Final report

Marshall Plan Scholarship Program

"Unraveling the Mystery of Epigenetics and Cancer-

Investigating the Influence of the Oncometabolite 2-Hydroxyglutarate on Heterochromatin Stability and Carcinogenesis"

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Abstract

The genetic code alone is not able to explain the diversity of cellular and individual phenotypes. Epigenetics provides an additional layer of information that is not encoded in the DNA molecule but nonetheless influences its activity in a stable and heritable manner through cell division. Epigenetic pathways play a significant role in oncogenesis and the reversible nature of those aberrations has led to the emergence of the promising field of epigenetic therapy.

Mutations in isocitrate dehydrogenase 1 (IDH1) and IDH2 occur in a variety of cancers in humans and this enzyme fails to convert isocitrate to α-ketoglutarate. Instead D-2-hydroxyglutarate (D-2HG) accumulates in cells. This oncometabolite inhibits the TET family of DNA demethylases and Jumonji family of histone demethylases which leads to epigenetic alterations that effect gene expression. Nevertheless D-2-HG is a normal endogenous metabolite found in all human body fluids and is maintained at low levels through the activity of D-2-hydroxyglutarate dehydrogenase (D2HGDH). The actual effect of this metabolite is still not known. That is why it is of critical importance to find out more about the role of D-2-HG in the cell in order to find therapies for cancer comprising IDH mutations in the future.

To tackle this research task a suppressor screen was performed in S. cerevisiae. The strains which were used comprise mutations in D-lactate dehydrogenase2 (dld2) and dld3. These enzymes are the yeast orthologs of human D2HGDH. Therefore, the cells accumulate D-2-HG. It was shown that growth of these cells is inhibited on EtOH. Three different suppressors found in the screen were analyzed and sequencing results showed mutations in RPD3, SIN3, and UME1. Rpd3 is a histone deacetylase, Sin3 and Ume1 are catalytic components of the Rpd3 histone deacetylase complex. The results suggest that histone deacetylase inhibitors (HDACi) might be an effective treatment for cancers comprising IDH mutations.

Kurzfassung

Der genetische Code allein ist nicht in der Lage die Vielfalt zellulärer und individueller Phänotypen zu erklären. Epigenetik liefert eine zusätzliche Informationsebene, welche nicht in der DNA kodiert ist, aber deren Aktivität trotzdem stabil und in vererbbarer Art und Weise beeinflusst. Epigenetische Regulation spielt eine entscheidende Rolle in der Onkogenese und die reversible Charakteristik dieser Abberationen führte zum Aufkommen der vielversprechenden epigenetischen Therapie.

Mutationen in Isocitrat-Dehydrogenase 1 (IDH1) und IDH2 treten in vielen verschiedenen humanen malignen Tumoren auf. Dabei ist das Enzym nicht mehr in der Lage Isocitrat in α-Ketoglutarat umzuwandeln und es kommt stattdessen zur Akkumulation von D-2-HG in den Zellen. Dieser Onkometabolit inhibiert sowohl die Familie der TET DNA-Demthylasen als auch jene der Jumonji Histondemethylasen. Die Folge sind epigenetischen Alterationen welche die Genexpression beeinflussen. Nichtsdestotrotz ist D-2-HG ein normaler endogener Metabolit welcher in allen Körperflüssigkeiten vorkommt und durch die Aktivität von D-2-Hydroxyglutarat Dehydrogenase (D2HGDH) in niedriger Konzentration aufrechterhalten bleibt. Die eigentliche Rolle dieses Metaboliten in der Zelle wurde bis dato noch nicht erforscht. Es ist daher von grösster Wichtigkeit mehr ueber die Aktivität von D-2-HG herauszufinden um in Zukunft effective Therapien fuer maligne Tumore welche IDH Mutationen aufweisen zu entwickeln.

Um dieser Forschungsaufgabe nachzugehen wurde ein Suppressor Screen in S. cerevisiae durchgeführt. Hierfür wurden Kulturen mit einer Mutation in der D-Laktat Dehydrogenase2 (dld2) und dld3 eingesetzt. Diese Enzyme sind die Hefeorthologe der humanen D-2-Hydroxyglutarate Dehydrogenase (D2HGDH), wodurch die Zellen folglich D-2-HG akkumulieren. Es wurde gezeigt, dass das Wachstum von Zellen mit der besagten Mutation durch Ethanol inhibiert wird. Drei verschiedene Suppressoren wurden im Screen gefunden, analysiert und sequenziert. Die Ergebnisse zeigen Mutationen in RPD3, SIN3 und UME1. Rpd3 ist eine Histondeacetylase und Sin3 bzw. Ume1 sind katalytische Komponenten des Rpd3 deacetylase Komplexes. Diese Ergebnisse deuten darauf hin, dass Histon-Deacetylase-Inhibitoren (HDACi) eine effective Therapie für Krebszellen welche IDH Mutationen aufweisen sein könnten.

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Finally, I would like to thank the Marshall Plan Foundation for their support. This endeavor would have not been possible without their generous funding.

1 Introduction

1.1 What is Epigenetics

The genetic code alone is not able to explain the diversity of cellular and individual phenotypes. Epigenetics provides an additional layer of information that is not encoded in the DNA molecule but nonetheless influences its activity in a stable and heritable manner through cell division. Epigenetic regulation can convert a developmental or transient environmental signal into a stable transcriptional response and explains how, starting from a unique genome, the pluripotent embryo can generate a variety of tissues and maintain their identity throughout development (Bourc'his D, 2010). Epigenetic changes regulate gene expression by hindering the availability of transcription factors towards DNA. This effect results from variations in chromatin states and is induced by biochemical modifications targeting the DNA molecule or associated histone proteins. (Subhankar Biswas, C. Mallikarjuna Rao, 2017). There are three epigenetic mechanisms present in human cells: DNA methylation, histone modifications and microRNAs (miRNAs) which are described below.

DNA methylation (see Figure 1) is probably the most extensively described modification of chromatin. It plays an important role in genomic imprinting, inactivation of X-chromosome in females, maintaining the stability of the genome, regulation of transcription and also in the developmental process of an organism (Robertson KD, Jones PA, 2000). Methylated DNA is present primarily in repetitive genomic regions (including satellite DNA, like micro and mini-satellites), within centromeres, telomeres and parasitic elements such as short interspersed transposable elements (SINEs) and long interspersed transposable elements (LINEs) where they function to silence genes and non-coding genomic regions. The majority of DNA methylation occurring on cytosine residue is present in the CpG dinucleotide distributed throughout the genome and is also densely found in regions known as CpG islands (Jones PA, Takai D, 2001). In normal cells, the promoter regions of genes, especially those preceded by CpG islands are usually unmethylated, allowing transcription factors and other associated proteins to interact with the gene and facilitate their expression. In contrast, the genomes of gametes and cells whose promoter regions are less enriched with CpG islands are frequently methylated during early development. However, we should bear in mind that these genes exhibit a distinct expression control during development and are always tissue specific. (Subhankar Biswas, C. Mallikarjuna Rao, 2017).

The conversion of cytosine into 5-methyl cytosine (5mC) is carried out by the catalytic activity of a group of enzymes called DNA methyltransferases (DNMTs). These enzymes use S-adenosyl methionine (SAM) as a key methyl group donor which transfers methyl group to cellular elements like DNA, lipids and proteins. SAM is converted into S-adenosyl homocysteine (SAH) after the transfer of methyl group by DNMTs. There are two major categories of the DNMTs in mammalian cells, a maintenance

methyltransferase and a de novo methyltransferase. The original DNA methylation pattern in a cell is greatly maintained by the catalytic activity of DNMT1, which prefers hemi-methylated DNA in place of non-methylated DNA as a substrate during replication, most likely with the support of UHRF1 (Ubiquitin like with PHD and ring finger domain 1) which also recognizes hemi-methylated sites, suggesting a role in maintaining the methylation patterns during cell division (Qin et al., 2015). In contrast, new DNA methylation pattern are established in the developmental phase of a cell utilizing DNMT3A and DNMT3B, which are expressed all over the cell cycle and shows equal preference for both hemi and unmethylated DNA making them de novo methyltransferase. Another enzyme, DNMT3L has been identified which is deficient in the conserved catalytic domain commonly associated with DNA methyltransferase. Although it is accepted that DNA methyltransferase are specific in their functions and non-overlapping, yet recent evidence suggests the overlapping role of de novo methyltransferases with maintenance methyltransferase (Walton EL et al., 2016). DNA methylation silence gene expression directly by impeding the binding of various transcription factors and indirectly by enrolling methyl-CpG binding domain (MBD) proteins. The MBD family contains five core proteins which include MBD1, MBD2, MBD3, MBD4 and the methyl cytosine binding protein 2 (MECP2). Apart from these, other MBD containing proteins are MBD5/6, SETDB1/2 and BAZ2A/B. The MBD protein employs histone modifying enzymes and chromatin remodeling complexes in methylated sites and facilitates transcriptional repression. Chromatin remodeling complex like NuRD binds with MBD2 protein and methylate DNA (Du Q, Luu PL, Stirzaker C, Clark SJ, 2015). These mechanisms play a central role in establishing the critical role of DNA methylation in epigenetic gene regulation. Although enzymes catalyzing DNA methylation has been well established, recent research has also identified mechanisms involved with the removal of methyl group. The discovery of ten-eleven translocation (TET) [which derives its name based on a recurrent chromosomal translocation and activation-induced cytidine deaminase (AID) family of enzymes has provided unprecedented information in our understanding of DNA demethylation (Scourzic L, Mouly E, Bernard OA, 2015). DNA demethylation can be achieved by two processes involving passive and active demethylation. Passive demethylation occurs by the failure of maintenance DNMT enzyme to methylate DNA after replication. Whereas, active DNA demethylation utilizes TET and AID family of enzymes to hydroxylate, oxidize or deaminate 5mC. Three TET family members have been identified so far including TET1, TET2 and TET3 and each of them are involved in distinct cellular process. Hydroxylation of 5mC by TET proteins produces 5-hydroxy methylcytosine (5hmC) and its subsequent conversion into 5- formylcytosine (5-fC) and 5-carboxylcytosine (5caC) followed by deamination and entry into the subsequent base excision repair pathway (Zhao H., Chen T., 2013).

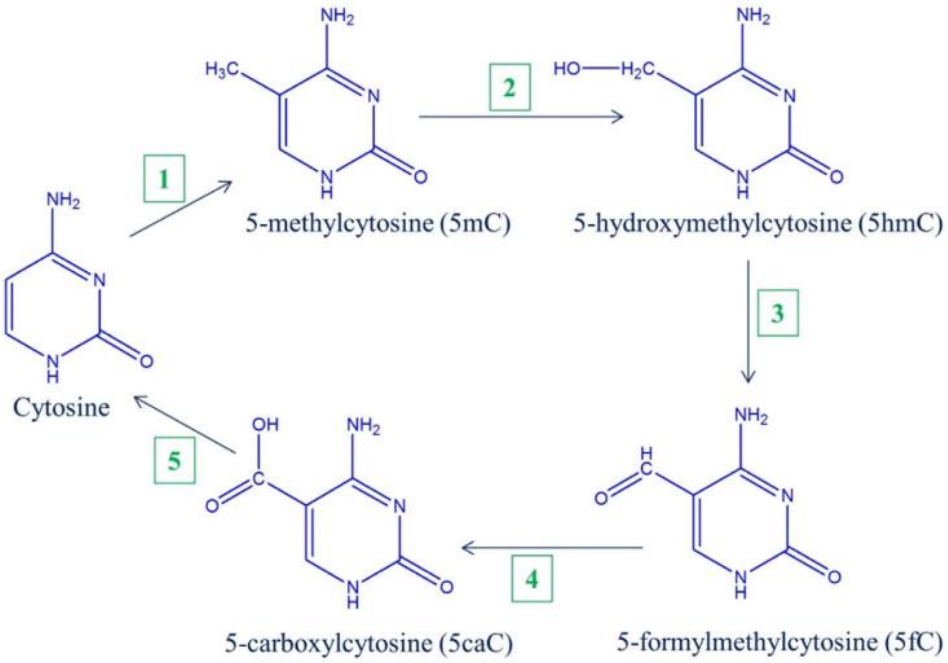

Figure 1: **Modification of cytosine residue.** The enzyme DNA methyltransferase methylates cytosine residue present in DNA at C5 position to form 5mC (1). TET1-3 enzymes then oxidize 5mC (2) to form 5hmC. Over-activity of TET enzymes (3,4) can further oxidize 5hmC into 5fC and 5caC. The enzyme Thymine-DNA glycosylase (5) removes the carboxyl group from 5caC following which base excision repair pathway (5) converts it into unmodified cytosine *(Subhankar Biswas, C. Mallikarjuna Rao, 2017)*.

Histone modifications: The basic element of chromatin, the nucleosome core particle, wraps 147 base pair of DNA around an octamer of four core histone proteins, see Figure 2. The basic histone proteins comprise an inherent positive charge which provides efficient binding with negatively charged DNA. The four core histones are present as H2A-H2B dimer and H3-H4 tetramer in association with a linker histone H1, which joins nucleosomes together. The sequence of amino acids comprising the histone proteins vary substantially among different species. But the histone proteins are made up of a common structural domain called the "histone fold". These folds comprise of a long central helix linked with two helix-strand-helix motifs at the opposite ends (Subhankar Biswas, C. Mallikarjuna Rao, 2017). The N-terminal tails of these proteins are highly flexible and are rich in lysine and arginine residues which can be extensively modified by a large number of cellular systems (Ramakrishnan V, 1997). Posttranslational, covalent modifications on those tails include methylation, acetylation, ubiquitination, sumoylation and phosphorylation on specific residues (Shikhar Sharma,Theresa K. Kelly, and Peter A. Jones1, 2009). Probably the most studied histone modifications include acetylation and methylation of lysine residues on the N-terminal tails of histone. Acetylation of lysine residue of histone tails is highly prevalent and their levels associate with transcriptionally active chromatin. Acetylation removes the net positive charge on the histone proteins by acetylating the ε-amino group of lysine residues using acetyltransferases (HATs) which utilizes acetyl-CoA as the acetyl group donor (Subhankar Biswas, C.

Mallikarjuna Rao, 2017). These enzymes are generally categorized into two different types: Type A, which are found in the nucleus and Type B, which are found in the cytoplasm (Brownell JE, Allis CD., 1996). However, evidence has suggested various functions of HAT which are beyond these classifications (Ruiz-García AB et al., 1998). Nucleosomal histones within the nucleus are acetylated by Type A HAT, whereas housekeeping role are associated with Type B HAT where they are involved in acetylating newly synthesized histones present in the cytoplasm (Ruiz-Carrillo A, Wangh LJ, Allfrey VG., 1975). Methylation of histone proteins is generally found on arginine and lysine residues, however unlike acetylation there is no alteration in the charge of the histone protein. Furthermore, it should be kept in mind that this type of modification has a different level of intricacy. Three different forms of methylation have been observed on the lysine residues viz. mono-, di- and tri-methyl whereas arginine can be mono-methylated and symmetrically or asymmetrically di-methylated (Bedford MT, Clarke SG., 2009). A wide range of information is available because of the multiple methylation states associated with lysine and arginine. For example, H3K4me3 i.e. trimethylation of lysine 4 on histone H3 is abundant at active gene promoter, whereas H3K9me3 is associated with transcriptionally repressed gene promoters (Kouzarides T, 2007). Histone methylation is catalyzed by three distinct families of enzymes namely, the SET-domain containing protein family, the non-SET domain protein family and the PRMT1 (protein arginine methyltransferases) family. There is no doubt that apart from acetylation and methylation various other mechanisms prevail to modify histone tails and it is widely accepted that phosphorylation plays a major role in amending protein structures. The amino acids serine, threonine and tyrosine residues on the histone tails are prone to phosphorylation. It has been observed that phosphorylation of serine 139 on histone variant H2AX plays a major role in generating the initial stages of DNA damage response (DDR). Although phosphorylation of threonine is a less common phenomenon, it contributes a major portion in epigenetic control of chromatin structure. Studies have demonstrated the phosphorylation of threonine 119 on H2A by nucleosomal histone kinase-1 which plays a major role in cell cycle progression (Aihara H et al., 2004). Phosphorylation of H3S10, S28 and T11 is widely studied and it is associated with transcriptional activation. Studies have shown that H3S10 phosphorylation encourages acetylation of H3K14 (Lo WS et al., 2000). Phosphorylation of serine 1 of histone H4 is involved in the later stages of DDR and also in stabilizing new nucleosome structure by preventing their acetylation (Rossetto D, Avvakumov N, Côté J., 2012). The enzymes involved in phosphorylating the serine residues belong to the kinase family of enzymes among which Ribosomal S6 kinase (RSKs), Mitogen and stress activated protein kinase 1 and 2 (MSK1 andMSK2) and Aurora kinases are widely studied (Rossetto D, Avvakumov N, Côté J., 2012). They play a major role in phosphorylating various serine residues on histone H3. Addition of ubiquitin and small ubiquitin-related modifier protein (SUMO) on specific lysine residues is another prominent histone posttranslational modification. Ubiquitination of lysine 119 of Histone H2A and lysine 120 of H2B is one of the most important observations made in recent years (Jason LJ, Moore SC, Lewis JD, Lindsey G, Ausió J., 2002).

It is seen that for dimethylation and trimethylation of H3K4 and H3K79 to occur ubiquitination of H2B is mandatory and therefore considered as a gene activating mark (Cole AJ, Clifton-Bligh R, Marsh DJ, 2015). On the contrary, ubiquitination of H2A is associated with transcriptional silencing with the involvement of two different E3 ubiquitin ligases, Ring1B and 2A-HUB (Spivakov M, Fisher AG., 2007). Although ubiquitinaiton of a protein is generally directed towards proteasomal degradation the above role of ubiquitin on histone proteins suggests its epigenetic role. Almost analogous to ubiquitination on the basis of reaction mechanism and the class of enzymes used, sumoylation adds SUMO peptides to all the four core histone proteins. An important feature associated with sumoylation is its primary target which is lysine. Although lysines are the targets of various other modifications, sumoylation is usually related with repression of target genes (Nathan D, Sterner DE, Berger SL., 2007).

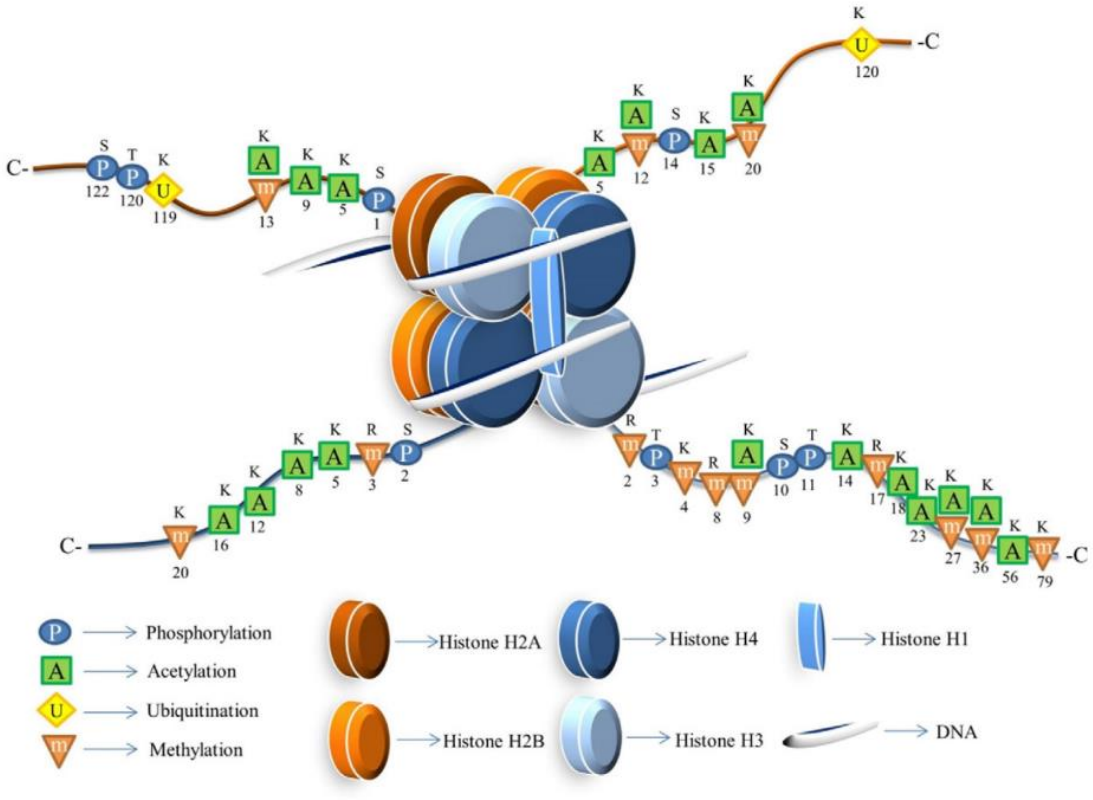

Figure 2: **Histone modifications**. The basic unit of DNA packaging in eukaryotes are nucleosomes. Each particle consists of an octamer of histone proteins including H2A, H2B, H3 and H4. Histones comprise N-terminal tails which play an important role in modulating nucleosome structure and function. Modifications on the different residues of histone tails are being shown here. S, T, K and R represent Serine, Threonine, Lysine and Arginine respectively. *(Subhankar Biswas, C. Mallikarjuna Rao, 2017).*

MicroRNAs (miRNAs) are small non-coding RNAs which are endogenous molecules and around 16 to 22 nucleotides long. Transcription of miRNAs is carried out by the enzyme RNA polymerase II to form the primary microRNA (pri-miRNA), which is processed by the microprocessor complex (DROSHA and DGCR8) to generate the precursor miRNA (pre-miRNA). The pre-miRNA is exported

into the cytoplasm by Exportin 5 in association with Ran-GTP. The pre-miRNA is further processed by DICER in the cytoplasm to generate a double stranded RNA. The double stranded RNA cleaves to form a mature miRNA, which associates with RNA induced silencing complex (RISC). The mature miRNA guides RISC to recognize the target mRNA leading to mRNA degradation or translation repression (Figure 3) (Subhankar Biswas, C. Mallikarjuna Rao, 2017) (Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM., 2006). The expression of miRNA is quite analogous to that of protein-coding genes as they are regulated by both genetic and epigenetic mechanisms. Recent research has mapped the presence of miRNA genes in the common breakpoint regions of oncogenes and tumor suppressor genes and fragile regions of the genome which are preferential site for deletion, translocation or amplification suggesting their involvement in driving the behavior of tumor growth. In addition, studies have found a link between epigenetics and miRNA, for example various miRNAs are able to modulate the activity of epigenetic modifying enzymes associated with carcinogenesis (Guil S., Esteller M., 2009). miRNA serves as a part of the regulatory network which takes part in silencing gene expression by methylation and modifying the structure of chromatin. Although a number of miRNA has been identified till date, mir-127 was the first epigenetically regulated microRNA associated with cancer (Saito Y et al., 2006).

Indeed, with the progress in the field of micro RNA it was possible to come across numerous microRNAs that are regulated by the epigenetic machinery viz. DNA methylation and histone modification. One of the most common epigenetic alterations includes DNA methylation and it has been observed that numerous microRNA genes are hypermethylated resulting in miRNA silencing. Among them miR-9, mir-148, miR-124, miR-137, miR-34, miR-127, miR-512 are frequently reported to be silenced in various types of cancer. Apart from methylation, histone modifications have also been associated with miRNA expression. Studies have identified a link between histone modifications (especially H3K27 and H3K9) and miR-212 gene contributing towards the development of lung cancer (Incoronato M et al., 2011).

On the other hand, a different outlook of miRNA has also been established in controlling DNA methylation and histone modification i.e. they are capable of targeting genes that regulate the epigenetic pathway creating a highly organized feedback pathway (Denis H, Ndlovu MN, Fuks F., 2011). An abnormal expression of these microRNAs, called epimiRNAs has been associated with various diseases. Studies have identified numerous miRNA that control chromatin structure by altering histone deacetylase enzymes and polycomb group related genes (Sato F, Tsuchiya S, Meltzer SJ, Shimizu K., 2011). For instance, miR-1 and miR-140 are involved in targeting HDAC4 isoenzyme whereas miR-449a binds to the HDAC1 and regulates their expression pattern (Noonan EJ et al., 2009). Also noted is the fact that the expression of EZH2 a catalytic subunit of the polycomb repressive complex 2 (PRC2) is altered by the epi-miRNA miR-101 (Varambally S et al., 2008). Moreover, a family of miRNAs (miR-29) regulates the expression of maintenance DNA methyltransferases DNMT3a and DNMT3b. Studies

have established that by repressing the activities of DNA methyltransferases, miR-29 suppresses tumorigenesis by altering the existing DNA methylation pattern in a cell (Morita S et al., 2013). Taken together the role of epi-miRNA in epigenetic control of gene expression is enormous and a lot remains to be uncovered.

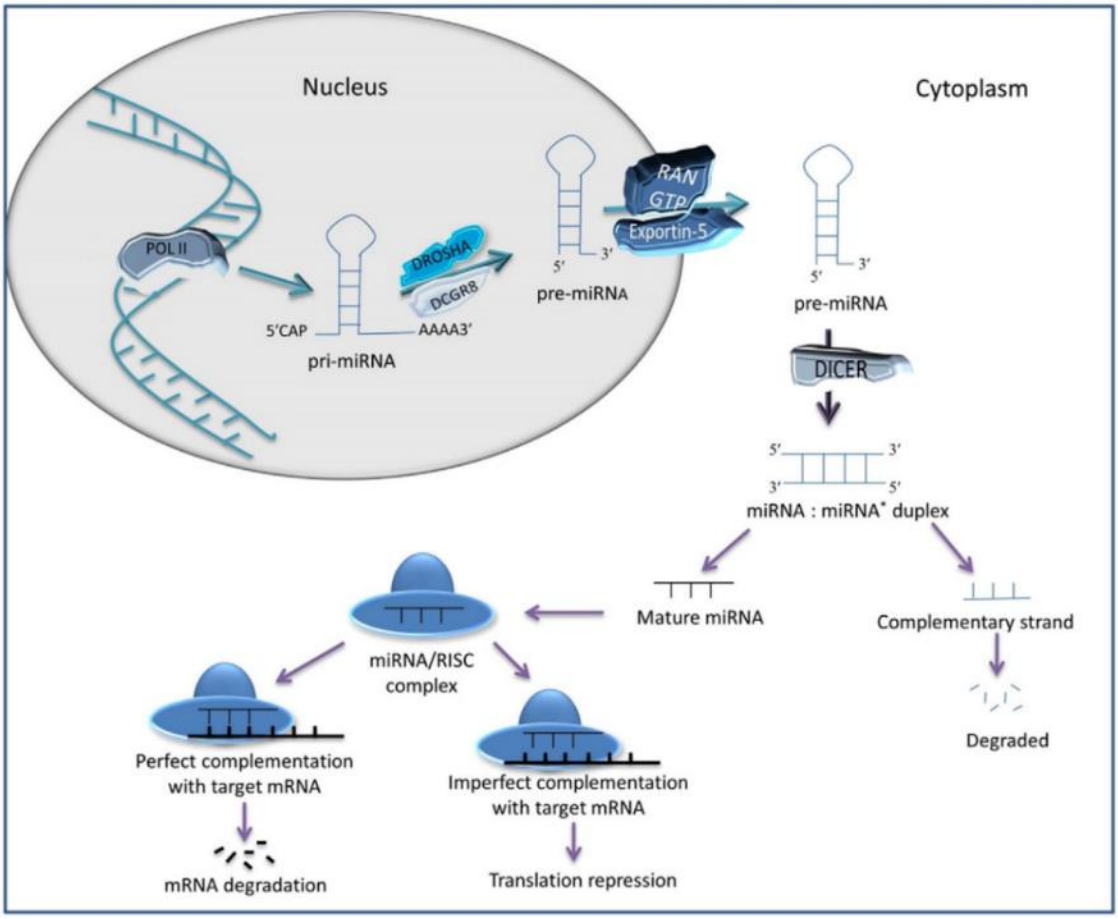

Figure 3: **miRNA biogenesis**. Most miRNA are transcribed by the enzyme RNA polymerase II to form the initial transcript which is known as primary microRNA (pri-miRNA). The primary miRNA contains an imperfect structure with a long sequence extending from the 5′ and 3′ end. The pri-miRNA is processed by microprocessor complex containing DROSHA and DGCR8 to form the precursor miRNA (pre-miRNA). The pre-miRNA is exported into the cytoplasm by Exportin 5 in association with Ran-GTP. The pre-miRNA is further processed by DICER in the cytoplasm which removes the stem loop to generate a double stranded RNA of 20-25 nucleotide long. The double stranded RNA cleaves to form a mature miRNA which associates with RNA induced silencing complex (RISC). The mature miRNA guides RISC to recognize the target mRNA leading to mRNA degradation or translation repression *(Subhankar Biswas, C. Mallikarjuna Rao, 2017)*.

1.2 Epigenetics and establishment of silencing in *S. cerevisiae* (Anne E. Dodson, 2016)

Yeast genetics and biochemistry studies have been extremely fruitful in illuminating many epigenetic modifications and their impact, most of which have proven to be widely conserved. Gene silencing in

Saccharomyces occurs by an epigenetic mechanism resulting from the assembly of heterochromatin at the loci to be silenced (Grunstein M, Gasser SM., 2013). The structural proteins of heterochromatin in Saccharomyces are the Silent Information Regulatory proteins Sir1, Sir2, Sir3 and Sir4 (Rine J, Herskowitz I., 1987). Silencing in Saccharomyces occurs by the recruitment of a complex of Sir2, Sir3 and Sir4 to regulatory sites called silencers, aided by the Sir1 protein bound to silencers. The Sir complex then deacetylates the H4K16-acetyl (H4K16-Ac) mark on neighboring nucleosomes, and such deacetylated H4 tails provide binding sites for additional Sir-protein complexes (Hecht A, Laroche T, Strahl-Bolsinger S, Gasser SM, Grunstein M., 1995). After rounds of deacetylation and binding of additional Sir-protein complexes, silenced domains of chromatin consist of hypoacetylated nucleosomes bound continuously by Sir-protein complexes, see Figure 5 below (Ellahi A, Thurtle DM, Rine J., 2015), (Thurtle DM, Rine J., 2014). Chromatin with this molecular topography blocks access of other site-specific DNA binding proteins to their cognate binding sites (Loo S, Rine J., 1994) (Steakley DL, Rine J, 2015). Sir2 is the founding member of the sirtuin family of NAD+-dependent protein deacetylases (Imai S, Armstrong CM, Kaeberlein M, Guarente L., 2000). The seven sirtuin paralogs of humans have been implicated in a rich variety of diseases including cancer (Imai S, Guarente L, 2014).

In eukaryotes, nuclear DNA winds around octamers of histone proteins to form chromatin, a structure that contributes to both the maintenance of genome integrity and the regulation of gene expression. Genetic and environmental cues shape the landscape of chromatin through the activity of chromatin modifiers and remodelers and through the binding of accessory proteins. As a result, chromatin comes in many flavors. One such flavor is heterochromatin. In 1928, Emil Heitz coined the term 'heterochromatin' to describe cytologically distinct regions of chromosomes that remain condensed throughout the cell cycle (Heitz 1928). Other defining features of heterochromatin include its inaccessibility to several DNA-interacting proteins, as well as its ability to silence transcription. Heterochromatin-mediated gene silencing is often heritable and thus provides a means for genetically identical cells to differentiate into distinct and stable cell types. Heterochromatin formation typically involves the addition or removal of modifications on certain histone residues (Grewal SI, Moazed D., 2003). Depending on the organism and the type of heterochromatin, the exact mechanism through which this occurs may involve sequence-specific DNA-binding proteins, RNA interference, DNA methylation, or long non-coding RNA. In budding yeast, heterochromatin assembly depends on specific DNA elements that recruit the Silent information regulator (Sir) proteins, including the conserved histone deacetylase Sir2 (Grunstein M, Gasser SM., 2013). This chapter reviews the function and formation of Sir-mediated heterochromatin in the budding yeast *Saccharomyces cerevisiae*.

Regulation of yeast mating types

To fully appreciate heterochromatin in *S. cerevisiae*, one should acquire a basic understanding of the different mating types. Heterochromatic repression serves an important role in mating-type determination. In fact, the discovery of Sir-mediated heterochromatin arose from the desire to understand certain curiosities of mating-type biology. Therefore, this review begins with an introduction to the three mating types: a, α and a/α.

Mating-type determination

The sexual life cycle of *Saccharomyces* requires the mating of two distinct cell types, referred to as a and α. Haploid cells exhibit either the a or α mating type and can mate with haploids of the opposite mating type to form an a/α diploid. The a/α status of the diploid permits the induction of meiosis, which completes the cycle by producing four spores, two that mate as a and two that mate as α. The mating-type (*MAT*) locus is the master controller of mating type (Strathern J, Hicks J, Herskowitz I., 1981). The two different wild-type alleles of this locus, *MATa* and *MATα*, code for proteins that regulate the expression of mating-type specific genes. *MATα* specifies the α mating type by encoding both α1, an activator of α-specific genes, and α2, a repressor of a-specific genes. In the absence of α2 protein, a-specific genes are constitutively active. Therefore, *MATa* cells (and *matΔ* cells) mate as a by default. In *MATa*/*MATα* diploids, the a1 protein encoded by *MATa* interacts with α2 to repress the expression of haploid-specific genes. Two additional loci, *HML* and *HMR*, each contain an exact copy of either the a or α mating-type allele (Figure 4). Unlike *MAT*, however, *HML* and *HMR* are transcriptionally silent. How do genetically identical loci adopt different states of expression? The answer lies within the flanking regions of *HML* and *HMR*. These regions contain cis-regulatory sites referred to as silencers that nucleate heterochromatin formation and thereby render *HML* and *HMR* transcriptionally inactive (Figure 4). Importantly, the heterochromatic repression of *HML* and *HMR* ensures that *MAT* is the sole source of mating-type information.

Mating-type switching

HML and *HMR* serve a critical role in mating-type switching, a process whereby haploids convert the *MAT* genotype from *MATa* to *MATα* or vice versa (Haber 2012). In the first step of mating-type switching, the site-specific endonuclease HO generates a double-strand break at the *MAT* locus (Kostriken R et al., 1983). Then, the homologous recombination machinery repairs the double-strand break using either *HML* or *HMR* as a donor template, as both loci share some sequence identity with *MAT*. The repair is typically a gene conversion event that copies the information stored at *HML* or *HMR* into the *MAT* locus, leaving the donor sequence unchanged. In the event that the donor allele differs from the original *MAT* allele (for example, cleavage of *MATa* is repaired using *HMLα* as the donor), the cell will switch mating types. Interestingly, selection of the donor template is nonrandom—a cells prefer

to use *HML*, whereas α cells prefer to use *HMR* (Figure 4). These preferences increase the probability that double-strand break repair will result in a mating-type switch, as most strains store α information at *HML* and a information at *HMR*. Donor preference is controlled in part by an extraordinary cis-acting element referred to as the recombination enhancer (*RE*) (Wu X, Haber JE., 1996). The *RE* locus resides on the left arm of chromosome III, approximately 17kb away from *HML* (Figure 4). In a cells, the *RE* activates recombination throughout the entire left arm of chromosome III and thereby promotes the selection of *HML* as a donor template. When the *RE* is absent or inactive, cells prefer to use *HMR* by default. For example, α cells prefer to use *HMR* as a donor because the α2 protein encoded by *MATα* represses *RE* activity (Szeto L, Broach JR., 1997). Hence, *HMR* is always available for recombination, whereas *HML* is only made available through the action of the *RE*. A few points regarding HO are worth mentioning here. First, *HO* expression, and therefore mating-type switching, occurs only in haploid mother cells during the G1 phase of the cell cycle (Nasmyth K, 1993). Second, HO recognition sites also reside at *HML* and *HMR*, but the heterochromatin-dependent positioning of nucleosomes over these sites protects them from HO cleavage (Weiss K, Simpson RT, 1998). Finally, most laboratory strains do not contain a functional HO and thus do not readily switch mating types. The recombination enhancer is still functional in laboratory strains, however.

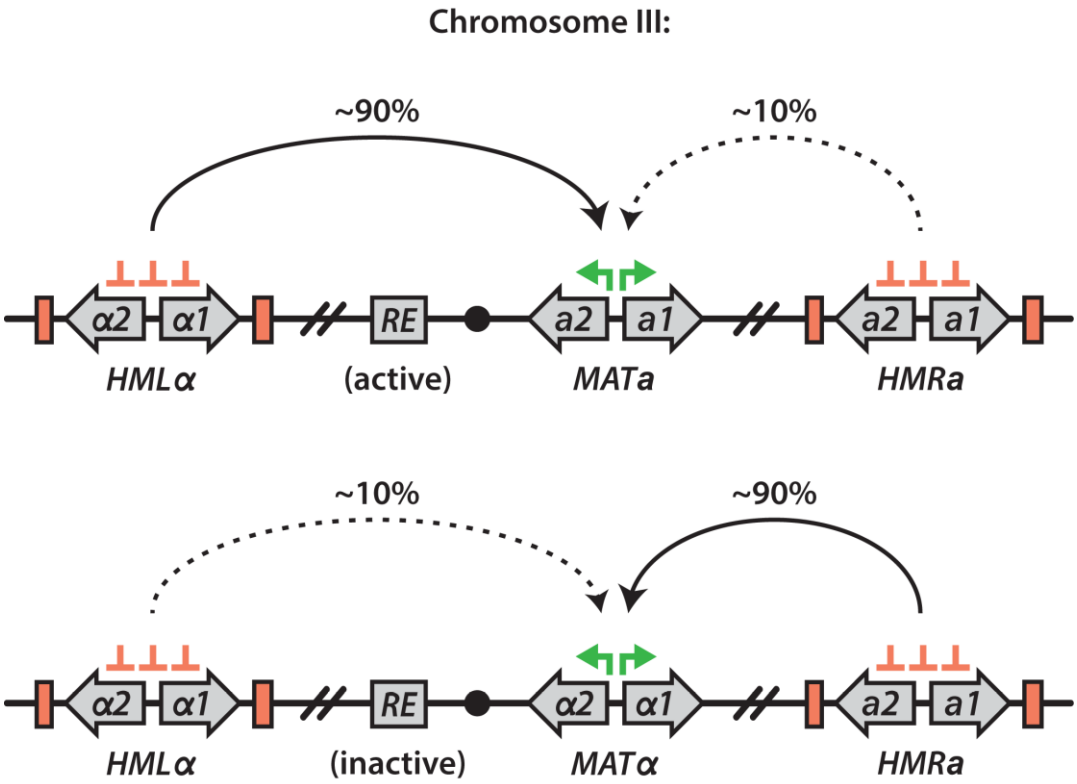

Figure 4: (Anne E. Dodson, 2016) *MATa* and *MATα* versions of chromosome III in S. cerevisiae. The centromere (black circle) demarcates the left and right arms. The silencers (red boxes) mediate the repression of *HML* and *HMR*. Percentages indicate how often *HML* or *HMR* is used as a donor template during repair of a HO-induced double-strand break at MAT. Donor preference differs between mating types due to the activity of the recombination enhancer (RE). Features are not drawn to scale.

Heterochromatin assembly

The identification of *HML* and *HMR* in early studies of mating-type biology marks the beginning of the quest to understand position-effect silencing in budding yeast. Today, a wealth of literature exists on this topic; yet, the mechanisms underlying certain fundamental aspects of silencing remain unclear.

- **Silencers**

Transcriptional repression of *HML* and *HMR* depends on the silencers, short DNA elements that border the left and right sides of each locus (Feldman JB, Hicks JB, Broach JR., 1984). Each silencer contains a binding site for the origin recognition complex (ORC), as well as a binding site for either Abf1 or Rap1, or both. Paradoxically, Abf1 and Rap1 often function individually in the transcriptional activation of many genes. In the context of a silencer, however, these proteins cooperate with ORC to recruit the Sir proteins and thereby silence transcription.

- **Sir proteins**

The four *SIR* genes were identified in a forward genetic screen designed to isolate mutants that aberrantly express *HML* and *HMR* (Rine J, Herskowitz I., 1987). Loss-of-function mutations in *SIR2*, *SIR3* or *SIR4* result in full derepression, meaning that the genes present at *HML* and *HMR* are expressed at the same level as their counterparts at *MAT*. By contrast, loss-of-function mutations in *SIR1* result in an extraordinary bistable phenotype—some cells exhibit full repression, other cells exhibit full derepression, and each state of expression is heritable for multiple cell divisions (Pillus L, Rine J., 1989). The existence of *sir1* cells in the silenced state demonstrates that Sir1, unlike the other Sir proteins, is not essential for silencing. In addition to silencing transcription at *HML* and *HMR*, the Sir proteins also silence a subset of subtelomeric genes. Placing a reporter gene next to certain telomere constructs results in the variegated expression of that gene—a fraction of cells silence the reporter, and that silencing depends on Sir2, Sir3 and Sir4 (Gottschling DE et al. , 1990).Given that Sir1 does not contribute to this effect (Aparicio OM et al., 1991), it is interesting to note that telomere-proximal reporter genes exhibit two distinct, semistable states of expression, much like the genes at *HML* and *HMR* in a *sir1Δ* background. Most studies of telomeric position effect were performed using truncated telomeres that contain the terminal TG1-3 repeats but lack more internal features such as the X and Y' elements. In their native context, telomeres vary in the degree to which they mediate heterochromatic repression (Pryde FE, Louis EJ, 1999). In fact, the Sir proteins silence less than 10% of subtelomeric genes. Sir2, the only Sir protein with known enzymatic activity, is a highly conserved protein deacetylase. As a member of the sirtuin family of NAD+-dependent deacetylases, Sir2 couples protein deacetylation with the breakdown of NAD+ (Tanny JC, Moazed D., 2001). Specifically, Sir2 cleaves NAD+ into nicotinamide and ADP-ribose and transfers the acetyl group on the substrate to ADP-ribose to produce 2'-*O*-acetyl-ADP-ribose. Sir2 preferentially targets acetylated lysine 16 of histone H4 (H4 K16-ac), as well as acetylated lysines 9 and 14 of histone H3 (H3 K9-ac and H3 K14-ac) (Landry J, Sutton A et al.,

2000).Independent of the other Sir proteins, Sir2 suppresses intrachromosomal recombination between ribosomal DNA repeats as a component of the RENT (regulator of nucleolar silencing and telophase exit) complex (Gottlieb S, Esposito RE., 1989).

- **Establishment of the silenced state**

Since wild-type cells are programmed to constitutively silence *HML* and *HMR*, they rarely encounter the need to establish silencing de novo. However, analyses of the establishment process have greatly informed our understanding of heterochromatin assembly. To study the de novo establishment of silencing, one must be able to conditionally inactivate and reactivate silencing. Therefore, temperature-sensitive *SIR* alleles, small-molecule inhibitors of Sir2, and cleverly designed assays that control the state of silencing have all been particularly useful for dissecting the individual steps of heterochromatin assembly. The establishment of silencing begins with the loading of Sir proteins onto the silencers. Sir1 localizes to the silencers through a direct interaction with the ORC subunit Orc1 and assists in recruiting the other Sir proteins (Triolo T, Sternglanz R., 1996). The Sir1-Orc1 interaction is stabilized by the presence of Sir4, which binds Sir1, Sir2, Sir3 and Rap1 (Triolo T, Sternglanz R., 1996). Sir3, in turn, interacts with Rap1 and possibly Abf1 (Moretti P, Shore D., 2001). Thus, the silencers weave a web of interactions between Sir proteins and silencer-binding proteins. Although most of these interactions are weak in isolation, they stabilize one another when combined. Once the Sir proteins assemble at the silencers, they recruit additional Sir proteins that associate throughout the locus. This step requires the deacetylation of histone residues by Sir2 and the binding of nucleosomes by Sir3 and Sir4 (Rusche et al., 2002). Sir3 preferentially binds histones in the hypoacetylated state (Carmen et al., 2002); therefore, Sir2 helps create high-affinity binding sites for Sir3. Deacetylation of H4 K16-ac by Sir2 seems particularly important, as a crystal structure of the Sir3-nucleosome interaction predicts that acetylation of H4 K16 would disrupt several electrostatic contacts (Armache et al., 2011). Chromatin immunoprecipitation (ChIP) analyses show co-enrichment of Sir2, Sir3 and Sir4 at the silencers and at internal sites of *HML* and *HMR*, whereas Sir1 associates only with the silencers (Rusche et al., 2002). Therefore, Sir1 does not spread across *HML* and *HMR* like the other Sir proteins do. Transcriptional silencing marks the completion of heterochromatin formation. Surprisingly, this last step is separable from the deacetylation and binding of nucleosomes by Sir proteins (Lau et al. 2002; (Kirchmaier AL, Rine J., 2006). Induction of the establishment process in G1-arrested cells leads to the spreading of Sir proteins across *HMR*, yet transcription of the *a1* gene at *HMR* persists until the cells are allowed to pass through S phase (Kirchmaier AL, Rine J., 2006). Therefore, the establishment of silencing involves an additional, unknown step that occurs during S phase (Miller AM, Nasmyth KA., 1984). This step does not require the passage of a replication fork through the locus (Kirchmaier AL, Rine J., 2006), and it is less important for the silencing of *HML* than it is for the silencing of *HMR* (Ren et al., 2010). A second cell-cycle requirement for silencing exists in G2/M under certain conditions, and disruption of sisterchromatid cohesion allows cells to bypass this requirement (Lau et al., 2002). The de novo

establishment of silencing typically occurs within one to two cell divisions, and mutations known to affect certain histone modifications either shorten or lengthen this timeline (Katan-Khaykovich Y, Struhl K., 2005). For example, mutants defective in the methylation of histone H3 at lysine 79 (H3 K79) establish silencing faster than wild type (Katan-Khaykovich Y, Struhl K., 2005). These data suggest that H3 K79 methylation antagonizes one or more steps in the establishment process. Given that H3 K79 methylation reduces the affinity of Sir3 for nucleosomes (Altaf M et al., 2007), this modification probably impedes the spreading of Sir proteins throughout *HML* and *HMR*. Enzymes that modify H4 K16 and lysine 4 of histone H3 (H3 K4) also contribute to the kinetics of silencing establishment (Katan-Khaykovich Y, Struhl K., 2005).

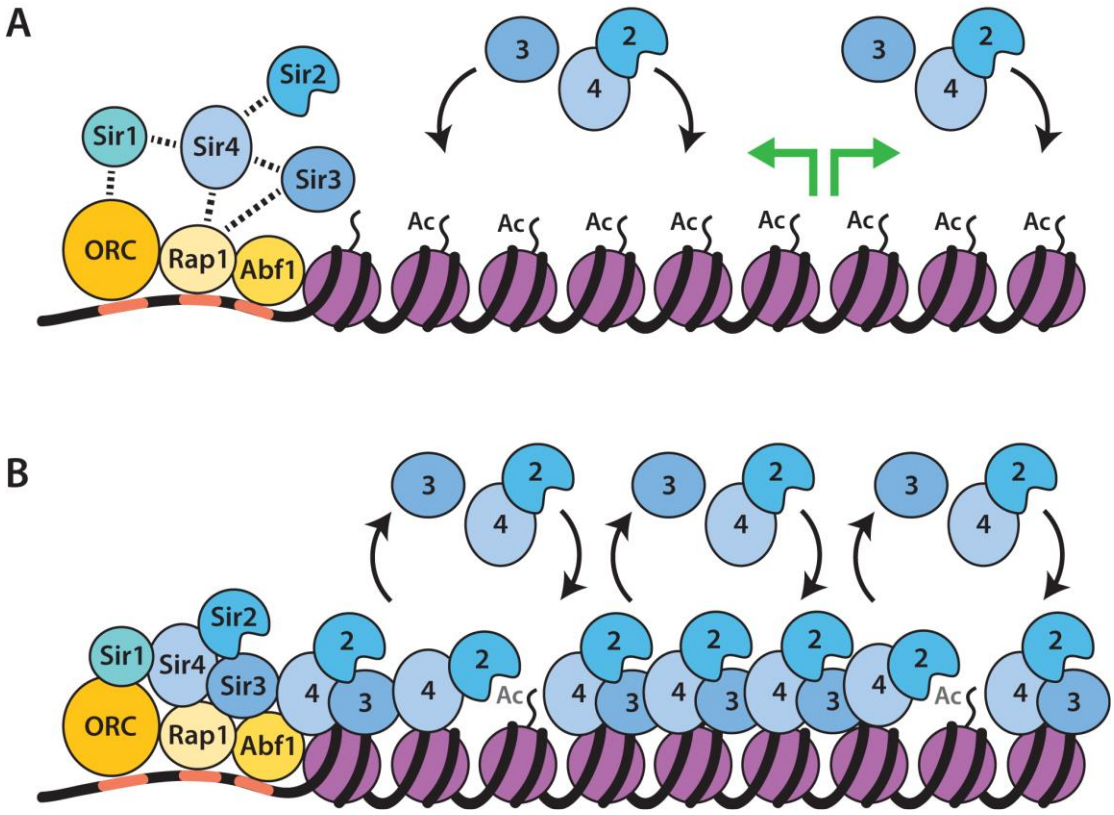

Figure 5: **Establishment and maintenance of silencing.** (Anne E. Dodson, 2016) A- Establishment of silencing. Silencer binding proteins (ORC, Rap1, Abf1) recruit the sir proteins (sir1, sir2, sir3, sir4). Dashed lines indicate direct interactions. When sir proteins are assembled at the silencers more sir proteins get recruited to the locus to be silenced. First sir2 deacetylates H4K16 acetyl mark, then sir3 and sir4 bind to nucleosomes. B- Sir2 mediated deacetylation and recruitment of more sir protein continuously reinforces the silenced state.

1.3 Epigenetics, Metabolism and Cancer

Cancer metabolism is one of the oldest areas of research in cancer biology. Metabolic activities are altered in cancer cells relative to normal cells. These alterations support the acquisition and maintenance

of malignant properties. Some altered metabolic features are observed quite generally across many types of cancer cells and reprogrammed metabolism is therefore considered a hallmark of cancer. Changes in metabolite levels can affect cellular signaling, epigenetics, and gene expression through posttranslational modifications such as acetylation, methylation, and thiol oxidation (Ralph J. DeBerardinis and Navdeep S. Chandel, 2016). This was also confirmed by other members of the UC Berkeleys Rine Lab like Dr. Ryan Janke. He found out recently that the oncometabolite D-2-hydroxyglutarate (D-2-HG) enhances gene silencing through inhibition of specific H3K36 histone demethylase in yeast (Ryan Janke, Anthony T Iavarone, Jasper Rine, 2017).

1.4 The oncometabolite D-2-hydroxyglutarate (D-2-HG) and its role in cancer development

Cancer-associated isocitrate dehydrogenase (IDH) 1 and 2 mutations gain a new function of reducing α-KG to produce D-2-HG, see Figure 5 (Shenghong Ma et al., 2015). α-KG plays crucial roles in the TCA-cycle, amino acid synthesis and nitrogen transport (Ryan Janke, Anne E. Dodson, Jasper Rine, 2015). D-2-HG is proposed to function as an oncometabolite by inhibiting α-KG dependent dioxygenases like the TET family of DNA demethylases and Jumonji family of histone demethylases. This leads to epigenetic changes and altered gene expression (Ryan Janke, Anthony T Iavarone, Jasper Rine, 2017). Figure 5 shows in which metabolic pathways D-2-HG is involved in. Somatic mutations in IDH1 and 2 occur in multiple types of human cancers (Ming Yang, Tomoyoshi Soga, Patrick J. Pollard, 2013).

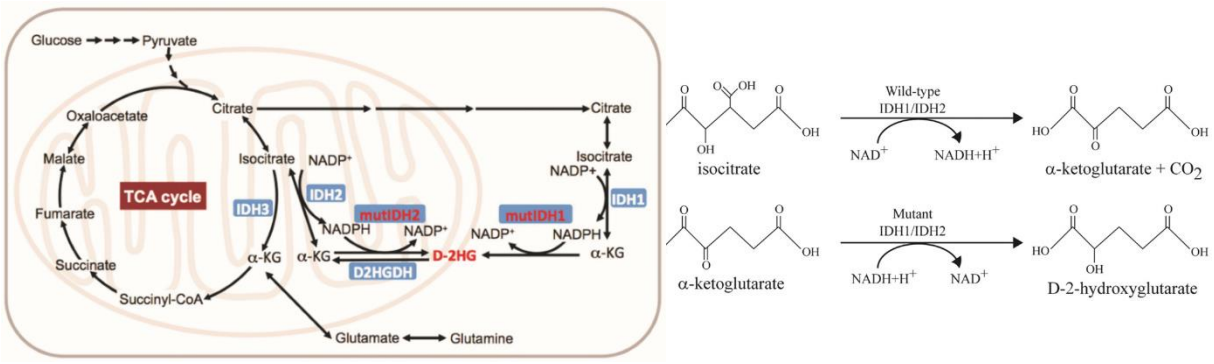

Figure 6: **Scheme of metabolic pathways involved in D-2-HG metabolism and enzymatic reactions catalyzed by wild-type and mutant IDH enzymes.** α-KG is a key intermediate in the TCA cycle for energy metabolism and also acts as an entry point for amino acids to enter the TCA cycle *(Shenghong Ma et al., 2015)*. Mutated IDH1 and IDH2 reduce α-ketoglutarate to D2HG while converting NADH and H+ to NAD+ *(Nadine F. Voelxen et al., 2016)*.

1.5 Goals of this work

The primary goal of this work was to find a potential approach to treat cancer that comprise IDH mutations. These mutations are found in various types of cancers like, gliomas, secondary glioblastomas, acute myelogenous leukemia, cholangiocarcinoma, cartilaginous tumors, prostate cancer, papillary breast carcinoma, acute lymphoblastic leukemia, angioimmunoblastic T-cell lymphoma, and primary myelofibrosis. This indicates that IDH mutations are important players in multiple types of cancers (Adam Cohen, Sheri Holmen, and Howard Colman, 2013).

Finding out more about the oncometabolite D-2-HG (which accumulates in cells with mutations in IDH) and its role in the cell, performing a suppressor screen was the first logical step towards being able to combat a variety of malignant tumors in the future.

2 Materials and Methods

2.1 Yeast strains

The strains and oligonucleotides used in this study are listed in Table 1 and Table 2, respectively at the end of this chapter. All strains were derived from the W303 background. Deletions were made using one-step integration of gene disruption cassettes and confirmed by PCR. This process is described in more detail in the following references (Longtine MS1, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR., 1998), (Goldstein AL, McCusker JH., 1999), (Wach A, Brachat A, Pöhlmann R, Philippsen P, 1994).

2.2 Suppressor screen

A suppressor screen (as shown in Figure 7) is used to identify suppressor mutations which alleviate or revert the phenotype of the original mutation (in this study *dld2Δdld3Δ*), in a process defined as synthetic viability. Suppressor mutations can be described as second mutations at a site on the chromosome distinct from the mutation under study, which suppresses the phenotypic effect of the original mutant (Jonathan Hodgkin, 2005).

To define the function of biochemical pathways within a cell and the relationships between different biochemical pathways, suppressor mutations are extremely useful (Fabio Puddu, Tobias Oelschlaegel et al., 2015).

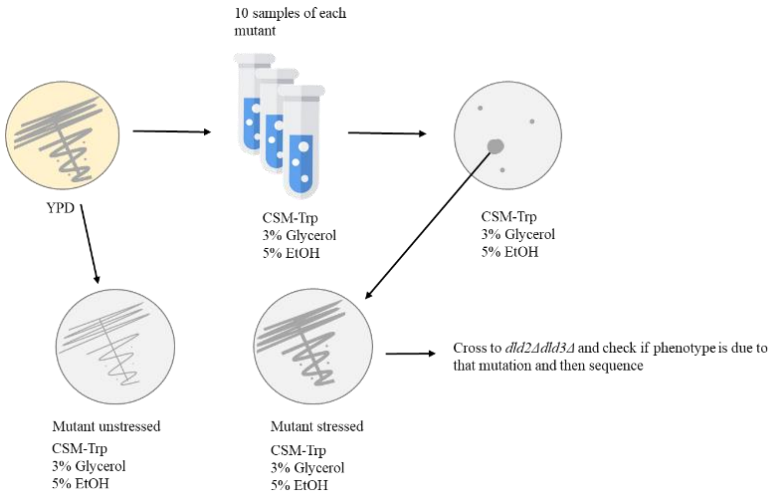

***Figure 7*: Schematics showing the procedure of the suppressor screen.** Description can be found in text below.

Procedure of the screen

Ten single colonies of each mutant (*dld2Δ, dld3Δ, dld2Δdld3Δ*) were grown until saturation in CSM-Trp Glycerol 5% EtOH liquid media and plated with different dilutions on CSM-Trp glycerol 5% EtOH plates. These liquid cultures were incubated at 30˚C for a few days until colonies were growing. Colonies that stood out, meaning grew into bigger colonies than the rest were selected and suppression phenotype was verified doing a drop assay. This process was repeated until real suppression phenotypes were found in the drop assay.

The next step was mating the suppressor found to the original mutant (in this case *dld2Δdld3Δ*) and to check the segregation pattern. Also, a complementation test was performed to determine whether mutations were in the same, or in different genes. Each suppressor (haploid) found was mated to each suppressor to get diploids and phenotype was examined.

A test for dominance was carried out afterwards to check whether mutations are dominant. Each suppressor candidate was mated back to the original mutant (*dld2Δdld3Δ*) and diploids were reviewed.

2.3 Whole genome sequencing and data analysis

DNA for whole-genome sequencing analysis was isolated following the yeast DNA extraction protocol in (Charles S.Hoffman, Fred Winston, 1987).

The data received from the UC Berkeley DNA Sequencing Facility were 100bp paired end. Each sample was mapped to the SacCer3 reference genome using data available at *yeastgenome.org* and the mapping results were sorted according to their position along the genome.

Then, a command called *mpileup* that generates a table of all sites in the genome, and scores how often they are counted, or how often there is a mutation at that position was generated. The generated file was fed into a program called *bftools*, which identified sites that have real mutations (rather than sequencing errors). A separate list of sites that have mutations for each mutant pool and for the wild-type pool was made, then a custom code in the programming language *R* was written by Gavin Schlissel to compare them and identify the mutations that are present in the "suppressed" pools but not in the wild-type pools. In the last step, those mutations were fed into a program called *SnpEff*, and *SnpEff* returned the genes in which each mutation occurs, and a prediction for how severe the mutation was.

2.4 Immuno-blotting (Ryan Janke, Anthony T Iavarone, Jasper Rine, 2017)

Cells were grown in liquid cultures of CSM-Trp-3% glycerol shaking at 30°C until mid-log phase. Ten OD units of cells were harvested from each culture, pelleted, frozen with liquid nitrogen, and stored at −80°C. Pellets were resuspended in 200 µl of 20% w/v trichloroacetic acid and transferred to 2-ml screw cap tubes. Cell extracts were prepared by addition of an equal volume of 0.5 mm zirconium ceramic beads (*BioSpec* Products, Bartlesville, OK) followed by bead beating using a *Millipore MP-20 FastPrep* (*EMD Millipore*, Billerica, MA) on setting 5.5 with 20 s cycle duration. A total of five cycles were performed with 2-min incubations on ice between each run to prevent overheating of samples. Recovered precipitate was dissolved in 200 µl of 2X *Laemmli* buffer and the pH adjusted by adding 30 µl of 1.5 M Tris pH = 8.8. Samples were heated at 65°C for 10 min and insoluble material was pelleted by centrifugation. An equal amount of the soluble portion of each sample was run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in Li-Cor Odyssey Blocking Buffer (LI-CORE Biosciences, Lincoln, NE) and the following primary antibodies were used for immunodetection: anti-acetyl histone H4 (Lys12) polyclonal antibody was from *Millipore*, anti-phosphoglycerate kinase antibody (22C5D8) from *ThermoFisher* (Rockford, IL.). Membranes were incubated with infrared dye-conjugated secondary antibodies, IRDye800CW goat anti-mouse and IRDye680RD goat anti-rabbit antibodies (*LI-CORE* Biosciences, Lincoln, NE) and imaged on a *LI-CORE Odyssey* imager in the 700 nm and 800 nm channels. All washing steps were performed with Tris-buffered saline – 0.05% Tween-20. Quantitative analysis of immunoblots was performed using *LI-CORE* Image Studio software (*LI-CORE* Biosciences, Lincoln, NE).

2.5 CRASH Assay

The CRASH assay (Cre-Recombinase Assessment of Stability of Heterochromatin), involves the Cre-recombinase coding sequence placed under the control of a promoter silenced by heterochromatin at the HML or HMR locus. In such cells, a genetic cassette consisting of a gene encoding red fluorescent protein (RFP) is inserted under control of a constitutive yeast promoter, and a promoterless gene encoding green fluorescent protein (GFP) downstream of the RFP gene. Lox sites, at which the Cre-recombinase can act, are positioned such that if Cre is ever expressed, the RFP coding sequence is looped out of the chromosome, permanently fusing the GFP gene to the constitutive promoter, as shown in Figure 8 (Anne E Dodson, Jasper Rine, 2015). Thus, transient losses of silencing results in a switch of a red cell and all its descendants into green cells. Therefore, the number and size of sectors reflect epigenetic stability. Loss-of-silencing events at HML occur in 1.6×10^{-3} cell divisions. This rate also

allows for advanced microscopic and flow cytometry applications to interrogate the molecular events in these cells with single-cell resolution (Anne E Dodson, Jasper Rine, 2015).

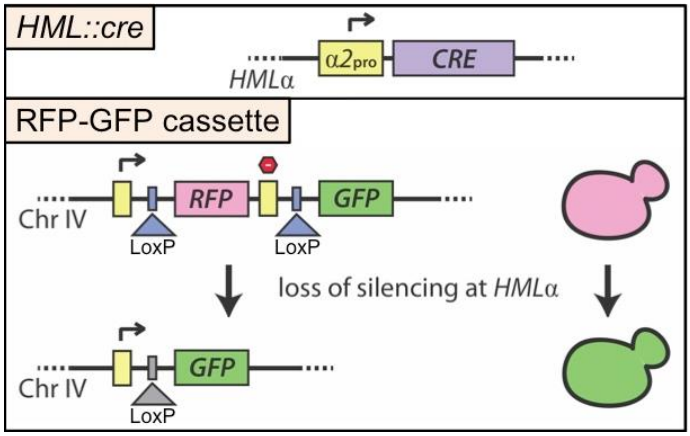

Figure 8: Schematics of CRASH-Assay *(Anne E Dodson, Jasper Rine, 2015)*

2.6 Flow Cytometry

Quantification of Silencing Loss by Flow Cytometry

For each CRASH strain, ten single colonies were inoculated separately into 1 ml of CSM-Trp 3% Glycerol media in 96-deep-well plates and grown overnight to saturation at 30°C on a low orbital shaker. Overnight cultures were diluted into 1 ml of fresh media at a density of 105 cells/ml in 96-deep-well plates and were grown at 30°C on a low orbital shaker until mid-log phase. For each culture, a minimum of 50,000 events were collected using an *Attune NxT Flow Cytometer* (by Life Technologies). Scatterplots of forward scatter (height) and forward scatter (width) measurements were generated and gating was established to include only singlets (unbudded) and budded cells and exclude debris as well as clumped cells for further analysis. Gating was used to separately measure the number of GFP-positive cells and the number of RFP-positive cells. Finally, a *Boolean logic gate* 'RFP+ AND GFP+' was used to determine the number of cells that were both GFP and RFP fluorescent. Such cells were inferred to have just undergone the Cre-mediated recombination event leading to GFP expression, yet retained RFP expressed in the recent past. The frequency of switching was calculated by dividing the number of cells in a population that had very recently lost silencing (cells that are both GFP- and RFP-fluorescent) by the number of cells in the population that had the potential to lose silencing (cells that are only RFP-fluorescent + cells that are both GFP-and RFP-fluorescent). Boxplots were generated where the median value was calculated from at least 10 cultures. The boxes in Figure 17 represent the 25th and 75th percentile. Whiskers represent the range of values within 1.5-times the interquartile range. Unpaired

two-sided (Student's) t tests were used to determine whether differences in frequency of silencing loss were statistically significant.

Table 1: Strains used in this study. All strains are so called "CRASH strains". A more detailed description can be found in section 2.5 or in the following publication *(Dodson A, Rine J, 2015)*.

Strain	Genotype
RHJY42	*MATα* ADE2, lys2, TRP1, hmlα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:Hygmx:loxP:yEGFP:tADH1 dld2Δ::URA3 (C. albicans)
RHJY43	*MATa* ADE2, lys2, TRP1, hmlα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:KanMX:loxP:yEGFP:tADH1 dld2Δ::URA3 (C. albicans)
RHJY63	*MATa ADE2, lys2, TRP1, hmlα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:hygMX:loxP:yEGFP:tADH1*
RHJY65	*MATα ADE2, lys2, TRP1, hmlα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:hygMX:loxP:yEGFP:tADH1*
RHJY165	*MATa ADE2, lys2, TRP1, hmlα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:hygMX:loxP:yEGFP:tADH1 dld3Δ::kanmx dld2Δ::URA3 (C. albicans)*
RHJY212	*MATα* ADE2, lys2, TRP1, hmlα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:hygMX:loxP:yEGFP:tADH1 dld2Δ::URA3 (C. albicans) dld3Δ::KANMX
RHJY297	*MATa* ADE2, lys2, TRP1, hmlα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:hygMX:loxP:yEGFP:tADH1 dld3Δ::kanmx dld2Δ::URA3 (C. albicans) *rpd3Δ::natMX*
RHJY296	*MATa ADE2, lys2, TRP1, hmlα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:hygMX:loxP:yEGFP:tADH1 rpd3Δ::natMX*

***Table 2*: Oligonucleotides used in this study.**

Oligonucleotide	Sequence (5'-3')
RPD3 KO For	TACAAAACATTCGTGGCTACAACTCGATATCCGTGCAGGCATAGGCCACTAGTGGATCT
RPD3 KO Rev	TCACATTATTTATATTCGTATATACTTCCAACTCTTTTTTCAGCTGAAGCTTCGTACGC
RPD3 co For	AAGTAATATCAACTCAGAGCGTATAGGTAAATTTGTAAAT
RPD3 co Rev	GTTTAGATAGTAATTACAATAGAAATACAACTGTTTCAAT

3 Results

3.1 Sensitizing conditions and suppressors found

The mutants under investigation (*dld2Δ, dld3Δ, dld2Δdld3Δ*) have never been subject of a suppressor screen. Therefore, different carbon sources and then different stresses were tested, see Table 3.

***Table 3*: Carbon sources and stresses tested in the suppressor screen.**

Carbon sources	Stresses
- Glucose	- H2O2
- Raffinose	- Sorbitol
- Galactose	- Hydroxyurea
- Glycerol	- Ethanol

The strains turned out to be sensitive to EtOH as can be seen in Figure 9.

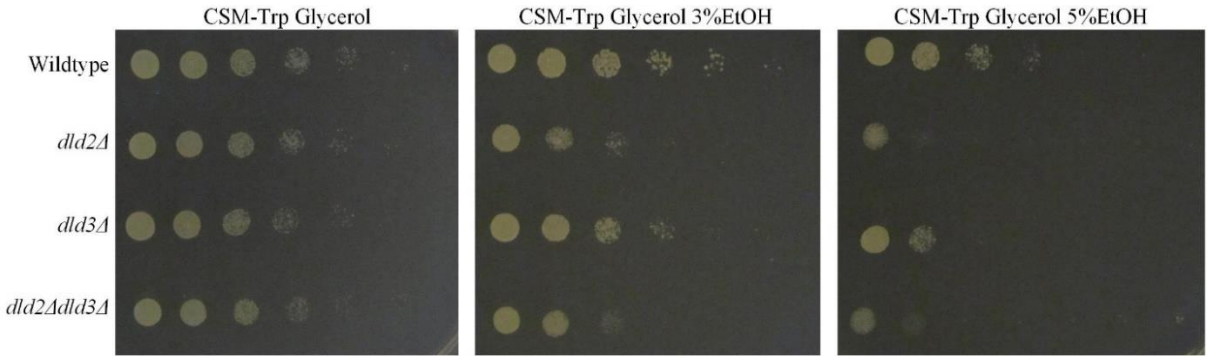

***Figure 9:* Sensitizing conditions found.** Fivefold serial dilutions of yeast were spotted on plates to determine their growth phenotype. Plated on CSM-Trp 3% Glycerol for the control and 3% or 5% EtOH added to the media to check for a difference in growth. All the strains are *MATa*.

Performing the screen as described in the method section, three suppressors were found. All of them in the *dld2Δdld3Δ* background. Candidates are shown in Figure 10 below.

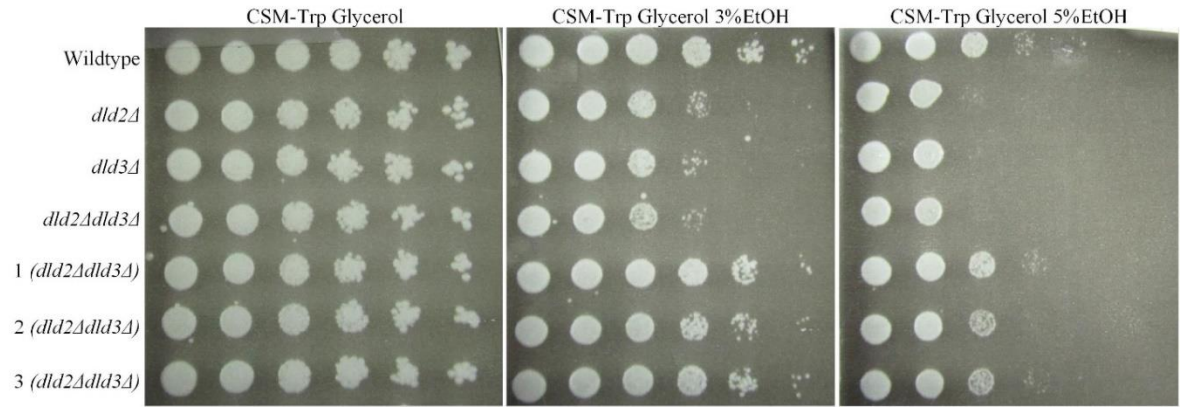

***Figure 10:* Suppressors found.** Fivefold serial dilutions of yeast were spotted on plates to determine their growth phenotype. Number 1-3 with a *dld2Δdld3Δ* background are the 3 candidates found in the suppressor screen. All strains are *MATa*.

3.2 One mutated gene in each candidate found in the screen is responsible for suppression phenotype

After having found three candidates, which suppress the growth phenotype of ethanol sensitivity, the next step was to determine whether a single gene is responsible for that phenotype. Therefore, the suppressor candidates were mated back to the original mutant (*MATα dld2Δdld3Δ*) and dissected. Two tetrads of each candidate were tested on a drop assay. Every drop assay showed a segregation pattern of 2:2. One typical example is displayed in Figure 11.

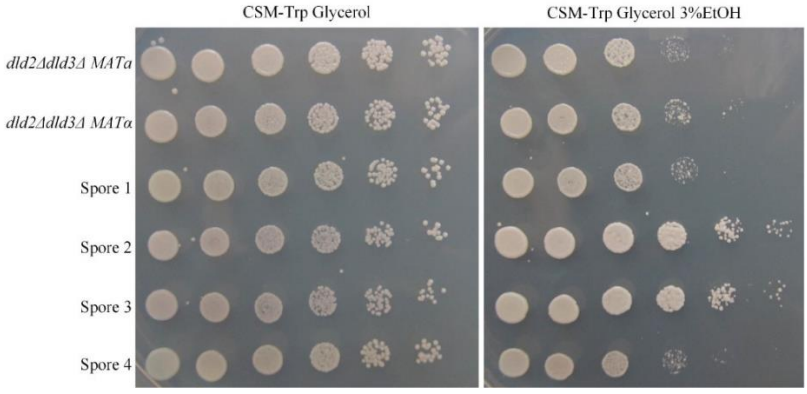

***Figure 11:* One mutated gene responsible for growth phenotype**. Fivefold serial dilutions of yeast were spotted on plates to determine their growth phenotype. Suppressor candidate with a *dld2Δdld3Δ* background was mated to *MATα dld2Δdld3Δ* and dissected. The first two rows show the controls *MATa* and *MATα* respectively. Row 3-6 display the dissected spores.

A complementation test is the next logical step in a suppressor screen to determine whether the mutations in the suppressor candidates are in the same complementation group. The candidates found were mated to each other (1x2, 1x3, 2x3) and diploids were spotted on plates containing EtOH of different

concentrations. Figure 12 shows that the growth phenotype of the suppressors match the *dld2Δdld3Δ* diploid and not wildtype.

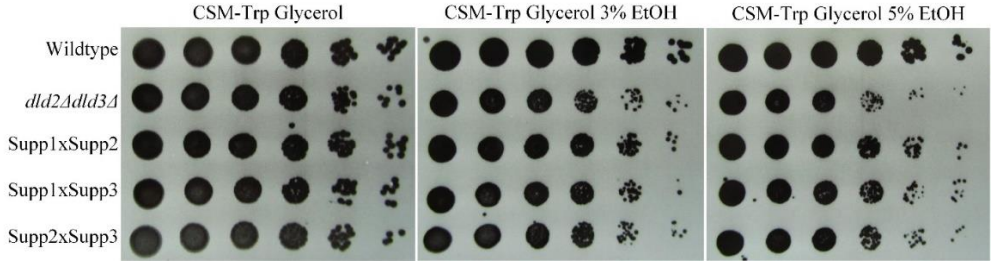

Figure 12: **Complementation assay.** Fivefold serial dilutions of yeast were spotted on plates to determine their growth phenotype. All strains displayed are diploids. Row 1 and 2 are the controls Wildtype and *dld2Δdld3Δ*, respectively. Row number 3 shows the diploid consisting of suppressor candidate no 1 and no 2. The diploid in row 4 displays candidate no 1 and 3 and in the last row no 2 was mated to no 3.

3.3 Test for dominance

To find out whether the mutation of the suppressor candidates are of dominant or recessive nature, a test for dominance was carried out. The three haploid suppressor candidates and the original mutant were mated to *MATα dld2Δdld3Δ* and diploids were plated. Different growth phenotypes are displayed in Figure 13. All suppressor candidates show less sensitivity to EtOH compared to the original mutant *dld2Δdld3Δ*.

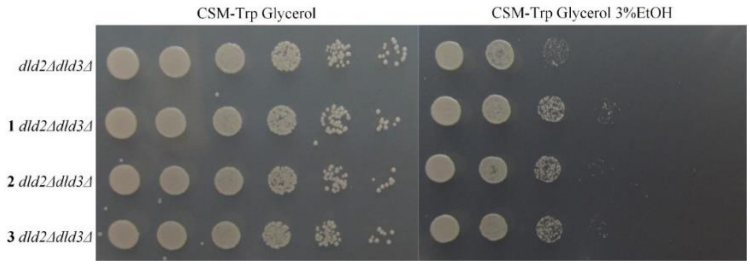

Figure 13: **Test for dominance.** Fivefold serial dilutions of yeast were spotted on plates indicated to determine their growth phenotype. Row 1 shows the original mutant *dld2Δdld3Δ*. Number 1-3 with a *dld2Δdld3Δ* background are the 3 candidates found in the suppressor screen. All the strains are diploids mated to *MATα dld2Δdld3Δ*.

3.4 *Set2Δ* phenocopies suppression phenotype but sequencing shows no mutation in SET2

Set2 is the only methyltransferase for H3K36 methylation. The chromodomain of Eaf3 recruits Rpd3S to nucleosomes methylated by Set2 on H3K36me3, leading to deacetylation of transcribed regions (Lee JS, Shilatifard A., 2007). To make sure the growth phenotype seen in the suppressors is not just due to a mutation in SET2, SET2 was deleted and a drop assay was performed on media indicated in Figure 14. In the drop assay one can see that an additional deletion in a *dld2Δ* mutant leads to the exact same growth phenotype. But results from sequencing do not show a mutation in the SET2 gene in any of the suppressor candidates.

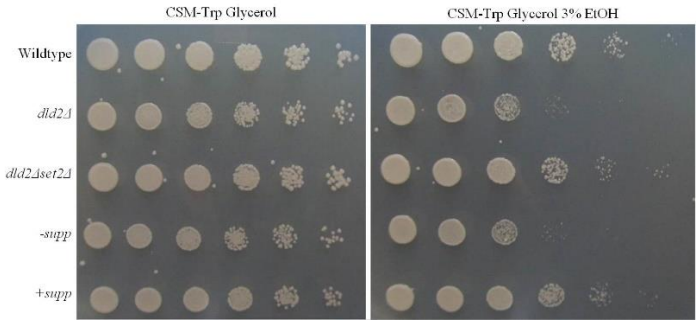

Figure 14: **Deletion of SET2 in a *dld2Δ* background phenocopies suppression phenotype.** Fivefold serial dilutions were spotted on indicated media to determine growth phenotype. All strains are *MATa*. The first two rows display the control wildtype and *dld2Δ*. The third row shows the *dld2Δset2Δ* double mutant. Row number 3 and 4 show the suppressor without the point mutation and suppressor with the point mutation respectively.

3.5 Sequencing results suggest that point mutations in RPD3, UME1 and SIN3 are responsible for the suppression phenotype and this was confirmed by knockouts

After having analyzed the results from the UC Berkeley DNA Sequencing Facility as described above in 2.3 , the first pool showed a point mutation in the RPD3 gene. The second pool showed a mutation in the UME1 gene and the third pool of suppressors comprises a mutation in the SIN3 gene. Rpd3 is a histone deacetylase responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Ume1 and Sin3 are catalytic components of the Rpd3 histone deacetylase complex (Xiao-Fen Chen, Benjamin Kuryan et al., 2012). To confirm these results, knockouts were made starting with *rpd3Δ*. In Figure 15 it can be seen that *rpd3Δ* looks like wildtype and that the triple mutant *dld2Δdld3Δrpd3Δ* clearly shows the suppression phenotype when compared to the *dld2Δdld3Δ* double mutant.

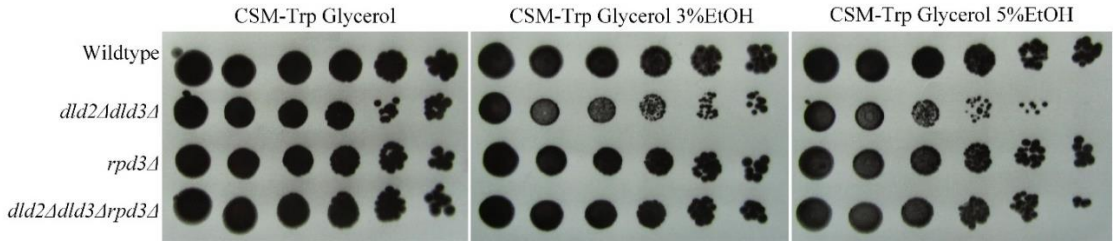

Figure 15: **RPD3 deletion in a *dld2Δdld3Δ* mutant leads to suppression phenotype.** Fivefold serial dilutions were spotted on indicated media to determine the growth phenotype. All strains are *MATa*. The first row shows wildtype and the second one displays the *dld2Δdld3Δ* double mutant. Row number 3 and 4 show the *rpd3Δ* single mutant and *dld2Δdld3Δrpd3Δ* triple mutant respectively.

3.6 Increase in acetylation levels on H4K12 shown in Western Blot

Rpd3 is a known deacetylase active in both core histones H3 and H4. H4K5-acetyl, H4K8-acetyl, and H4K12-acetyl are the known substrates on H4 for Rpd3 in yeast nucleosomes (Deborah M. Thurtle-Schmidt, Anne E. Dodson, and Jasper Rine, 2016). In this work only H4K12 acetylation levels were tested. In Figure 16 changes in acetylation are displayed in wildtype, *dld2Δdld3Δ*, *rpd3Δ*, *dld2Δdld3Δrpd3Δ*, and the suppressor candidate found in the screen. H4K12 is hyperacetylated not only in the *dld2Δdld3Δrpd3Δ* triple mutant and the suppressor candidate, but also in the *rpd3Δ* single mutant compared to wildtype. A difference in acetylation levels cannot be observed between wildtype and the *dld2Δdld3Δ* double mutant.

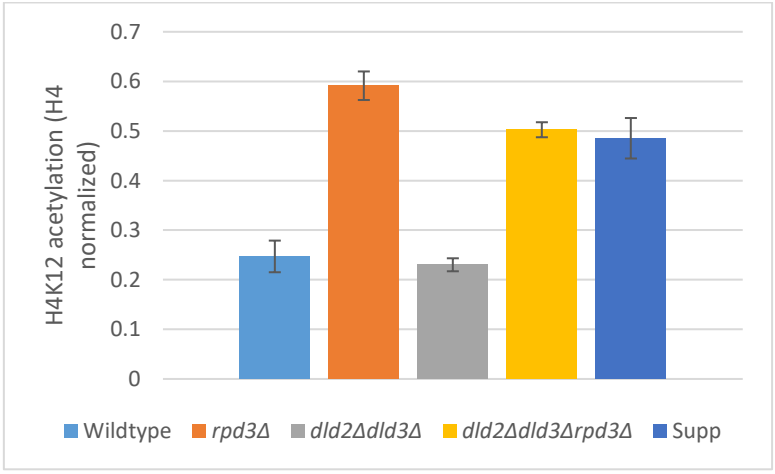

Figure 16: Acetylation levels of H4K12 in wildtype, *dld2Δdld3Δ*, *rpd3Δ*, *dld2Δdld3Δrpd3Δ*, Suppressor candidate found in the screen. P-value between wildtype and *rpd3Δ* is 0.0013, so significantly different. P-value between *rpd3Δ* and *dld2Δdld3Δ* is also 0.0013. Values are normalized to H4. Error bars are standard error of the mean.

3.7 Lower switching rate in *rpd3Δ* strains compared to wildtype measured in Flow Cytometry

Previous research has shown that RPD3 deletion results in increased genomic silencing (De Rubertis F, Kadosh D, Henchoz S, Pauli D, Reuter G, Struhl K, Spierer P, 1996) and therefore counteracts genomic silencing although histone acetylation (which should be increased when Rpd3 function is lost) is generally correlated with transcriptional activity. To check whether the additional mutation of *dld2Δdld3Δ* affects this phenotype, switching rates in Flow Cytometry were determined. In Figure 17 switching rates are displayed. It must be noted that wildtype and *dld2Δdld3Δ* double mutant are statistically different. Furthermore, *dld2Δdld3Δrpd3Δ* and the suppressor (with a point mutation in RPD3) found in the screen, show a significantly lower switching rate than *dld2Δdld3Δ* double and *rpd3Δ* single mutant.

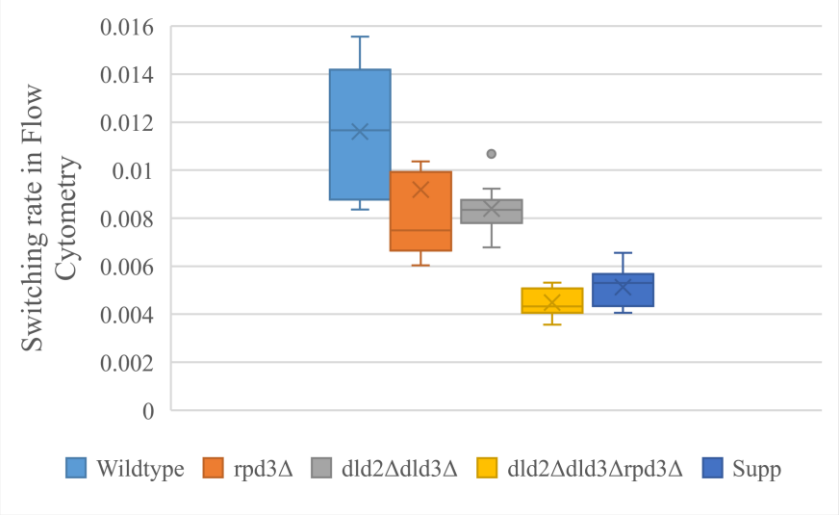

Figure 17: **Flow Cytometry** Error bars are standard error of the mean. P-value between *dld2Δdld3Δ* and *dld2Δdld3Δrpd3Δ* is less than 0.0001 and therefore the result is extremely statistically significant. Wildtype and *dld2Δdld3Δ* are also significantly different with a p-value of 0.0039. Unpaired two-sided (Student's) t tests were used to determine whether differences in frequency of silencing loss were statistically significant.

4 Discussion and Outlook

The oncometabolite D-2-HG accumulates in various types of cancers comprising IDH1 or IDH2 mutations. In this study a suppressor screen was performed to find out more about D-2-HG and its role in the cell to being able to combat a variety of malignant tumors in the future.

Three suppressor candidates were found in this study. Experiments like the complementation assay, confirmed that mutations were not in the same complementation group meaning different mutations in every candidate were responsible for the suppression phenotype. Also, segregation patterns of 2:2 indicate that one gene in each suppressor was responsible for the growth phenotype. Sequencing results revealed point mutations in RPD3, UME1, and SIN3.

The test for dominance showed that the mutations might be dominant but there is also a chance that it is just happloinsufficiency creating this phenotype. To answer this question a plasmid containing the RPD3 gene could be given back to the suppressor candidate. If the growth phenotype disappeared then happloinsufficiency would be responsible for the differences in growth.

In the Western Blot H4K12 acetylation levels were elevated when RPD3 was mutated. No difference was detected between wildtype and *dld2Δdld3Δ* double mutant. This might be due to the fact that a different acetylation site is responsible for the phenotype discovered. Different acetyl marks Rpd3 acts on need to be looked at and a Chip-seq experiment would be the next step forward.

In the flow cytometry experiment the switching rate in the *dld2Δdld3Δrpd3Δ* triple mutant was significantly lower than in *rpd3Δ* or *dld2Δdld3Δ* mutant alone. This indicates that the phenotypes might be additives. A *dld2Δdld3Δ* mutant accumulates D-2-HG leading to H3K36 hypermethylation and more stable heterochromatin (Ryan Janke, Anthony T Iavarone, Jasper Rine, 2017). As already known, Rpd3 counteracts genomic silencing. Rpd3S subunit gets recruited to H3K36 via the Eaf chromodomain, leading to deacetylation (Lee JS, Shilatifard A., 2007). In a *dld2Δdld3Δrpd3Δ* triple mutant hypermethylation of H3K36 occurs but no deacetylation via Rpd3, leading to even more stable heterochromatin.

In the drop assays, however, mutations in RPD3, SIN3 or UME1 (catalytic components of the Rpd3 histone deacetylase complex) in a *dld2Δdld3Δ* double mutant restored growth back to wildtype. SIN3 and UME1 knockouts still need to be investigated but nevertheless this is an interesting observation, which raises the question whether HDACi might be an effective treatment in cancers comprising IDH mutations.

5 List of Figures

6 List of Tables

7 Bibliography

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