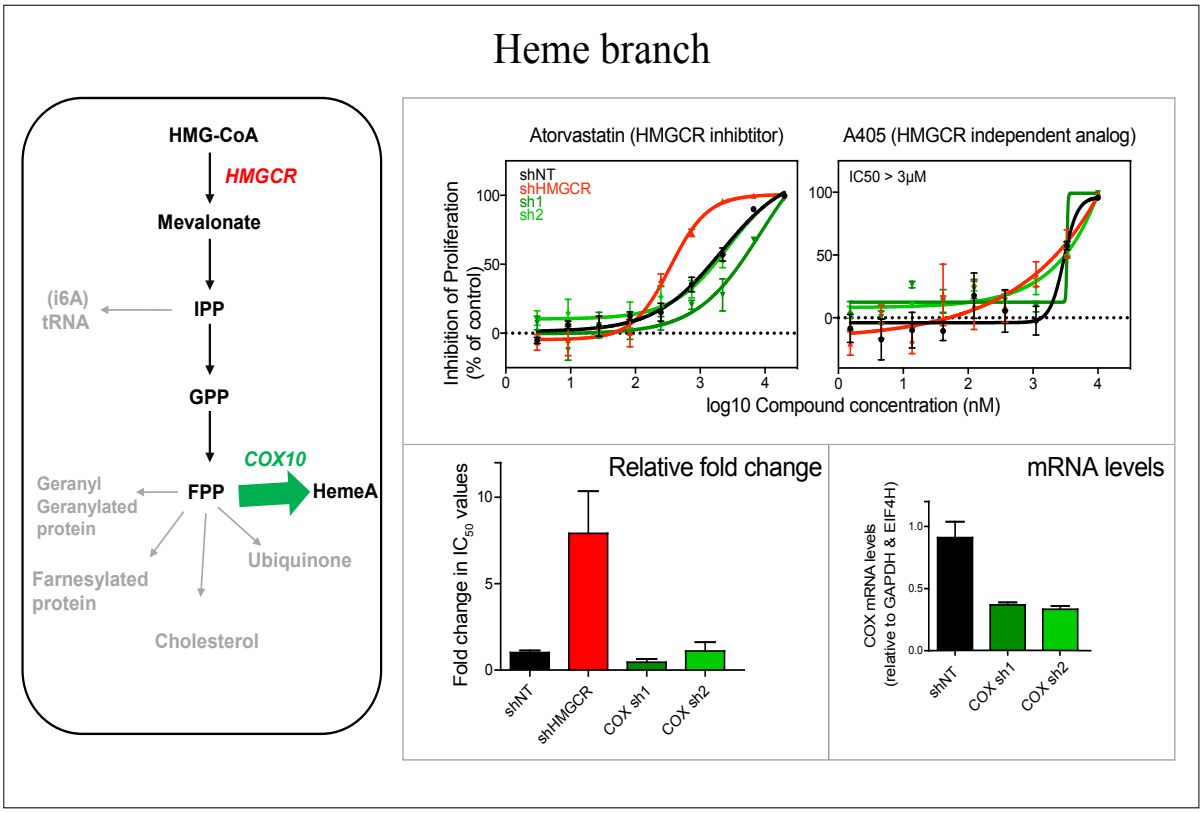




i^{6}A tRNA branch







Dose response curves of mevalonate pathway sub-branches from the RNAi screen. Schematic representation of the mevalonate pathway depicted in left panel. Large green arrow depicts branch targeted. Dose response curves of OCI-AML-5 cells upon knocking down HMGCR or genes indicated after 4 days of treatment with atorvastatin (top left) or A405 (top right). Horizontal axis represents inhibition of proliferation as a percentage of cells in control (DMSO treated) conditions. Assessment of knockdown efficiencies by qRT-PCR (bottom right) and fold change in IC50 values of dose response curves compared to control cells containing shRNA targeting Luciferase renilla (bottom left). Fold changes calculated by (IC50 value of shRenilla)/(IC50 value of shFNTA) (Done in quadreplicates, n=1 independent experiment)

Appendix II- RNAi and CRISPR sequences

shRNA

	97mer oligo
HMGCR sh2	TGCTGTTGACAGTGAGCGAAACAAGAATATTGTATGTTAATAGTGAAGCCACAGAT GTATTAACATACAATATTCTTGTTGTGCCTACTGCCTCGGA
HMGCR sh3	TGCTGTTGACAGTGAGCGCCAGCAGAGTTATTGAATCTTATAGTGAAGCCACAGATG TATAAGATTCAATAACTCTGCTGATGCCTACTGCCTCGGA
FNTA sh1	TGCTGTTGACAGTGAGCGCAAGGAAGACATTCTTAATAAATAGTGAAGCCACAGAT GTATTTATTAAGAATGTCTTCCTTATGCCTACTGCCTCGGA
FNTA sh2	TGCTGTTGACAGTGAGCGACAGCGTGATGAAAGAAGTGAATAGTGAAGCCACAGAT GTATTCACTTCTTTCATCACGCTGCTGCCTACTGCCTCGGA
FNTA sh3	TGCTGTTGACAGTGAGCGCAAGCATTAGAGTTATGTGAAATAGTGAAGCCACAGAT GTATTTCACATAACTCTAATGCTTTTGCCTACTGCCTCGGA
FNTB sh1	TGCTGTTGACAGTGAGCGATCCGAGTTCTTTCACCTACTATAGTGAAGCCACAGATG TATAGTAGGTGAAAGAACTCGGAGTGCCTACTGCCTCGGA
FNTB sh2	TGCTGTTGACAGTGAGCGAACCAAGATGAGTTCTCTGTAATAGTGAAGCCACAGAT GTATTACAGAGAACTCATCTTGGTGTGCCTACTGCCTCGGA
FNTB sh3	TGCTGTTGACAGTGAGCGCACAGGCAAAAGTAGAAGAAAATAGTGAAGCCACAGAT GTATTTTCTTCTACTTTTGCCTGTTTGCCTACTGCCTCGGA
PGGT1B sh1	TGCTGTTGACAGTGAGCGCAAGGAGATTGTTTGAAGACAATAGTGAAGCCACAGAT GTATTGTCTTCAAACAATCTCCTTTTGCCTACTGCCTCGGA
PGGT1B sh2	TGCTGTTGACAGTGAGCGCAAGGTAGAGTCTTACAATCAATAGTGAAGCCACAGAT GTATTGATTGTAAGACTCTACCTTTTGCCTACTGCCTCGGA
PGGT1B sh3	TGCTGTTGACAGTGAGCGCCAGAAGACAGATCAAATCTAATAGTGAAGCCACAGAT GTATTAGATTTGATCTGTCTTCTGTTGCCTACTGCCTCGGA
RABGGTB sh1	TGCTGTTGACAGTGAGCGAAAGGATGTTATTATCAAGTCATAGTGAAGCCACAGAT GTATGACTTGATAATAACATCCTTCTGCCTACTGCCTCGGA
RABGGTB sh2	TGCTGTTGACAGTGAGCGACCGGAGAAGTTACCAGATGTATAGTGAAGCCACAGAT GTATACATCTGGTAACTTCTCCGGCTGCCTACTGCCTCGGA
RABGGTB sh3	TGCTGTTGACAGTGAGCGCCAAGTCAGTTGCATCAAGTAATAGTGAAGCCACAGAT GTATTACTTGATGCAACTGACTTGTTGCCTACTGCCTCGGA

FDFT1 sh1	TGCTGTTGACAGTGAGCGCCAGTTAGATGTTTCCTAAGAATAGTGAAGCCACAGATG TATTCTTAGGAAACATCTAACTGTTGCCTACTGCCTCGGA
FDFT1 sh2	TGCTGTTGACAGTGAGCGACCGACTCAGACCCATCTTCTATAGTGAAGCCACAGATG TATAGAAGATGGGTCTGAGTCGGGTGCCTACTGCCTCGGA
FDFT1 sh3	TGCTGTTGACAGTGAGCGAAGCAGGTATGTTAAGAAGTTATAGTGAAGCCACAGAT GTATAACTTCTTAACATACCTGCTCTGCCTACTGCCTCGGA
COX10 sh1	TGCTGTTGACAGTGAGCGCCAGCTCAGTCAGTGAATACAATAGTGAAGCCACAGAT GTATTGTATTCACTGACTGAGCTGATGCCTACTGCCTCGGA
COX10 sh2	TGCTGTTGACAGTGAGCGCTAGAACAAGATTATAAACGAATAGTGAAGCCACAGAT GTATTCGTTTATAATCTTGTTCTAATGCCTACTGCCTCGGA
COX10 sh3	TGCTGTTGACAGTGAGCGCACCATAGTCCTTCTAACAATATAGTGAAGCCACAGATG TATATTGTTAGAAGGACTATGGTTTGCCTACTGCCTCGGA
PDSS1 sh1	TGCTGTTGACAGTGAGCGCCGGGTCAAAAGAAAATGAGAATAGTGAAGCCACAGAT GTATTCTCATTTTCTTTTGACCCGATGCCTACTGCCTCGGA
PDSS1 sh2	TGCTGTTGACAGTGAGCGCCACGATGACGTTATTGACGATTAGTGAAGCCACAGAT GTAATCGTCAATAACGTCATCGTGATGCCTACTGCCTCGGA
PDSS1 sh3	TGCTGTTGACAGTGAGCGCCCACCTGAATCTGTCATTCTATAGTGAAGCCACAGATG TATAGAATGACAGATTCAGGTGGTTGCCTACTGCCTCGGA
TRIT1 sh1	TGCTGTTGACAGTGAGCGCTCAGAAGAATGTTTCGGAAAATAGTGAAGCCACAGAT GTATTTTCCGAAACATTCTTCTGATTGCCTACTGCCTCGGA
TRIT1 sh2	TGCTGTTGACAGTGAGCGATCAGATGCTGTCAACACCATATAGTGAAGCCACAGAT GTATATGGTGTTGACAGCATCTGAGTGCCTACTGCCTCGGA
TRIT1 sh3	TGCTGTTGACAGTGAGCGATCCAATAAAGATGCCATACAATAGTGAAGCCACAGAT GTATTGTATGGCATCTTTATTGGAGTGCCTACTGCCTCGGA
FDPS sh1	TGCTGTTGACAGTGAGCGAAGCAGTGTTCTTGCAATATGATAGTGAAGCCACAGAT GTATCATATTGCAAGAACACTGCTGTGCCTACTGCCTCGGA
FDPS sh2	TGCTGTTGACAGTGAGCGCTCCTGCAGAGTTCCTATCAGATAGTGAAGCCACAGATG TATCTGATAGGAACTCTGCAGGAATGCCTACTGCCTCGGA
FDPS sh3	TGCTGTTGACAGTGAGCGCCCAGATCCTGAAGGAAAATTATAGTGAAGCCACAGAT GTATAATTTTCCTTCAGGATCTGGTTGCCTACTGCCTCGGA
GGPS1 sh1	TGCTGTTGACAGTGAGCGAACCGATTAGCTTTGAAGTTTATAGTGAAGCCACAGATG TATAAACTTCAAAGCTAATCGGTCTGCCTACTGCCTCGGA

GGPS1 sh2	TGCTGTTGACAGTGAGCGCAACACTGACTATAGAAACAAATAGTGAAGCCACAGAT GTATTTGTTTCTATAGTCAGTGTTATGCCTACTGCCTCGGA
ICMT sh1	TGCTGTTGACAGTGAGCGACAGAGTGTAAGTAAAGGATAATAGTGAAGCCACAGAT GTATTATCCTTTACTTACACTCTGGTGCCTACTGCCTCGGA
ICMT sh2	TGCTGTTGACAGTGAGCGCCAGCTCCTGTTGTCAGTGATATAGTGAAGCCACAGATG TATATCACTGACAACAGGAGCTGATGCCTACTGCCTCGGA

sgRNA

	Sense strand
HMGCR sg1	AGAGAGATAAAACTGCCAGA
HMGCR sg2	AGGGTTCGCAGTGATAAAGG
HMGCR sg3	GTCCAGGTCAGGGGATGCCA

Appendix III- qPCR primers and probes

mRNA target	Accession #	Probe ID	Left primer	Right Primer	Slopes derived from standard curves
3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR)	NM_000859.2, NM_001130996.1, NM_001364187.1	85	gacgcaacctttatatccgttt	ttgaaagtgctttctctgtaccc	3.16 (Obtained from the IRIC genomic platform)
Homo sapiens tRNA isopentenyltransferase 1 (TRIT1)	NM_017646.5, NM_001312691.1, NM_001312692.1	46	acaaacgcctaagccaggt	ttcctgtttcttcaaaaacttgc	-3.138 ± 0.2158
Homo sapiens farnesyltransferase, CAAX box, alpha (FNTA)	NM_002027.2	68	acttcagaaggatctacatgagga a	tactcgcctatgatgccaaa	-3.356 ± 0.05896
Homo sapiens farnesyltransferase, CAAX box, beta (FNTB)	NM_002028.3	16	ggaggtgaggtggatgtgag	ggagtgatgatgttggtcagc	-3.378 ± 0.2198
Homo sapiens protein geranylgeranyltransferase type I subunit beta (PGGT1B)	NM_005023.3	24	aaaagccatcacctatattagaagga	aggcaatgccacaaaaagtt	-3.198 ± 0.04871
Homo sapiens Rab geranylgeranyltransferase beta subunit (RABGGTB)	NM_004582.3	67	aaaggtctacagaaagaagatggttc	ccgcacaaaaagagaatcttg	-3.631 ± 0.1062
Homo sapiens COX10 heme A:farnesyltransferase cytochrome c oxidase assembly factor (COX10)	NM_001303.3	72	ggaaagagatgaagctgcaag	tgcagtggtacttacaaccagag	-3.309 ± 0.08428

Homo sapiens decaprenyl diphosphate synthase subunit 1 (PDSS1)	NM_001321979.1, NM_001321978.1, NM_014317.4	80	gatcatgcgac ggttcagt	ggttgtttgttgca caccat	-3.550 to -3.148
Homo sapiens farnesyl-diphosphate farnesyltransferase 1 (FDFT1)	NM_004462.4, NM_001287747.1, NM_001287748.1, NM_001287751.1, NM_001287756.1, NM_001287742.1, NM_001287743.1, NM_001287744.1, NM_001287749.1, NM_001287750.1	26	tctgaacagga gtgggacaa	aaagacgggaaa ggccaat	-3.371 ± 0.02159
Homo sapiens farnesyl diphosphate synthase (FDPS)	NM_001135821.1, NM_002004.3, NM_001135822.1, NM_001242825.1, NM_001242824.1	18	tctactccttcta ccttcctatagct g	tctgaaagaactc ccccatc	-3.361 ± 0.07833
Homo sapiens geranylgeranyl diphosphate synthase 1 (GGPS1)	NM_001037277.1	55	agcactccggtc ctttttct	tggttaaaaagca aaacttgtgg	-3.090 ± 0.1593
Homo sapiens isoprenylcysteine carboxyl methyltransferase (ICMT)	NM_012405.3	66	tcttggttagagt tcacacttgaaa	cagacattctccg aagaccac	-3.345 ± 0.08594