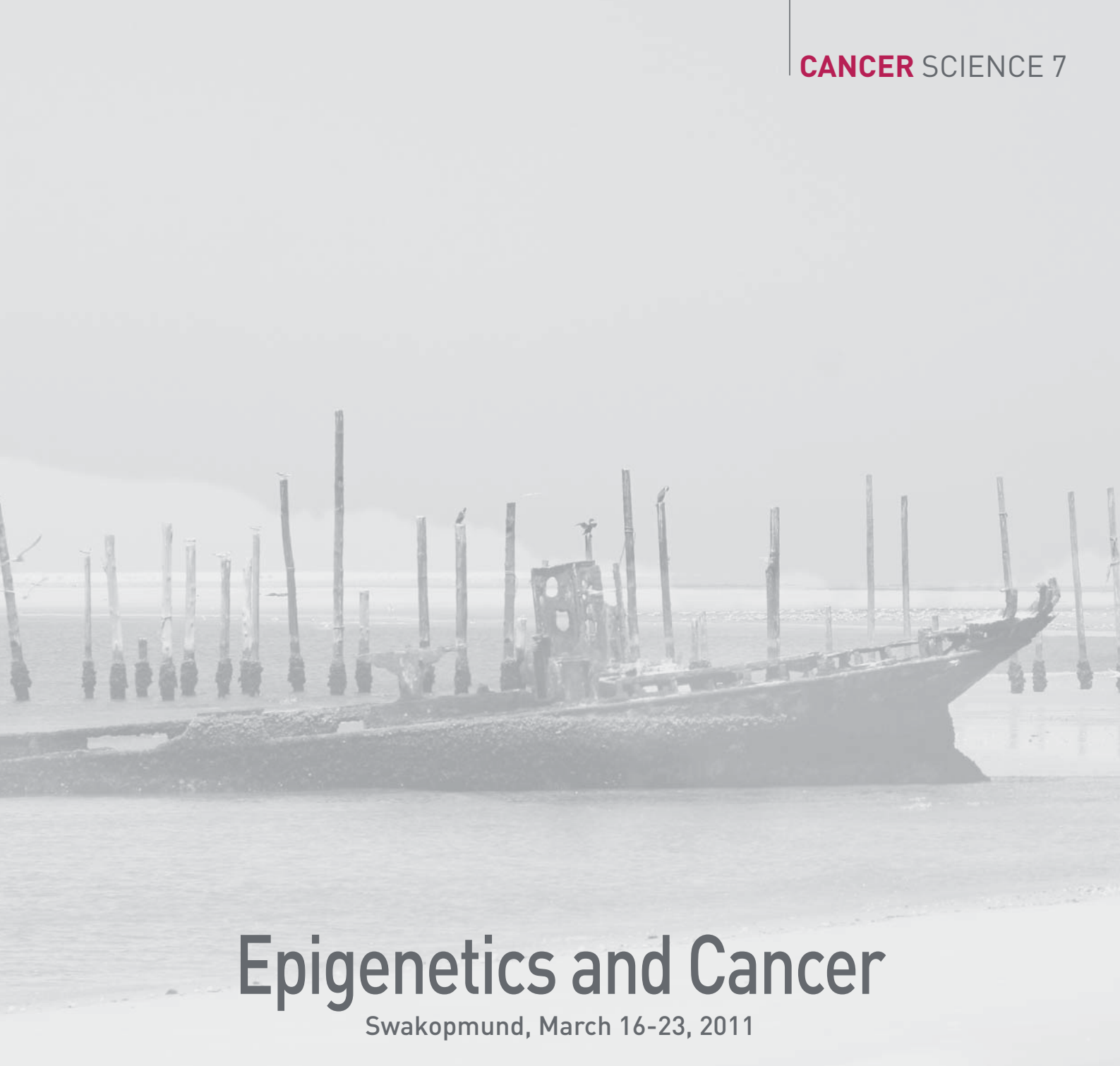
CANCER SCIENCE 7



Epigenetics and Cancer

Swakopmund, March 16-23, 2011

COLLOQUES MÉDECINE ET RECHERCHE

FONDATION IPSEN

SCIENTIFIC REPORT BY APOORVA MANDAVILLI

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CONTENTS

FOREWORD by INDER M. VERMA

The word 'epigenetics' refers to potentially heritable modifications in gene expression that do not involve a change in DNA sequence. The combinatorial pattern of DNA and histone modifications constitutes an epigenetic 'code' that shapes gene expression patterns by enabling or restricting the transcriptional potential of genomic domains. Another view is that epigenetics is a fancy way of saying 'gene expression.'

Because epigenetic research is especially active in the field of cancer science and has obvious potential applications in cancer management, we chose the to hold the 7th Ipsen Cancer Series meeting on Epigenetics and Cancer in Swakopmund, Namibia, an unusual country with breathtaking scenery and limitless lengths of sand dunes.

The nucleosome is the fundamental building block of chromatin and is the integrator of epigenetic information. One key question is how modifications of histones, the major protein components of chromatin, mark functional regions of the genome. Using pericentromeric heterochromatin as a model system, **Geneviève Almouzni** presented data on higher-order chromatin assembly and maintenance of chromatin structure during DNA replication.

DNA methylation patterns are known to be replicated in a semi-conservative fashion during cell division, but whether and how histone modifications are inherited is unclear. **Xiaodong Cheng** presented a model that could partly explain how DNA methylation patterns and histone modifications are maintained simultaneously.

Peter A. Jones described a novel mechanism for epigenetic control, in which the level of DNA modifying enzymes, such as the DNA methyl transferase DNMT3, may be regulated by the levels of their products, thus preventing spurious DNA methylation from taking place during the process of cell division.

Transcription is an epigenetic balance, reflecting the combined weight of transcriptional activation and repression machinery. Active and repressive cistromes are nucleosome-concentrated and dynamic, and so the cistrome changes continuously in monitoring the environment of the cell. **Ron Evans** discussed the importance of BCL-6 in maintaining this balance.

Abnormal DNA methylation provides genes with tighter transcriptional repression, and this could help evolve a stem-like state for key sub-populations that initiate and perpetuate tumors. **Stephen B. Baylin** reported that chronic exposure of cells during tumorigenesis to the formation of a complex that contains DNA methyltranferases, SIRT1 and polycomb-group proteins may be a key step in rendering genes vulnerable to abnormal promoter DNA methylation in cancer cells. He presented encouraging data that showed how drugs like 5-azacytidine, known to cause DNA demethylation at low concentrations, can stop tumor growth.

Repressive polycomb-group protein complexes are involved in the dynamic maintenance of proper gene expression patterns during development, acting at the level of chromatin structure. **Maarten van Lohuizen** discussed the maintenance of polycomb repression in normal and cancer stem cells.

Joseph Ecker reported the first whole-genome profiles of DNA methylation at single-base resolution in five human induced pluripotent stem (iPS) cell lines, along with methylomes of embryonic stem (ES) cells, somatic cells and differentiated iPS and ES cells.

Genetic screens have revealed that ES cells are highly sensitive to reduced levels of mediator, cohesin and condensin. These proteins co-occupy different promoters in different cells, thus generating cell-type-specific DNA loops linked to the gene expression program of each cell. **Richard Young** provided mechanistic insights into a variety of diseases caused by mutations in these proteins.

In normal cells, the bulk of the genome is methylated, but CpG island-associated promoters of active or bivalent genes remain unmethylated. Among the best defined of cancer-related abnormalities is the silencing of hundreds of genes, often associated with DNA hypermethylation of promoter CpG islands.

In both mouse and human genomes, CpG density correlates positively with the degree of H3K4 trimethylation, supporting the hypothesis that these two are mechanistically interdependent. **Adrian Bird** proposed that the ability of CpG density to influence chromatin modification directly via CFP1 suggests that this is an important function common to CpG islands.

Susan Clark presented data showing that epigenetic reprogramming in cancer is not just limited to single genes, but can occur across large domains, resulting in the repression or activation of domains of cancer genes.

Strong concerted epigenetic change is also seen in CpG Island Methylator Phenotypes (CIMP) that have been found in several types of cancer. There are striking associations between specific genetic mutations and CIMP. **Peter Laird** presented two interesting examples of synergy between cancer genetics and epigenetics.

There is much evidence that tumor-specific increase or loss of genomic DNA methylation may play a prominent role in both genetic and epigenetic changes related to cancer. **Rudolf Jaenisch** proposed that DNMT3A, counter to expectation, acts like a tumor suppressor gene in lung carcinogenesis.

Several researchers presented evidence of a role for well-known cancer genes in epigenetic regulation.

For example, the proteins encoded by the MYC gene family function in multiple normal physiological roles and are profoundly involved in the etiology of a wide range of cancers. **Robert Eisenman** presented research on the role of MYC in large-scale changes in chromatin.

Kristian Helin proposed that the major function of TET1 is to regulate the fidelity of DNA methylation. **Craig Thompson** presented IDH1/2- and TET2-mutant leukemias as a biologically distinct disease subtype, linking cancer metabolism with epigenetic control of gene expression. And I proposed that the role of BRCA1 in maintaining global heterochromatin integrity accounts for a major part of its tumor suppressor function.

Chromosomal translocations that fuse mixed lineage leukemia 1 (MLL1) to any one of a large number of translocation partners are indicative of a poor clinical outcome in acute leukemias. **Ali Shilatifard** discussed the molecular role of MLL-chimeras in leukemic pathogenesis.

Epigenetic abnormalities in cancer also present great potential for therapy. Drugs that target epigenetic modifiers have proved effective in subsets of patients with cancer. **Jean-Pierre Issa** reported that an unbiased screen of FDA-approved drugs reveals that up to 5% of all drugs in use in the clinic may have epigenetic effects.

As in the past, the wonderful science was interspersed with short excursions, this time revealing the captivating beauty of the Namib desert. The organization of this meeting in this distant land was no exception to the high quality the participants have come to expect from Jacqueline Mervaillie and her tireless staff. We are again indebted to Apoorva Mandavilli for pulling together the multiple talks, covering a wide swath of cancer biology, in a very readable monograph.

Inder Verma

PART I: Chromatin assembly and maintenance

Epigenetic challenges in centromere inheritance during the cell cycle

A report on a lecture by
Geneviève Almouzni
Institut Curie, Paris, France

Chromatin organization in the nucleus is known to provide a large repertoire of information. One key question is how histones, the major protein components of chromatin, mark functional regions of the genome with variants and modifications. Understanding the mechanisms of chromatin assembly, from the nucleosome to higher-order structures in the nucleus, is relevant for all major DNA transactions during replication, repair, recombination and transcription. Potential errors in these mechanisms can lead to dysregulation of genome function, with implications for various diseases including cancer. There are several key H3-H4 chaperones involved in nucleosome assembly including CAF-1, HIRA, ASF1a and ASF1b, and a new chaperone called HJURP, which is involved in the deposition of the centromeric variant of histone H3. CAF-1 is a marker for cell proliferation in breast cancer, and ASF1b has a strong prognostic value for metastasis risk. Beyond nucleosomal organization, pericentromeric heterochromatin serves as a good model for studying higher-order chromatin assembly and maintenance of chromatin structure during DNA replication. During development, and particularly after fertilization, this domain undergoes major rearrangements. In the model for this domain, SUMOylation of HP1, through a specific association with a major forward transcript, seeds further HP1 localization. Using pericentric heterochromatin as a model system, ***Geneviève Almouzni*** *explored the general principles for the organization of nuclear domains.*

DNA in eukaryotic cells is organized into chromatin, and the basic unit of chromatin is the nucleosome. Each nucleosome consists of 147 base pairs of DNA, wrapped around a histone core comprised of a H3-H4 tetramer flanked by two H2A-H2B dimers.

Nucleosomal arrays are organized into distinct higher-order structures and domains. The specific structure depends on a number of factors, which make the nucleosome rich in variation. There are several different histone variants, each of which can be modified — by methylation, acetylation, phosphorylation or ubiquitination — generating a diverse repertoire at that level.

It is important to understand how these different modules form and contribute to the particular landscape of a cell. There are also other important components — such as chromatin-binding proteins and RNA — that contribute to the variation.

Chromatin assembly factors are involved in the deposition of histones, which affects higher-order chromatin assembly. The pericentromeric heterochromatin domain serves as

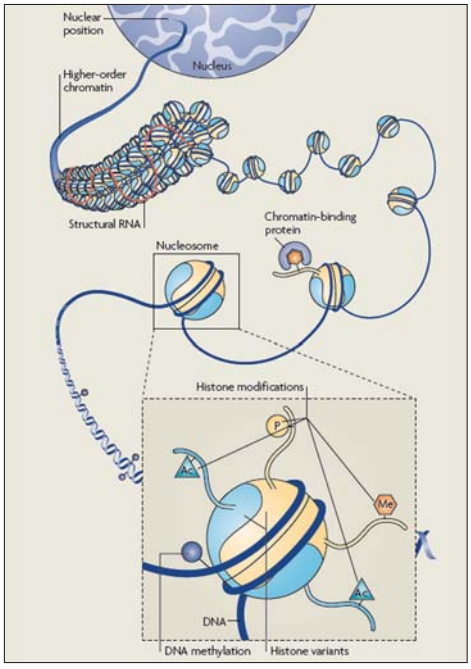

Figure 1
Chromatin domains and their dynamic assembly.

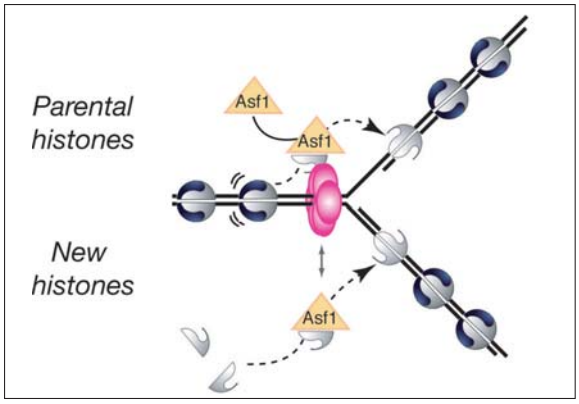

Figure 2
ASF1 promotes unwinding of the parental chromatin template.

a good model for studying higher-order chromatin assembly and maintenance of chromatin structure during DNA replication.

At the nucleosomal level, there are three classes of factors to consider: remodeling complexes, histone-modifying enzymes and the lesser-known class of chaperones. Together these contribute to chromatin assembly, modification, disassembly and movement. Histone chaperones can be defined as factors that associate with histones and stimulate a reaction involving histone transfer, without being part of the final product. Histones are small, charged basic proteins that can potentially stick to anything with the opposite charge, so a chaperone can also be seen as a factor that keep histones under control.

These chaperones have various functions, including transfer of histones from one place to another. Histone chaperones are often associated with other factors, such as histone-modifying enzymes or remodeling factors, to help load the histone on or off DNA.

They are also involved in storage of histones in histone pools during development, and in ensuring their availability and positioning at key steps. Chaperones can help buffer transient histone overload by tightly regulating their production. They can also help select a particular histone variant for a metabolic pathway and regulate histones in different cell types, during the cell cycle, and in any other situations in which histone dynamics are important.

Multiple variants:

There are several histone H3 classes in mammals. One set of replicative histones, H3.1 and H3.2, peak in S phase, providing the load needed during genome replication. A set of replacement variants, H3.3, differ by only 4 to 5 amino acids from the others, but these are expressed in all phases, including G0.

H3.3 variants accumulate in regions that are actively transcribed, but they also have roles beyond transcription. For example, H3.3 is important at the time of fertilization, when sperm chromatin needs to be rearranged.

The most extreme H3 variant, CENP-A, specifically marks the region corresponding to the centromere, indicating specification of a region that serves to build the kinetochore in mitosis. It also illustrates how the use of a particular histone variant can contribute to defining a functional chromosomal region.

In contrast with mammals, *Saacharomyces cerevisiae* has a single histone H3, which is most similar to the mammalian H3.3.

Purification of complexes associated with the variants can help define selectivity for a particular variant. Using cell lines in which the different variants can be expressed, the different forms of soluble complexes or those associated with chromatin can be distinguished.

One particular set of chaperones is key for interacting with the pathway associated with H3.1: CAF1, or chromatin assembly factor 1, is required for histone deposition coupled to DNA synthesis. CAF1 acts during replication or at sites of nucleotide excision repair, for example.

Histone H3.3 is similarly associated with a factor called HIRA. There are also other components, such as ASF1 or anti-silencing function 1. ASF1 was first identified in yeast, and has two isoforms in mammals, one of which specializes in proliferation. With these and other factors, there is an assembly line of regulated histone deposition to ensure a controlled flow.

In the context of chromatin replication, as the replication fork progresses, one nucleosome ahead of the fork needs to be displaced and can then be recycled. At the same time, a nucleosome needs to be assembled *de novo*. The recycling and *de novo* assembly must be coordinated with forward progression.

It is important to understand which factors recognize nucleosomes, handle histones and modify them at each step. For example, parental histones may carry histone-based information, and new histones may also have specific modifications that need to be characterized. How much is maintained, and how much can be changed?

CAF-1 is involved in *de novo* assembly by incorporating H3 and H4. A significant proportion of ASF1 is tightly associated with chromatin, and could potentially play a role in histone transfer[1].

Biochemical purification of different fractions shows that ASF1 is associated with the MCM complex in the chromatin fraction, particularly during the S phase. In this model, the helicase moves forward interacting with histones, and a bridge of these histones with ASF1 then transfers them onto the double strand.

ASF1-MCM allows DNA unwinding ahead of the fork to be coupled to histone supply and usage behind the fork. Recycling of parental histones must be integrated with the *de novo* deposition of new histones and the progression of fork. In keeping with this model, depletion of ASF1 induces defects in progression.

The ASF1 isoform ASF1b turns out to be specialized for proliferation. In breast cancer samples from the Institut Curie, ASF1b, but not ASF1a, is clearly connected to proliferative status and is predictive of the outcome[2].

Key chaperones:

The centromere is key to segregation of chromosomes. Cancer is associated with many segregation defects, so it is important to understand this function.

Centromeres could be considered epigenetically defined loci because, except in budding yeast, they are not defined by a specific sequence. The current view is that centromeres are dictated by their organization, with the H3 variant CENP-A as a key component.

CENP-A is found in the inner part of the centromere, and is always embedded in the heterochromatin environment where there is an accumulation of HP1 protein. Using the same approach as for H3.1 and H3.3, purified complexes have identified HJURP, or the Holliday Junction Recognizing Protein, as a key CENP-A chaperone. When HJURP is depleted, CENP-A cannot be loaded at centromeric regions.

There are two aspects to pericentromeric organization: the role of RNA and

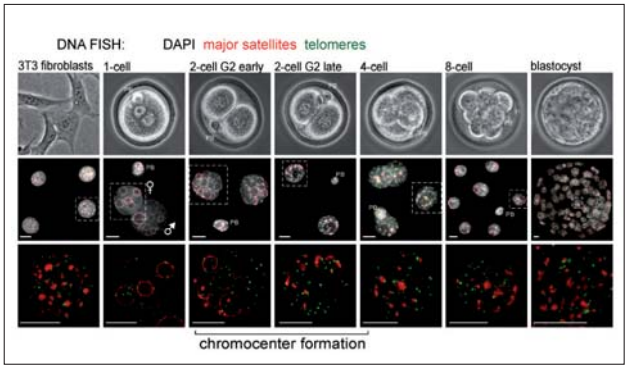

Figure 3
Pericentric domains undergo an important structural reorganization during the first cleavage stages.

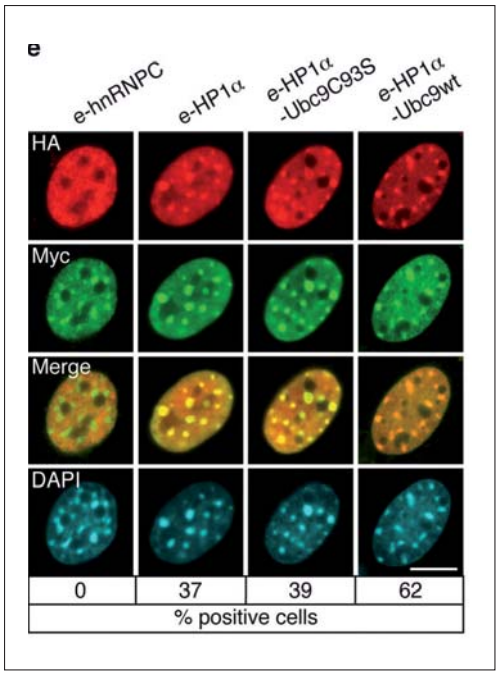

Figure 4
Enhancing e-HP1a SUMOylation to assess targeting and accumulation at pericentric heterochromatin.

heterochromatin during early development, and the *de novo* targeting of HP1-α[3].

There is much attention given to histone dynamics during replication, but the cycle of CENP-A is more interesting at other times. With the help of HJURP, CENP-A is deposited only at the end of mitosis, for example.

Pericentric transcripts are required for early heterochromatin. In mammals, constitutive heterochromatin corresponds to regions that are density-stained throughout the cell cycle. Both maternal and paternal chromosomes behave in the same way in most cell types.

Non-coding RNA may have a role in establishing and organizing these higher-order chromatin domains. One model of choice to study is mouse pericentric heterochromatin, which is comprised predominantly of major satellite repeats, AT-rich sequences arranged in tandem repeats, and which plays a role as part of the centromere in chromatin cohesion. In interphase nuclei, pericentric domains from different chromosomes cluster into chromocenters, as revealed by DNA FISH.

Pericentric heterochromatin has a characteristic signature of a series of marks identified over the years, including H3K9 di- or tri-methylation, H4K20 tri-methylation, accumulation of H2A.Z, DNA methylation and binding of HP1 proteins.

Initially thought to be stable and transcriptionally inert, in *S. pombe* heterochromatin organization has been linked to transcription of this pericentric repeat. This transcription, dynamically regulated during the cell cycle, is connected with small RNA-mediated silencing pathways in the maintenance of heterochromatin.

In mouse, pericentric satellites sequences are known to be transcribed and cell-cycle regulated, but their precise role in heterochromatin organization is unclear. In any event, sophisticated mechanisms clearly contribute to heterochromatin maintenance when it is challenged during replication, involving mutual interplay between the machineries setting DNA and histone marks.

Pericentric domains:

In the case of specialized gametes, distinct organization and nucleoprotein complexes come together to induce major reprogramming and organizing of heterochromatin at fertilization. Mouse pre-implantation development provides a remarkable model to study how this heterochromatin is first established, particularly at the paternal pericentromere domain, which undergoes major changes.

Pericentric domains undergo important structural changes during the first cleavage stages. They form rings at the one-cell stage and, at the two-cell stage, they organize and form chromocenters.

There is an important burst of transcription of pericentric satellites during a specific developmental time window, but not at other times of the cell's life, and coincides with chromocenter formation. This burst is

critical, controlled in space and time, and is strand- and parent-specific. Interfering with this pericentromeric transcript precludes chromocenter formation and blocks development.

Aberrant over-expression of satellite repeats may be a marker in pancreatic and other epithelial cancers, according to a new study. New methods make it possible to analyze these repeat sequences.

The HP1 protein is key in the organization of nuclear architecture. HP1-α is SUMOylated, which is important for *de novo* targeting to pericentric heterochromatin[4].

HP1-α recognizes H3K9, and because HP1 recognizes more HP1, it leads to its accumulation at pericentric heterochromatin. But surprisingly, HP1-α has not been not found among the proteins interacting with centromeric RNA.

SUMO modification is involved in the maintenance of heterochromatin in fission yeast. HP1 is SUMOylated *in vivo* and defective SUMOylation of HP1 results in a significant reduction in heterochromatin stability. There is also a SUMO-like protease in the HP1-α complex isolated from mouse cells.

Using N-ethylmaleimide, a SUMO isopeptidase inhibitor, shows that a major satellite — but not the minor satellite — in the forward orientation retrieves SUMO-1-modified HP1-α. Shifted by 11 kilodaltons, it represents a small fraction of the HP1 protein that associates with the RNA.

Based on mass spectrometry analysis, the delineating domains in HP1-α that are subjected to SUMO modifications are in the hinge domain, which represents a major target.

UBC9 fusion-directed SUMOylation can help assess the importance of HP1-α SUMOylation by increasing the amount of SUMO-HP1-α and bypassing the requirement for the E3 ligase.

In a maintenance assay in which NIH3T3 cells are transfected, there's no advantage to having UBC9 promote SUMOylation. By contrast, in SUV39 double null cells — which lack a key H3K9me3 methyltransferase — there is no HP1 localized at pericentric heterochromatin.

In those cells, co-transfecting HP1-UBC9 fusion creates spots of *de novo* HP1 targeting at sites of pericentric heterochromatin. Grafting the UBC9 catalytic dead mutant doesn't create this effect, suggesting that only UBC9, but not the catalytic mutant, promotes *de novo* targeting. The hinge domain and SUMOylation are both critical for HP1-α *de novo* targeting at pericentric heterochromatin.

In this model, SUMOylated HP1-α interacts with RNA, helping to target it to the right sites, and is followed by a maintenance step. There could be a particular major RNA transcript that helps build up a domain in the nucleus.

In summary, histone chaperones act as escorts because many of them interact with other factors that feed into metabolic pathways important for the dynamics of nucleosomes during the cell cycle. They may potentially be useful as markers or perhaps even targets for cancer management.

Non-coding RNA is important in nuclear organization. HP1 modification and RNA interaction set up *de novo* targeting, and other mechanisms then take over for maintenance. These processes also contribute to the formation of a domain of accumulation.

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Coordinated chromatin control: structural and functional linkage of DNA and histone methylation

A report on a lecture by
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The combinatorial pattern of DNA and histone modifications constitutes an epigenetic 'code' that shapes gene expression patterns by enabling or restricting the transcriptional potential of genomic domains. DNA methylation and histone methylation may be coordinately maintained through mitotic cell division, allowing for the transmission of parental DNA and histone methylation patterns to be copied to newly replicated chromatin. It is well accepted that DNA methylation patterns are replicated in a semi-conservative fashion during cell division, but one fundamental unresolved question is whether and how histone modifications are similarly 'inherited'. Higher organisms have evolved coordinated mechanisms of deposition and transmission of repressive chromatin marks to both DNA and histones. The DNA methyltransferase-like protein DNMT3L binds to H3K4me0 and recruits DNMT3A to regions of chromatin where H3K4 is unmethylated. ***Xiaodong Cheng*** *presented a model that could partly explain how DNA methylation patterns and histone modifications are maintained simultaneously.*

The analyses of genome-scale DNA methylation profiles suggest that DNA methylation patterns highly correlate with histone methylation patterns, even down to the resolution of single nucleosomes[1]. In particular, DNA methylation is strongly associated with the absence of histone H3 lysine 4 methylation (H3K4me0) and the presence of histone H3 lysine 9 methylation (H3K9m).

DNA methylation might be related to gene silencing, whereas unmodified CpG islands relate to activation. The modification status, either of DNA or of histones, could also be a component of signaling.

The goal is to understand the mechanisms that establish the signals and maintain the correlations. For example, how anti-correlation of DNA methylation and histone H3K4 modification are established, how the reciprocal methylation of H3K4 and H3K9 are set up and how the so-called binary switch, from H3K9 and H3S10, can be applied to non-histone proteins as well.

One member of the DNMT3 family, DNMT3L, on its own does not have enzymatic activity for DNA methylation. Still, DNMT3L knockout shows that it is important for establishing maternal imprint. DNMT3L has a ring structure with an approximately 100 Angstrom channel in the middle of those rings, the perfect size for a nucleosome to fit in.

Tagging DNMT3L pulls down active members DNMT3A and DNMT3B, as well as histones. The N-terminus of H3, particularly the unmodified lysine on the histone tail, is important for these interactions.

DNMT3L binds to the unmodified H3K4 peptide; the bonding is lost with methylation. Two acidic residues of DNMT3L are important for H3K4me0 interactions.

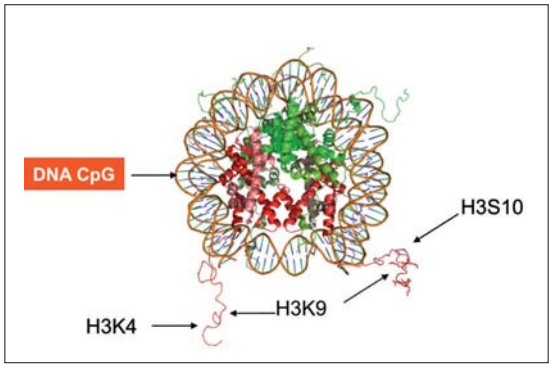

Figure 1
Regulation of epigenetic signaling.

In this model, the DNMT3L-DNMT3A complex has enzymatic activity on DNA. It recognizes H3K4 status and ensures that the methylation is on K4 unmodified nucleosomes.

Several pieces of data support this model. First, the LSD1 and LSD2 enzymes are required for demethylation of H3K4. When the H3K4 demethylase is knocked out, there is a change in K4 methylation, but also more profoundly a change in DNA methylation.

LSD1 deletion induces progressive loss of global DNA methylation and LSD2 is required to establish maternal genomic imprints. It's possible that LSD1 removes the methylation on the K4, DNMT3L follows on unmodified K4, then enhances the silencing signal by methylating DNA.

Another piece of data comes from experiments in which mouse DNMT3L-DNMT3A was expressed in *Saccharomyces cerevisiae* with no DNA methylation background. Expression of DNMT1 and DNMT3 individually in *S. cerevisiae* results in a low level of DNA methylation, but when DNMT3A and DNMT3L are combined, the level of DNA methylation increases. Further, when the complex responsible for H3K4 methylation is knocked out, DNA methylation is widely distributed through the entire genome.

Model complexes:

In this model, DNMT3A and DNMT3L form a tetramer complex. The DNMT3A molecules form a dimer with DNMT3L on the outside. DNMT3L binds H3 tails that connect to the histone core.

Like the complex of DNMT3A and DNMT3L, DNMT3A and 3B can also be expressed together and stay together. DNMT3B has many splice variants, some of which are mutated in the catalytic domain. DNMT3B also has the so-called ICF mutation. When DNMT3A is replaced with DNMT3B because the sequences are highly similar, those ICF mutations are located at the interface with DNMT3L. Those mutations could be important for the interactions of the heterodimer with DNMT3B or with other proteins.

The SET1 complex is essential for H3K4 methylation. CFP1 protein binds to unmodified DNA and is important for SET1 activity on H3K4[2]. Depletion of CFP1 results in a marked reduction in H3K4me3 genome-wide. This suggests that unmethylated CpGs recruit CFP1 and the associated methyltransferase SET1 creates new marks of H3K4me3 on the local chromatin.

The same is true for the H3K4 methyltransferase MLL1 complex. MLL1 doesn't have a partner like CFP1, but MLL1 and MLL2 have the same CXXC domain that binds to unmodified DNA, and is essential for K4 methylation. Disruption of the MLL1 gene in mice results in the loss of H3K4 methylation and *de novo* DNA methylation at some HOX gene promoters.

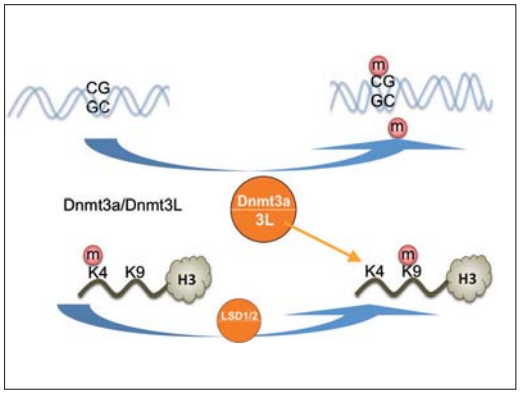

Figure 2
The DNMT3L-DNMT3A complex has enzymatic activity.

Another signal is H3K9 methylation, which has been established in different organisms, ranging from *Neurospora crassa* to higher-order organisms. H3K9 methylation is the marker for the follow-up DNA methylation. Surprisingly, the MBD family interacts with the H3K9 methyltransferases. SETDB1 has its own MBD domains.

H3K4 and H3K9 are only five residues apart, and they are maintained with opposite methylation status rather than being methylated or demethylated at the same time. LSD1 is the enzyme responsible for removing K4 methylation, but there is no evidence that K9 methylation influences demethylation of K4 by LSD1.

A protein called PHF8 has the Jumonji domain and functions as a demethylase. The Jumonji domain alone can demethylate K9, K27 and K36. The PHD domain can also bind to K4 in its di- and tri-methylated state.

When the full-length proteins are expressed with K9 methylation as a substrate, the enzyme is active but quite slow. However, if the substrate is K4 methylation together with K9 methylation, the enzyme reacts very fast.

PHF8 has not just K9 activity, but also binds to methylated K4, and interacts with the components of the MLL complex. This might be important because the MLL complex first settles the K4 methylation signal. It then interacts with PHF8, binds to K4 and then ensures that the K9 methylation is removed.

Sequence similarity:

In the same family, there is another protein called KIAA. At a sequence level, this protein is like PHF8, and has a Jumonji domain in the middle and a PHD domain at the end. When K4 and K9 are methylated together, KIAA has no activity. Rather, it has activity on K27. This is very unexpected because on a sequence level, it is so similar to the PHF8.

If the two structures are superimposed together, the domains in PHF8 are closed, whereas KIAA is in open conformation. And that open conformation allows K4 and K27 enough space to bind.

Although K4 can also bind to the PHD domain, however, K9 is too short to reach the active site. These results suggest how a simple enzyme can ensure that K4 and K27 are not methylated at the same time.

In a more complicated case, HOTAIR non-coding RNA binds to LSD1, removes K4 methylation, and then binds to another complex, PRC2, to make sure that K27 is methylated. In this particular case, there are two enzymes involved to demethylate K4 and add a methyl group to the K27[3].

LID2 in *S. pombe* — the mammalian enzymes have different names — is a component of CLR4 complex, which lays the methylation mark on K9 and then makes sure K4 methylation is removed. In this case, one enzyme reaction follows another, rather than a single enzyme turning the switch on and off.

SUV39H1/2 is also a K9 methyltransferase. H3S10 is known for phosphorylation, which affects K9 methylation. K9 methylation and K9 acetylation both affect H3S10 phosphorylation. This was thought to be a binary switch.

A lot of non-histone proteins have been found to also be subject to other modifications. Many lysines are subject to acetylation, and some are also subject to ubiquitination and methylation. So, for example, methylation of a lysine can prevent its ubiquitination and promote protein stability.

The estrogen receptor-alpha protein has a DNA-binding domain, a ligand binding domain and a Hinge. It's known for a variety of modifications. It has a lysine subject to acetylation and ubiquitination, and a serine that is subject to phosphorylation. In the Hinge region, K302 can also be modified by SET7 with methylation to promote the stability of ER.

Right next to this is K303, which is subject to acetylation. There's a breast cancer-associated mutation at that site, which changes the lysine to arginine. Surprisingly, K303 acetylation prevents methylation of K302, but K303-arginine mutation actually promotes K302 methylation[4].

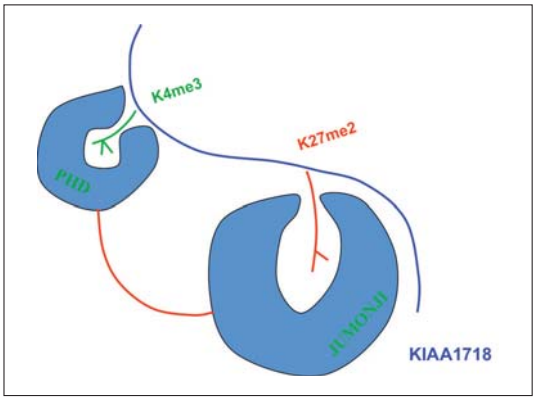

Figure 3
Model for KIAA1718 activity.

Methylation mark:

DNMT1 is itself subject to methylation at the N-terminal K142 by SET7. Right next to that is a known phosphorylation site for AKT1. K142 methylation in DNMT1 promotes degradation of the protein, rather than its stability, and the phosphorylation of S143 prevents methylation of K142. This switch between methylation and phosphorylation at an adjacent lysine and serine determines human DNMT1 stability.

In mammals, there are between 80 and 100 so-called SET domain proteins. Not all of those work on histones, in fact many don't. SETD6 methylates NF-κB at K310, right next to a known phosphorylation site for PKC. Phosphorylation at S311 prevents the methylation at K310.

NF-κB forms a dimer with p50 and p65. In this model, the methylation site is between the dimerization domain and the activation domain. In the dimer, only the p65 is methylated, but p50 is not.

G9a-like GLP, a H3K9 methyltransferase, has an ankyrin repeat that recognizes this particular modification of lysine in the p65 subunit of NF-κB. GLP is bound to mono-methylated K301, but S311 phosphorylation prevents the binding.

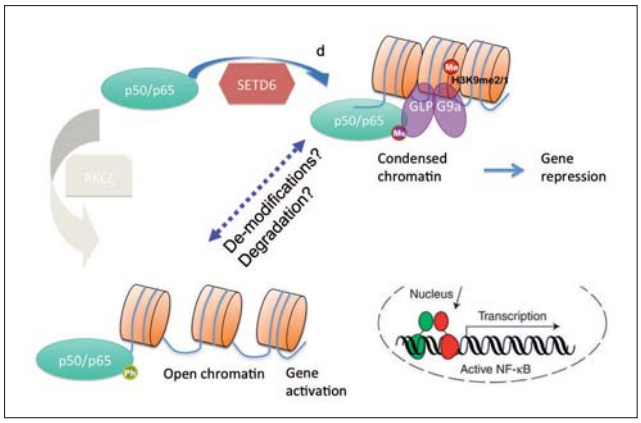

Figure 4
Model of interplay between SETD6, GLP and PKCζ in the regulation of NF-κB signaling.

In this model, NF-κB in the nucleus has to be either methylated or recognized by the silencing complex, resulting in condensed chromatin and gene repression, versus the phosphorylation on the residue right next to it. It can also bind to DNA and result in gene activation.

In summary, there is an anti-correlation of DNA methylation and histone H3K4 modification. Enzymes are able to reciprocally methylate H3K4 and H3K9. And there is a binary switch of adjacent methylation and phosphorylation.

There are a few examples of residues right next to each other that can regulate the fate of protein stability, or create a signaling pathway resulting in different consequences. These binary switches could occur more widely. Overall, there are reactions that occur together that enhance the message.

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How nucleosomes integrate and execute epigenetic information

A report on a lecture by
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Understanding how the epigenome functions requires a coordinated appreciation of the way that the chemical modifications interact with nucleosomal remodeling machines. The nucleosome is the fundamental building block of chromatin and is the integrator of epigenetic information. Nucleosomes have to be moved or evicted to expose DNA and make it available for transcription. The DNMT1 enzyme is only loosely associated with nucleosomes and can be disassociated from them by high salt concentrations. On the other hand, DNMT3A and 3B are firmly anchored to nucleosomes and cannot be disassociated from them even at 0.3M sodium chloride concentrations. Indeed the anchoring of DNMT3A to nucleosomes is essential to ensure the stability of the protein. In cells with markedly decreased levels of DNA methylation, the level of DNMT3A protein is strongly depressed even though there is evidence for increased transcription of the gene. These results suggest a novel mechanism for epigenetic control in which the level of chromatin enzymes such as the DNMT3s may be regulated by the levels of their products. ***Peter A. Jones*** *described how the enzyme levels do not allow for spurious DNA methylation to take place during the process of cell division.*

DNA methylation, nucleosome remodeling and histone variants and modifications collaborate together and communicate with each other to cause heritable gene silencing. But how they talk to each other is not well understood.

Unexpectedly, the cancer genome atlas project (TCGA) has shown that many of the enzymes orchestrating these processes are mutated in human cancer and nearly all of the new mutations discovered in human cancer relate to epigenetic processes. This is important because geneticists have been skeptical that epigenetics has an important role in carcinogenesis.

The nucleosome has an amazing biological structure: 146 base pairs of DNA wrapped around a histone octamer. Nucleosomes are the building blocks of chromatin. Whereas chromatin is an amorphous substance, nucleosomes are very distinct, discrete structures and their location is important in controlling gene expression.

Roger Kornberg's classic paper in 1987 showed very clearly that a nucleosome at the transcription start site (TSS) blocks the initiation of transcription, making it the ultimate transcriptional repressor.

It is critical for nucleosomes to be out of the way of TSS if transcription is to occur. This is achieved by chromatin remodelers and other factors, allowing transcription to start. The function of epigenetic 'marks' such as DNA methylation and histone modifications is to dictate the regions occupied by nucleosomes. Standard methods for finding nucleosomes are old and rely on nuclease digestion. A new method digitizes the location of nucleosomes.

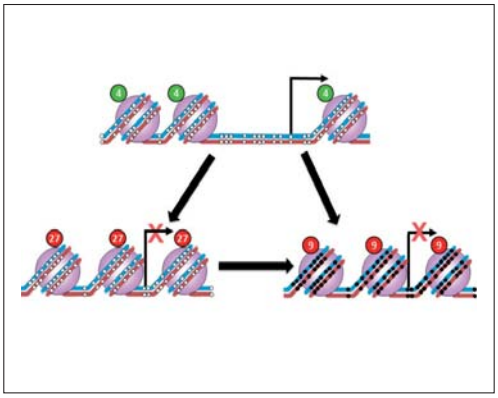

Figure 1
Three states of CpG islands.

In a stretch of DNA with nucleosomes on it, the endogenous methylation is all in the CpG sequence. However, it also has GpC sites, and an enzyme that methylates these sites (CviPII) is commercially available. If nuclei from a cell are treated with this enzyme for 15 minutes, all the accessible GpC sites are methylated.

However, this enzyme can't methylate where there are nucleosomes. Fingerprints of nucleosome occupancy can be generated on a single DNA molecule, and it is possible to get a readout of endogenous sequence CpG methylation and where there are patches accessible to the GpC enzyme.

Genome-wide analysis shows that CpG islands are largely unmethylated at TSS and there is a clear reciprocal accessibility to the CviPII enzyme. Unmethylated CpG islands are in nucleosome-depleted regions. Silencing by the polycomb repressive complex, which methylates H3K27, involves the presence of nucleosomes at the TSS.

There are two ways to silence genes, both of which involve nucleosomes. One silencing mechanism involves a nucleosome marked H3K27me3 at the TSS[1]. A more stable system of silencing employing DNA methylation also involves a nucleosome at the TSS and can be somatically inherited. During tumorigenesis, there is sometimes a so-called "epigenetic switch," in which one silencing mechanism is replaced by the other.

Stable patterns:

DNA methyltransferases are the orchestrators of DNA methylation. DNMT3A and DNMT3B are the *de novo* enzymes, and show equal preference for hemimethylated and unmethylated DNA. By contrast, DNMT1 is a "maintenance" enzyme and preferentially methylates hemimethylated DNA. DNMT3L stimulates DNMT3A/3B activity in embryonic stem (ES) cells[2].

However, no system can copy itself with 100% fidelity over a period of time. In fact, DNMT3A and 3B work in somatic cells to keep methylation patterns stable. This is consistent with the idea of a complex between DNMT3L and DNMT3A, which has as its substrate not DNA but nucleosomal DNA (see Cheng, page 15). This complex needs a nucleosome in order to methylate DNA.

In somatic cells, classic sucrose density gradient analysis of fractionated nucleosomes shows that DNMT1 is distributed with nucleosomes on the gradient at low-salt concentrations. But at high salt, it comes off, suggesting that it does not associate with nucleosomes very strongly. However, DNMT3A and 3B are very tightly associated with nucleosomes[3].

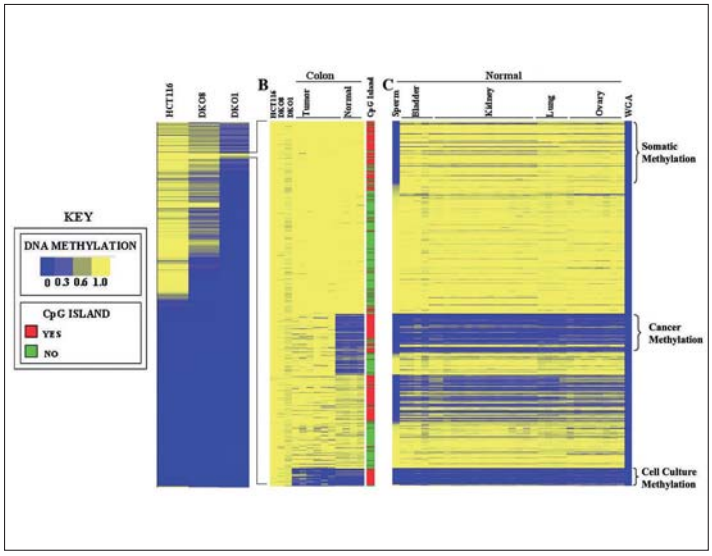

Figure 2
Retained DNA methylation profile in DKO1 cells.

This binding of DNMT3A/3B requires intact nucleosomes. If the nucleosomes are unwound by ethidium bromide, the enzymes dissociate and migrate differently on the sucrose gradient. DNMT3A/3B bind specifically to methylated CpG islands and repeats, but less avidly to unmethylated DNA.

Interestingly, other enzymes that modify histones — for example, G9a and SUV39h1, which put

methyl groups on H3K9 — also bind tightly to nucleosomes. This fits very well with a 2003 hypothesis that proposed that, in replicating nucleosomes, the enzymes that apply the marks are ready to ensure modification of new nucleosomal components because they are localized to specific regions. This explains in some way how epigenetic marks are replicated.

OCT4 is a well-known transcription factor that is an auto-regulatory factor, and there is incontrovertible data that nucleosome occupancy precedes methylation on OCT4 during differentiation. If ES cells are induced to differentiate using retinoic acid, *de novo* methylation goes from 0% up to 41%. But nucleosomes arrive first because the whole region becomes occupied by nucleosomes prior to *de novo* DNA methylation. The Nanog proximal promoter shows the same time course of inactivation.

Silent patches:

An auto-regulatory loop maintains DNA methylation levels within a cell. When DNMT3B is knocked out and DNMT1 is knocked down, the DNA loses 95% of its cytosine methylation and the cells grow very slowly. The cells also increase the expression of DNMT3A mRNA, but Western blots strangely show a much reduced level of protein.

DNMT3A has to be bound to a nucleosome in order to be stabilized. So the level of the product dictates the level of the protein. This is unusual: normally when the substrate levels increase, the enzyme levels also increase. But DNMT3A is all sequestered on nucleosomes, there is little free DNMT3A in the cells. Any DNMT3A that cannot bind to nucleosomes is proteolysed.

The cells lose 95% of their DNA methylation level and analysis of about 25,000 CpG sites with an Infinium array shows that this loss is not genome-wide. Patches of methylation on some essential genes, which can only be silenced by methylation, are maintained.

These genes may be ones to which cells become "addicted" in order to keep living. There is a somatic methylation pattern in which a set of genes is methylated in normal kidneys, lungs, ovaries and so on, except in sperm. Comparing these cells to cancer cells, some genes remain methylated even when the methyltransferase is removed.

The ADAM2 gene is expressed in sperm during germ cell development and silenced by DNA methylation in all somatic tissues and all cancer cells. It is also still silenced in the cells that have lost 95% of DNA methylation. Demethylation of this gene is possibly lethal. Similarly, genes such as ARMCX1 and MEOX2 remain methylated, illustrating the focality of the loss.

Interestingly, one gene that is methylated in all cell lines — but not in cancer tissues — is the cystic fibrosis gene CFTR, which regulates chloride transport.

Gene expression meta-analysis of the three identified clusters can show whether the methylated genes are actually down regulated or silenced. For example, a set of genes that includes ADAM2 and SYCP3 are expressed only in germ cells. Functional analysis shows that many of the genes are unmethylated in sperm and methylated in every other human tissue.

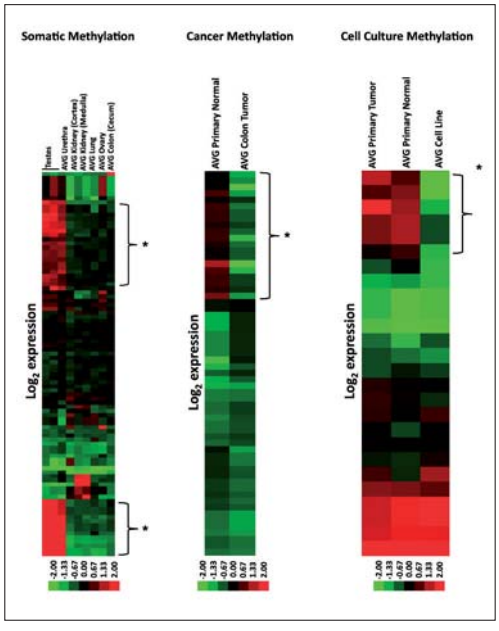

Figure 3
Gene expression meta-analysis of the three identified clusters.

Persistent methylation:

There are about 77 interesting genes that are persistently methylated in cancer, such as IRAK3, GPR34 and P2RY12. There are about 25 others that are only methylated in cell cultures, such as HIST1 and H2BH.

IRAK3 is down regulated in many different tumors, including colon, lung, prostate and skin. It is methylated in tumors, such as the human bladder cancer TCC, and can be detected in urine sediments. IRAK3 is an interesting gene because it inhibits IRAK1, which controls two key pathways — NF-κB and MAPK — that keep cells alive. Methylating IRAK3 would allow these pathways to keep going, perhaps explaining why cells become addicted to its methylation.

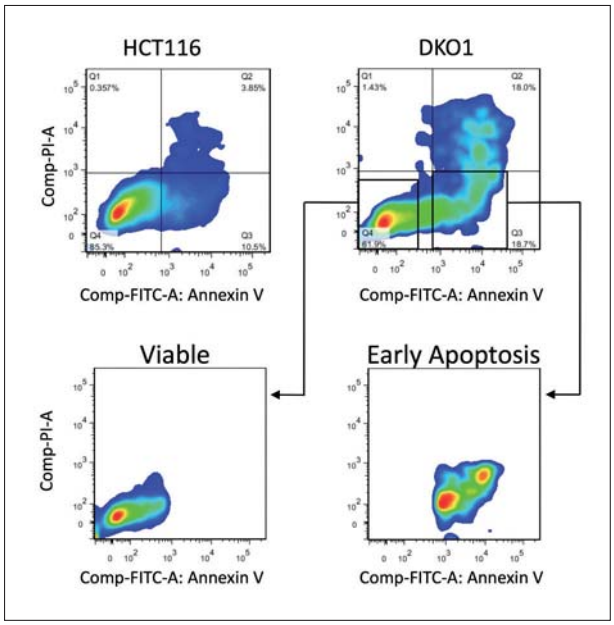

Figure 4
DKO1 cells are under constant selective pressure.

To see whether there is a requirement for residual DNA methylation in the DKO1 cells, their growth rate can be compared with that of wild type HCT116 cells. DKO1 cells, which have a low level of methylation, are constantly dying and they grow very slowly. Compared with sorted viable DKO1 cells, the ones in early apoptosis show a loss of DNA methylation.

Gene body methylation also plays a role in DKO1 cells. CpG islands in exons of genes are highly methylated in normal colonic epithelium and this becomes enhanced in cancers. Methylation of the promoters is rare; by contrast, gene body methylation is common. Cells become addicted to this methylation as well.

These are regions downstream of the TSS that are methylated in many tissues, and which maintain that methylation in the face of 95% decrease in DNA methylation. The fact that they're so regularly methylated even in normal tissues is interesting. However, on the Infinium array, there is a heavy bias toward promoters, so many of them may not yet be identified.

In summary, the nucleosome has a central role in gene expression. DNMT1 does the bulk of the heavy lifting in the cell in replicating DNA methylation. As DNA exits from the replication fork, together with UHRF, it does most of the work of copying the patterns of methylation.

Occasionally, there are sites that get missed. DNMT3A can also function after DNA has left the replication fork. The level of enzyme is exquisitely regulated by binding to the 5 methylcytosine generated marks. In ES cells, this is very different because the level of the protein is substantially increased. There, DNMT3A and DNMT3L together hug the nucleosome, which causes *de novo* DNA methylation[4].

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Cistromic competition controls macrophage-mediated inflammation

A report on a lecture by
Ron Evans
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In the macrophage, toll-like receptors are key sensors that trigger signaling cascades to activate inflammatory programs via the NF-κB gene network. However, the genomic network targeted by TLR/NF-κB activation and the molecular basis by which it is restrained to terminate activation and re-establish quiescence is poorly understood. Based on chromatin immunoprecipitation sequencing, the NF-κB cistrome is comprised of 31,070 cis-acting binding sites responsive to lipopolysaccharide-induced signaling. The transcriptional repressor B-cell lymphoma 6 (BCL-6) regulates nearly a third of the TLR4-regulated transcriptome, and 90% of the BCL-6 cistrome collapses following TLR4 activation. BCL-6-deficient macrophages are acutely hypersensitive to LPS. Based on comparative ChIP-seq analyses, BCL-6 and NF-κB cistromes intersect, within nucleosomal distance, at nearly half of BCL-6-binding sites in stimulated macrophages to promote opposing epigenetic modifications of the local chromatin. ***Ron Evans*** *presented a genomic strategy for controlling the innate immune response, in which repressive and inductive cistromes establish a dynamic balance between macrophage quiescence and activation via epigenetically marked cis-regulatory elements.*

The epigenome is highly dynamic. There is an epigenetic checkpoint in chronic inflammation that involves an important, but often overlooked, molecule: B-cell lymphoma 6 (BCL-6).

At a fundamental level, inflammation is driven by alterations in transcription. At the center of this story is NF-κB, which drives many different events in cells, contributing to a wide variety of diseases. The NF-κB pathway contributes to cancer, autoimmunity, insulin resistance, metabolic disease and heart disease, and each of those intersects with a nuclear receptor regulated signaling pathway. Nuclear receptor therapy relies on ligands such as glucocorticoids to subvert chronic inflammatory events, directly or indirectly managing the NF-κB signaling pathway.

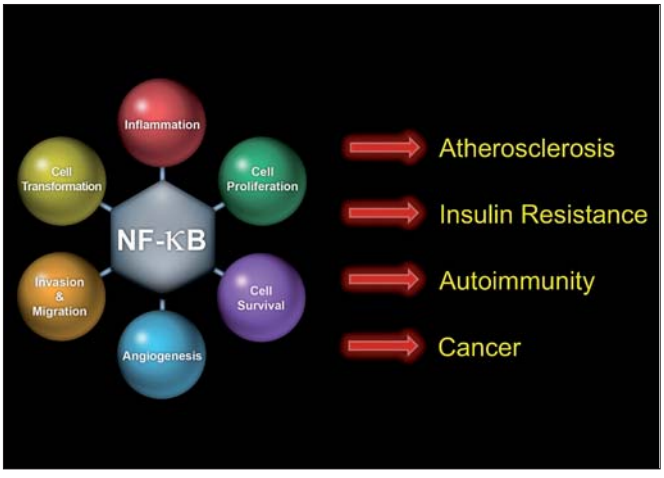

Figure 1
NF-κB drives disease.

If NF-κB is a key genomic player, the macrophage is the key cellular player. One reason for this is that macrophages are recruited to so called 'tumor stroma', where they help to fuel the pipeline of inflammation that nurtures tumorigenesis. Macrophages also directly contribute to inflammatory events linked to chronic obesity, which promotes insulin resistance and, ultimately, type 2 diabetes.

Tumor stroma is important for early events in tumorigenesis and cancer progression. It represents a critical

component in providing stimulus for both cell growth, and an environment rich with cytokines and interleukins that nurtures tumor progression.

Transcription is an epigenetic balance, reflecting the combined weight of transcriptional activation and repression machinery. NF-κB activates the process in a signal-dependent manner. In the absence of a signal, BCL-6, the most frequently targeted proto-oncogene in non-Hodgkin's lymphomas, keeps the macrophage in a repressed or off state, controlling the inflammatory response.

BCL-6, though creatively restricted in its expression, is an important regulator because it is active in both the myeloid and B-cell lineage. In B-cell precursors, BCL-6 is turned off as the B cell matures. If BCL-6 fails to turn off, as due to translocation, proliferation continues and leads to non-Hodgkin's lymphoma.

BCL-6 achieves the repression by recruiting a co-repressor complex, the SMRT nuclear receptor co-repressor[1]. BCL-6 interacts with SMRT, NCoR, a plethora of HDACs and BCoR, a B cell-specific co-repressor. These bind through a common penta-peptide sequence; if the sequence is mutated, none of them bind[2].

A decoy SMRT peptide effectively blocks the recruitment of repressive activity to BCL-6 and is being developed as a therapy. BCL-6 has a DNA-binding component and also recruits many other chromatin modules.

Stellate cells:

In addition to macrophages and B-cells, a third pro-inflammatory cell that expresses BCL-6 is the hepatic stellate cell, which is quiescent. Normally in the liver, it's the storage cell for vitamin A and vitamin D. Under inflammatory stimulus, it dumps vitamin A and D and becomes a pro-inflammatory fibrotic cell.

During fatty liver disease, the stellate cell becomes activated and produces an extensive amount of collagen and fibrin, which leads to liver fibrosis, liver cirrhosis and, ultimately, to liver cancer. A related cell in the pancreas also expresses BCL-6 and is thought to be a critical component of the pancreatic tumor stroma.

When bone marrow taken from BCL-6 null newborn pups is transplanted into an irradiated wild type adult mouse, that mouse does not die, and appears to be relatively normal. However, a closer look at the macrophages shows that BCL-6 loss alters the balance of the inflammatory process triggered by lipopolysaccharide (LPS).

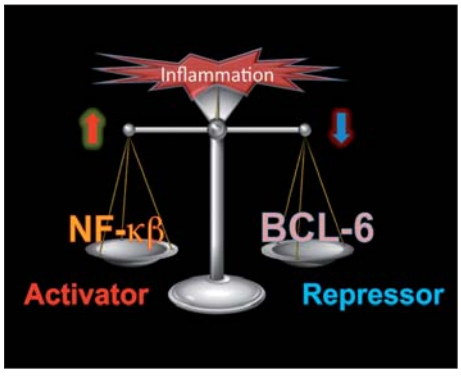

Figure 2
Tipping the epigenetic balance.

First, giving LPS to wild type mice increases interleukin 1-α (IL1- α) levels 50-fold or so in 2 hours and more in 6 hours. In the BCL6 null mice, by contrast, the levels are nearly 1000-fold above the level in the absence of LPS, and can go up to 100,000-fold.

Normally, BCL-6 keeps the NF-κB activation in check. But in the absence of BCL-6, some genes show high levels of induction. In another example, CCl2 or MCP1 show a 4- or 5-fold induction in the wild type. But without BCL-6, they start out with nearly a 1,000-fold induction and can cruise up to 3,000-fold. There is a dramatic increase in lymphokine production, both in cultured macrophages and in circulating levels in animals.

About half of the genes altered by loss of BCL-6 are inflammatory genes, followed by genes involved in differentiation, apoptosis, cell signaling and metabolism. BCL-6 regulates about one-third of LPS-affected genes. In about 60% of cases, BCL-6 loss and LPS affect individual genes in a directionally similar manner: loss of BCL6 de-represses and LPS induces expression.

Using chromatin immunoprecipitation-sequencing (ChIP-Seq) to define BCL6-binding sites across the genome reveals a series of interesting findings. At more than 30,000, NF-κB binding sites outnumber the 6,500 sites for BCL-6. NF-κB and BCL-6 co-localize at the IL-1 gene cluster.

BCL-6 and NF-κB cistromes are both not concentrated near promoter sites, they are mostly promoter distal. These are bonafide enhancer sites, with enhancer marks such as RNA Pol II present.

Inflammatory exposure:

In the absence of LPS, NF-κB level is basically zero because it's in the cytoplasm. The NF-κB cistrome is entirely inducible, and there is reciprocal appearance and disappearance of BCL-6 and NF-κB. Yet, ChIP-Seq shows that BCL-6 and NF-κB co-localize within nucleosomes at more than 2,400 binding sites. That suggests that the nature of the opposition is probably most effective when it's direct and proximal.

NF-κB piles into the nucleus following LPS signaling, and there may be many p300-inducible sites. For example after LPS signaling, there is a dramatic induction of p300 presence at inflammatory sites. Another example of the co-localization is in the CCL2 cluster. CCL2, 7 and 11 are all coordinately regulated.

With LPS, there is typically a reduction or loss of BCL-6 and the induction of NF-κB binding, all tracking at narrow sites with RNA Pol II, H3K4me3 and acetylation. This seems to lead to a dynamic interplay of histone deactylase and acetylase, concentrating at interlocking cistromes. BCL-6 is normally the dam that prevents that transcriptional flood; BCL-6 loss seems to unleash a mega over-inflammatory, chronic inflammatory situation.

IL6 is closely linked to many human cancers, including colorectal cancer. It has also been implicated in metabolic syndrome, and cancer risk is increased in individuals with metabolic syndrome. Interestingly, null mice are resistant to colitis-associated cancer. In intestinal epithelial cells, the downstream effector STAT3, which is also a BCL-6 target, contributes to colitis-associated colorectal cancer.

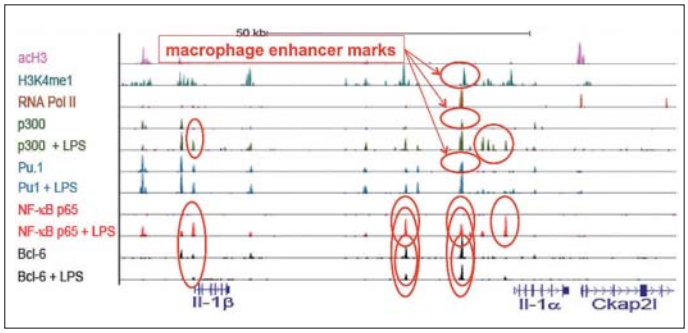

Figure 3
NF-κB and BCL-6 colocalize at the IL-1 gene cluster.

Following LPS stimulation for 3 hours, sequencing tracks for primary macrophages show a remarkable concentration of enhancer marks. H3K4me2 marks both enhancers and promoters. A peak at more than 60 kb upstream of the transcription start site for IL6 corresponds to an enhancer and is conserved among mammals. The IL6 transcription start site also has a peak, which is a mark at the promoter.

There is a dramatic clustering of pro- and anti-inflammatory regulators within the span of a nucleosome that controls IL6. The anti-inflammatory regulators include SMRT, HDAC3, BCL6 and GR, and the pro-inflammatory factors include NF-κB, AP1 and p300.

The competitive enhancers are also transcriptionally active. Because Pol II is there, these small regions can be activated and transcribed. So there is a co-repressor complex, a co-activator complex and a regulator of this process, the glucocorticoid receptor.

These findings suggest that negative regulation of enhancers is a key strategy for controlling the transcription of pro-inflammatory, pro-tumorigenic cytokines, by nuclear receptors such as GR, or by anti-inflammatory repressors including BCL6.

Large overlap:

BCL-6 affects a high percentage, about 25%, of the NF-κB target genes that are being controlled. It contributes to a much smaller component of pathways regulated by others, such as TL4, suggesting that BCL-6 really tracks with NF-κB. This overlap is, unsurprisingly, highly enriched for inflammatory genes.

In wild type cells, HDAC3 is highly induced by exposure to LPS, and BCL-6 recruits HDAC3 complexes to inflammatory genes, leading to a change in histone-based acetylation. This suggests that the inflammatory stimulus also brings in factors needed to suppress the transcriptional stimulatory event, limiting the overall extent of the ability of NF-κB as a potent activator.

The knockout cells show the opposite effects. That is, they have maximum histone acetylation because there are no HDACs present. This is not universal, there are exceptions, but the overall pattern is very clear: without BCL-6, there is a net genomic increase in acetylation, particularly at inflammatory sites.

Although BCL-6 is only expressed in a few cell types, those cells, such as macrophages, are everywhere in the body, meaning that BCL-6 can directly affect a wide variety of pro-inflammatory disease events.

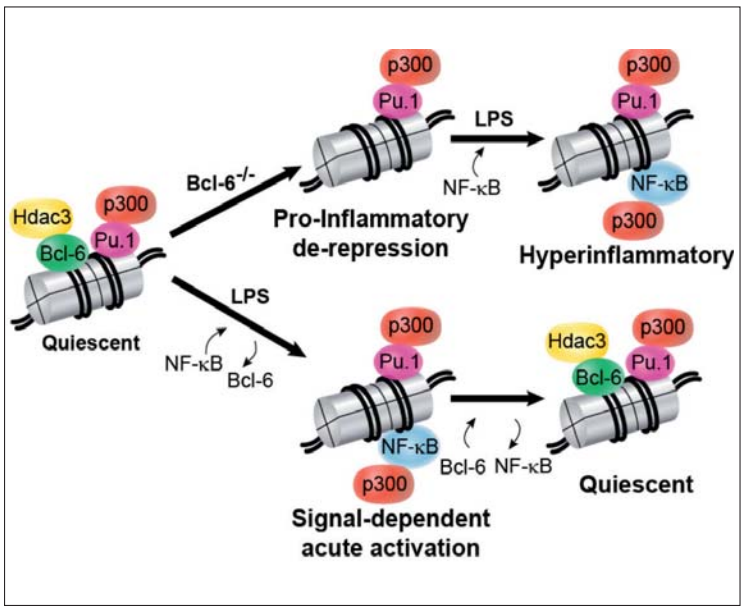

Figure 4
Model of BCL-6/NF-κB coordinated regulation of the inflammatory response.

A quiescent macrophage has BCL-6 and the HDAC complex. It appears to have PU1 and activators at some distal enhancer site. Exposure to LPS dismisses BCL-6, recruits NF-κB and there is signal-dependent activation. But NF-κB also induces BCL-6 and HDACs, allowing the activation to revert to quiescence.

When BCL-6 is lost, however, there is only a driving activator with nothing to counter the presence of PU1 and p300 activators. These null cells can't accomplish the reversion, and this leads to a super- or hyper-inflammatory state, which roughly mimics chronic inflammation. These null mice get profound inflammatory disease phenotypes[3]. For example, one mouse has severe inflammatory heart atherogenesis.

In summary, cistromic competition controls macrophage-mediated inflammation[4]. These cistromes don't act independently and the sub-cistromes communicate with each other. BCL-6 regulates one-third of the LPS transcriptome. BCL-6 and NF-κB act via promoter-distal cistromes as opposed to showing promoter importance.

Active and repressive cistromes are nucleosome-concentrated and dynamic, and so the cistrome changes continuously in monitoring the environment of the cell. Hyper-modified nucleosomes foster chronic inflammation in the absence of BCL-6. Nuclear receptors, though activators, restore repressive balance to revert chronic inflammation. Although it is unclear how that happens, it looks like it also occurs in context of the nucleosome.

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PART II: Epigenetics of stem cells

Joseph Ecker
Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells

Richard Young
Connecting gene regulation, chromosome morphology and disease

Maarten van Lohuizen
Role of polycomb repressors in stem cells, cancer and development

Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells

A report on a lecture by
Joseph Ecker
The Salk Institute for Biological Studies, La Jolla, USA

Induced pluripotent stem (iPS) cells offer immense potential for regenerative medicine and studies of disease and development. Somatic cell reprogramming involves epigenomic reconfiguration, conferring iPS cells with characteristics similar to those of embryonic stem (ES) cells. However, it remains unknown how complete the reestablishment of ES-cell-like DNA methylation patterns is throughout the genome. ***Joseph Ecker*** *reported the first whole-genome profiles of DNA methylation at single-base resolution in five human iPS cell lines, along with methylomes of ES cells, somatic cells and differentiated iPS and ES cells. iPS cells show significant reprogramming variability, including somatic memory and aberrant reprogramming of DNA methylation. iPS cells share megabase-scale differentially methylated regions proximal to centromeres and telomeres that display incomplete reprogramming of non-CG methylation. They also show differences in CG methylation and histone modifications. Finally, differentiation of iPS cells into trophoblasts reveals that errors in reprogramming CG methylation are transmitted at a high frequency, providing an iPS cell reprogramming signature that is maintained after differentiation.*

In the context of DNA methylation dynamics, methylation and active demethylation of sites are both important. For example, the Nanog promoter in embryonic stem cell development is actively demethylated.

Many cell types derive from a single genome, and transcriptional programs are obviously the driver in generating this phenotypic complexity. Processes that regulate the readout of genomic information, including epigenetic modifications, control these transcriptional programs.

Embryonic stem (ES) cells serve as an interesting model for understanding how the patterns and variability in the epigenome are written and read.

DNA methylation is a mitotically heritable layer superimposed on the genome that can manipulate the readout of underlying genetic information. DNA methylation has many roles, particularly in tumorigenesis. X chromosome inactivation involves DNA methylation and other mechanisms, such as RNA-guided chromatin silencing mechanisms.

A recent paper directly implicates DNA methylation in cancer, showing that mutations in DNMT enzymes, particularly DNMT3A, are involved[1]. More than 20% of cases of acute myelogenous leukemia harbor mutations in this enzyme. Comprehensive epigenomic maps of high resolution can help understand the importance of these changes.

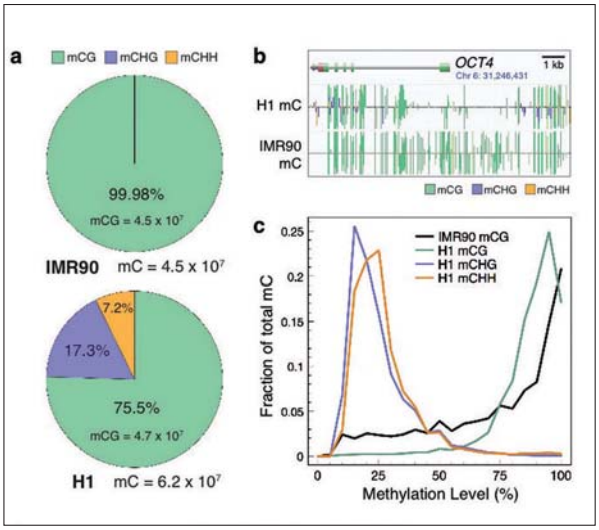


Figure 1
Composition of the human DNA methylome.

The NIH Roadmap Epigenome Project is an attempt to characterize how DNA methylation, chromatin modifications and RNA changes in different cell types drive the formation of many cell types from one genome[2]. Superimposed on this is the change in time, identifying the epigenome in normal cells at different time points.

Twin studies have shown, for example, that these marks change over time. In fact, chromatin modifications can be dynamic, sometimes changing every few hours. The epigenome can also change in response to the environment. Without comprehensive profiles of high resolution, however, it is very difficult to understand the prevalence of known processes and discover uncharacterized ones.

The limitation in seeing some these global differences is in part a lack of tools. Some of the tools that move away from arrays towards sequence-based approaches are browser-based, allow for dynamic browsing and distributed data hosting, have customizable track modules that can show information at megabase to nucleotide level[2].

Technical advances:

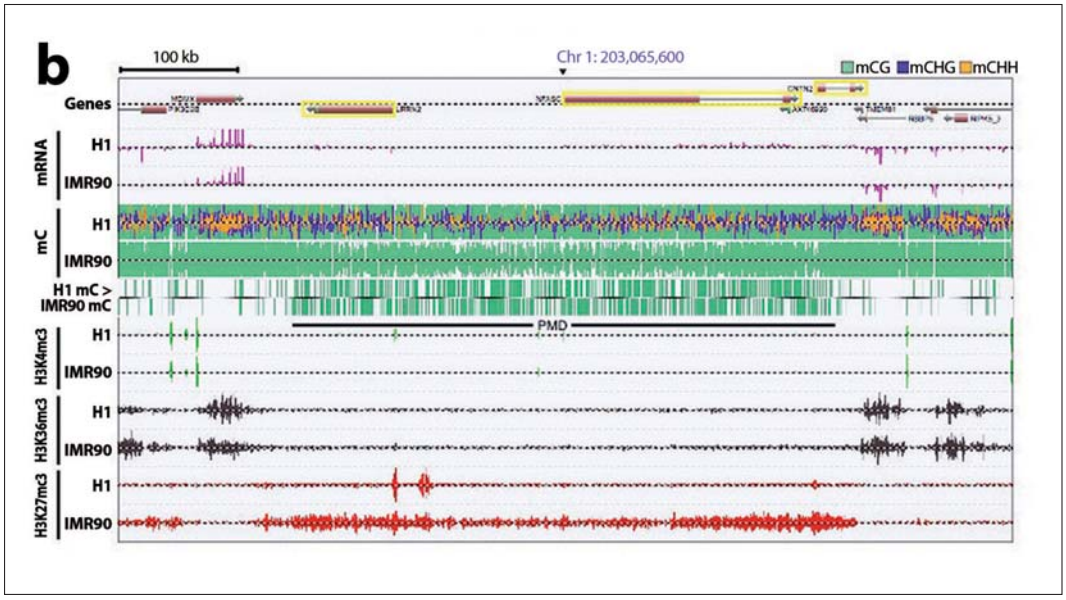
This kind of technology can be applied fairly easily now to the human genome, coupled to next-generation sequencing.

In this method, any C that is methylated is maintained as a C, any one that's not becomes converted to a U and a T after replication. This assay works for as little as 50 nanograms of DNA. Using this approach, about 5% to 7% of the genome is cytosine methylated. The method maps about 92% of all the cytosines in the genome.

Up until a few years ago, most laboratories were able to score between 0.003% to 1% of the entire methylome. That would be the equivalent of a geneticist looking at 1% of single nucleotide polymorphisms (SNPs) and trying to draw conclusions based on that.

Now, the roadmap covers 80-90% of the methylome and the high coverage delivers comprehensive reference epigenomes.

Figure 2
Partially methylated domains.



In parallel, it is also possible to read out the consequences of any differences in methylation, for example throughout development of an ES cell. A RNA-Seq assay also provides strand-specific information on promoter methylation or on scoring the dynamics of methylation and transcription.

Using these tools to look at a number of differentiation events, and next-generation sequencing, the San Diego Epigenome Center has covered about 90% of the genome in terms of the total cytosine content[3].

The first surprise from the results is that a large fraction of the genome in human ES cells has non-CG methylation, compared with standard embryonic lung fibroblasts, which have no non-CG methylation. Essentially, pluripotency and embryonicness correlate with a very high level of non-CG methylation in a CHH or CHG context. This accounts for about 25% of all the methylation in the human genome.

Typically, CGs are methylated, but before deep whole-genome sequencing, non-CG methylation was discounted as an artifact of incomplete bisulfite conversion. There's some regulation that maintains this methylation that is poorly understood.

Positive correlation:

ES cells are also known to have high levels of methyl transferases, including DNMT1, the *de novo* transferases and the regulatory subunit. In differentiated cells, the levels of these enzymes are dramatically reduced.

Non-CG methylation is enriched in gene bodies. If the genes are rank ordered from 1 to 25,000 and their amount of methylation noted, there is a positive correlation: the more the non-CG methylation in the gene body, the more the expression of that gene.

This is not true of CG methylation in ES cells. Rank ordering the genes with CG positions shows no correlation. However, in differentiated cells what looks like a correlation is, in fact, two distinct types of methylation. There are large regions of the genome that have less gene expression and also have less methylation at CG sites.

These regions, which constitute 30-40% of the genome, turn out to have partially methylated domains (PMDs). These domains don't exist at all in any ES cells or in induced pluripotent stem (iPS) cells, and are associated with lower mRNA levels and enrichment in histone H3K27me3 modification.

Enhancers are known to have K4 mono-methylation and K27 acetylation. There are H1 specific enhancers, IMR90 specific

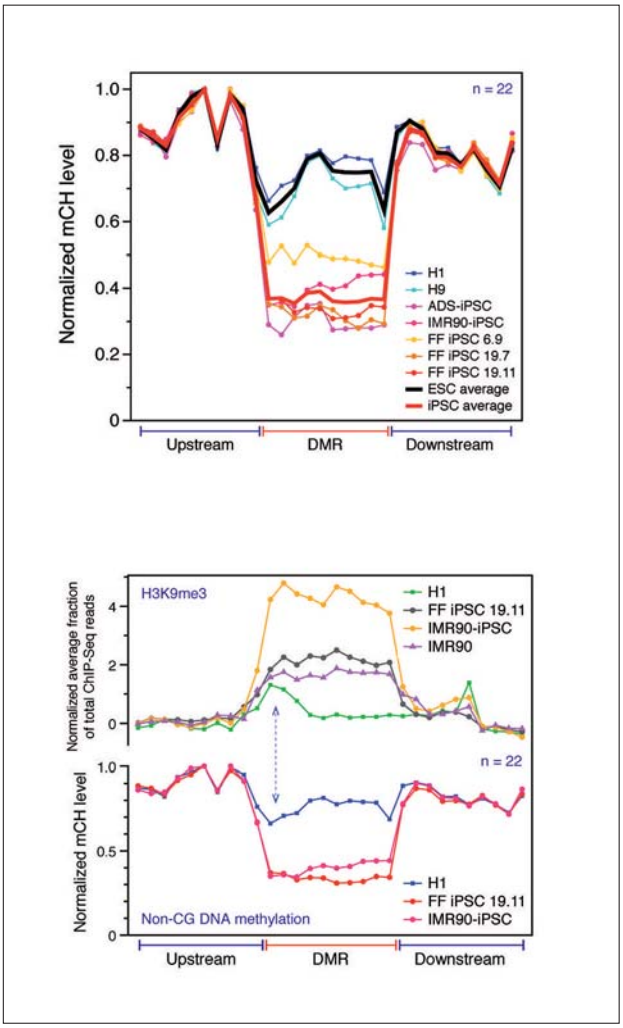


Figure 3
Non-CG mega-differentially methylated regions in multiple iPS cell lines.

enhancers and enhancers shared by both. Embryonic-specific enhancers have no inflection or change in the density of CG methylation. But regions that are marked by those two chromatin modifications in ES cells are depleted in non-CG methylation.

The opposite is true for IMR90 cells. There is no non-CG methylation in those cells, but the CG methylation is depleted across enhancers.

The enhancers shared by both types of cells show both types of effect: they're depleted in both CG methylation and non-CG methylation.

In summary, the first whole-genome human reference epigenomes have found abundant non-CG methylation in stem cells, enriched in genes and positively correlated with gene expression. Cell-specific methylation patterns of regulatory elements suggest that non-CG elements may have a role in ES cells of transcription, as read out by depletion of these ES cell-specific enhancers[3,4].

Cell comparisons:

Many of the comparisons so far between ES and iPS cells map to the laboratory conducting the research, but it is established that ES and somatic cell methylomes are distinct.

Comparing methylome changes between ES cells and iPS cells from a variety of sources can help understand what happens when somatic cells are reprogrammed to an ES-like state.

All of the somatic cells — including fat cells, adult stem cells and fibroblasts — all have PMDs, but none of the ES cells or iPS cells have them. Also, none of the somatic cells have non-CG methylation, but all of the pluripotent cells have a lot of non-CG methylation.

There are differences even in CG methylation. About 40% of the genome is in these PMDs that don't exist in embryonic cells. The genes in those regions are largely reduced in expression compared with the other cells.

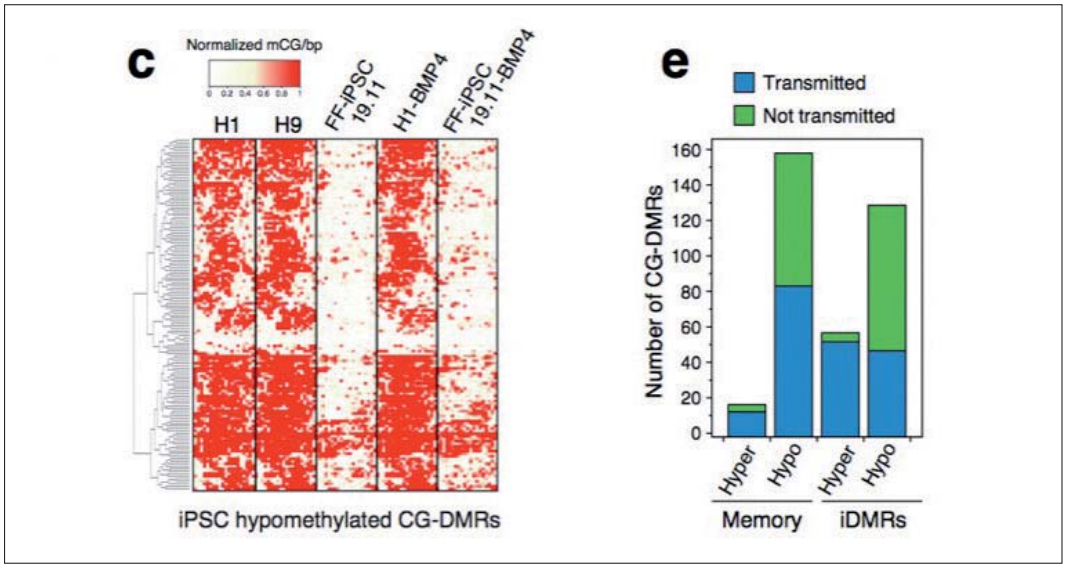


Figure 4
Transmission of CG-differentially methylated regions through differentiation.

Based on those data, all of the embryonic cells and iPS cells cluster together on a genome-wide scale, suggesting that the methylomes of ES and iPS cells are generally similar.

All of the differentiated cells have PMDs, with some very interesting features in cancer. But when the somatic cells are used to create iPS cells, those regions disappear, and gene expression in the regions is enhanced. The PMDs present in somatic cells are remethylated (on CG) to a high level in iPS cells. They are also associated with lower mRNA levels and enrichment in histone H3K27me3 modification.

There are other differences. For example, if a somatic cell is used to create an iPS cell that's very embryonic-like in terms of gene expression, some portion of the CG methylation from the parent is retained. In part, these reprogrammed cells 'remember' their somatic cell origin in terms of DNA methylation patterns.

Hundreds of CG differentially methylated regions (CG-DMRs) have been identified in each iPS line compared with ES cells. Aberrant CG methylation patterns can be categorized as a failure to reprogram certain somatic patterns, or as iPS cell-specific methylation (iDMR) found neither in ES cells nor in progenitor cells.

For example, a hyper-methylation mark that is present on a promoter in iPS cells, but not in ES cells, does not disappear when the iPS cells are differentiated into trophoblasts. These differential marks create a signature of "iPS-ness" that distinguishes iPS cells from ES cells.

There are also very large differences on the mega-base scale, dubbed non-CG mega DMRs, when comparing iPS cells to ES cell lines. These regions are millions of base pairs long and map to non-random locations in the genome. They're found near telomeres and centromeres, suggesting that something about these genes is refractory to complete reprogramming.

iPS cells show a depletion of non-CG methylation in regions that normally have it in ES cells. Non-CG DMRs are often shared between independent iPS cell lines and many are present in all iPS cell lines. The loss of non-CG methylation is associated with increased H3K9me3. Any gene that lies in one of these regions that's not reprogrammed is silenced or reduced in expression dramatically.

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Connecting gene regulation, chromosome morphology and disease

A report on a lecture by
Richard Young
Whitehead Institute for Biomedical Research, MIT, Cambridge, USA

Transcriptional activity and silencing involve essential changes in chromosome morphology, but the mechanisms that link gene regulation and genome structure are not fully understood. Genetic screens have revealed that embryonic stem cells are highly sensitive to reduced levels of mediator, cohesin and condensin. These proteins contribute to the structure of the genome by promoting interactions between some chromosomal sites while inhibiting others. The master transcription factors in ES cells generally recruit the mediator co-activator to active enhancers. Because mediator can also bind RNA polymerase II, the active enhancers are brought into proximity of the core promoter elements of these genes, thus forming DNA loops. Mediator is bound by NIPBL, which loads cohesin at these promoters. Prior to entry into mitosis, NIPBL also loads condensin at active promoters. After loading, cohesin and condensin translocate to different sites on chromosome arms, where they further contribute to interphase genome organization and chromosome compaction. Mediator, cohesion and condensin co-occupy different promoters in different cells, thus generating cell-type-specific DNA loops linked to the gene expression program of each cell. ***Richard Young*** *provided mechanistic insights into a variety of diseases caused by mutations in these proteins.*

Embryonic stem (ES) cells are a great model system for studying fundamental properties of cells. Some of the key themes probably also extend to other cell types.

Many embryonic pathways are misregulated in cancer. There are a small number of master transcription factors in ES cells that are essential regulators of the cell state[1]. They select the set of genes that are going to be transcribed as well as those that will be silenced. Some of them regulate initiation, others elongation, and in doing so, ultimately regulate chromatin state and even chromatin structure.

Nucleosome regulators may play essential roles not so much in the control of cell state, but in the coordination of transcription and genome packaging. They're particularly essential when cells move from one state to another.

All of active transcriptional control involves looped chromosome structures at promoters, where most control operations are probably carried out. That provides both insights and challenges in trying to interpret data.

It's useful to know what the global expression state of the genome looks like. Based on murine ES cell data, ChIP-Seq data for RNA polymerase II and histone modifications, there are four classes of genes: active, initiated, poised and silent. Most genes — not just in ES cells, but in somatic cells — experience transcription initiation. About 40% of genes are fully transcribed in ES cells. DNA binds the transcription factor, recruits cofactors and the polymerase goes down the transcription unit.

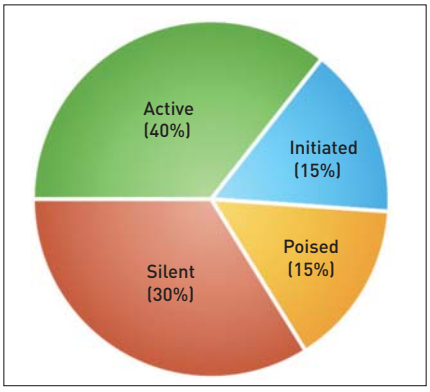

Figure 1
Four gene classes in embryonic stem cells.

Modifications by kinases to the C-terminal domain and modifications to the nucleosome by various types of modifiers are coordinate. And these events are stereotypical, they happen at every gene.

For 15% of genes, the process stops at polymerase II pausing, although it is unclear what prevents productive elongation.

Another 15% of genes are called poised genes, because when ES cells differentiate, this is the set that is most rapidly activated[2]. The master regulators may recruit some polymerase and make a small RNA, which is involved in recruiting polycomb. There are other regulators that are key to silencing this set of genes, and that includes SETDB1.

Finally, the silent set of genes is characterized by a variety of features, but the key commonality is H3K9 di- and tri-methylation by a variety of histone methyltransferases.

Genetic screens reveal key regulators of ES cell state, focusing specifically on transcription factors and co-activators, chromatin regulators and signaling proteins.

A short hairpin screen reveals all the genes known to control ES cells and many more. For example, the set of reprogramming factors for ES cells are: the transcription factors OCT4, SOX2, Nanog and cMYC; the co-activator mediator; the chromatin regulators SWI/SNF, TRXG, HDACs, PcG, SetDB1, cohesin and condensin; and the signaling pathways Wnt, Lif, TGFβ and Notch. Many of these, such as cMYC, mediator, Wnt, TGFβ and Notch, have cancer associations.

There is a discussion in the literature about the extent to which stem cell-like gene expression signatures occur in cancer stem cells. There is early evidence that the MYC transcriptional network accounts for these similarities, arguing against the hypothesis that cancer cells reactivate an ES cell signature.

Masterful controls:

Several labs have shown that, of the four transcription factors, OCT4 and SOX2 form a heterodimer that is important in ES cells. Loss of OCT4 or SOX2 causes loss of pluripotency and self-renewal. It is possible to reprogram cells that have SOX2 expression by adding OCT4.

Nanog is required to enter and maintain a stable pluripotent state. It cycles up and down in ES cells unless they are in their most naïve state. cMYC stimulates proliferation and self-renewal.

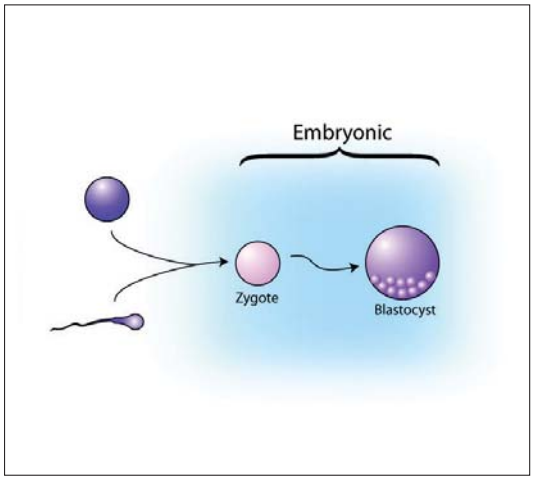

Figure 2
Embryonic stem cell master transcription factors.

The master transcription factors and their genes form an interconnected auto-regulatory loop. This is true for most cell types studied.

OCT4, SOX2 and Nanog are co-bound at enhancers that regulate at least 64% of actively transcribed genes in ES cells. Lowering the confidence value shows that, in ES cells at least, the master transcription factors regulate the majority of active genes.

Genes occupied by OCT4/SOX2/Nanog are also frequently regulated by c-MYC, which is bound to a large fraction of these active genes. At the genes with OCT4/SOX2/Nanog, TCF3, the TCF/LEF factor that Wnt targets, is always present, and provides repressor function for those enhancers.

It has long been known that RNA polymerase II initiates and then stops, and makes a short RNA. That happens at the majority of genes. It pauses because of two factors, NELF and DSIF: as long as these two proteins are in an unphosphorylated state, the polymerase is non-processive. cMYC recruits p-TEFb, which is essential for phosphorylating NELF and DSIF, licensing the RNA polymerase to elongate.

If c-MYC plays the dominant role in ES cells in polymerase II pause release at most of the active genes, it should interact with the pause release factor P-TEFb, and almost exclusively with actively transcribed genes.

cMYC might be expected to occupy regions near the promoter-proximal region, and its loss of function should not affect levels of Ser5P pol II (initiating) but should reduce levels of Ser2P pol II (elongating). Its loss of function should also have little effect on pol II initiation but cause reduced pol II elongation.

In biochemists' view of transcription, there is a pre-initiation complex, followed by elongation. However, that's not true *in vivo*. It's pretty clear that transcriptional pause and its regulation play key roles in development and cancer.

For example, *Drosophila* primordial germ cells are fully transcriptionally silent because PGC1 sequesters pTEFB and prevents elongation. TIF1γ controls hematopoietic differentiation through pause control, and there's some evidence that NF-κB may be involved. p53 may also communicate with mediator and regulate pause release at its target genes.

The super elongation complex is really critical in this process, and there is new R&D focus on p-TEFb, BRD4 and c-MYC at major pharmaceutical companies.

Nucleosome regulators:

There are several important nucleosome regulators, including SWI/SNF, TrxG, HDACs, PcG and SetDB1. With the exception of SetDB1, they're not essential for ES cell viability but, in their absence, ES cells tend to differentiate.

Transcription drives most nucleosomal regulation. Chromatin regulators provide the means to coordinate transcription with 'opening and closing' of packaged DNA.

For these two classes of genes, poised and silent, there is increasing evidence that transcription is probably required to establish and maintain most gene repression. Part of the reason for that is that most genes initiate transcription in both sense and anti-sense directions and produce small ncRNA species of between 50-200 nucleotides. Interestingly, the ncRNAs produced at CG islands can recruit polycomb complexes.

This suggests that ChIP data is not an accurate representation of the dynamics, it's a representation of the steady state. Dynamically, there is bidirectional transcription at all these promoters, the vast majority of which are CpG islands.

In the presence of activating transcription factors, co-activators and, ultimately, TrxG, are recruited. As part of their complexes, they recruit K27 demethylases. H3K27me3 is necessary for fully stabilizing polycomb in this model.

In summary, ES cells can dispense with most nucleosomal regulators, with only minor effects on

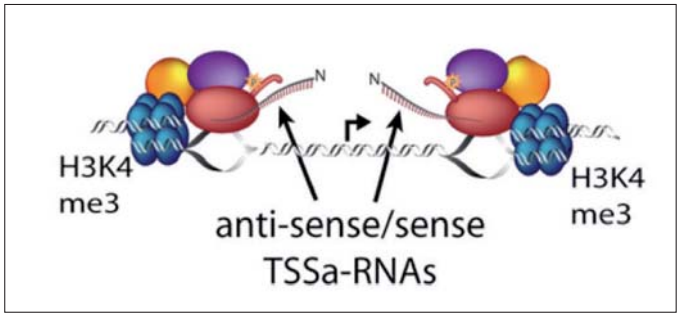

Figure 3
RNA molecules from GC-rich regions recruit and maintain PcG complexes.

transcription. Nucleosome regulators show profound effects on cells that are attempting to differentiate. Most nucleosomal regulators are essential for transition to new gene expression program during differentiation. The hypothesis is that the major changes in DNA packaging and chromosome morphology that accompany new gene expression programs require coordination.

Altered level of function of many of these regulators is implicated in cancer. For example, SETDB1 has been shown to be critical for metastatic melanoma[3]. However, many of the nucleosomal regulators are expressed generally and have wide-ranging effects, so targeting them with drugs could have serious adverse effects.

Major mediators:

Among the things that emerge from the screen are 15 subunits of the 30-subunit mediator. Mediator is a 6MD complex that binds both transcription factors and RNA polymerase II. It is present in all cells, but ES cells are very sensitive to reduced levels. The same is true for cohesin and condensin as well.

Transcription factors recruit and bind mediator, just like they do p300, which in turn binds to polymerase II. This is a critical complex: mouse MED21 and CDK8 nulls are embryonic lethal, and MED1 and MED24 nulls lead to impaired cardiac and neurological development. Mediator mutations are associated with Opitz-Kaveggia syndrome, Lujan syndrome, schizophrenia, Transposition of the Great Arteries syndrome, and colon cancer progression.

Mediator is recruited to active promoters and occupies active promoters bound by master regulators. It is associated with enhancers and core promoter elements. Data suggest models for mediator-facilitated looping of enhancers and promoters.

Cohesin functions in chromosome cohesion and regulation of gene expression[4]. Cohesin can encircle two DNA molecules and mediates sister chromatid cohesion.

It's long been known that there are hypomorphic mutations that do not affect chromosome segregation, but have profound effects on gene expression.

Cornelia de Lange Syndrome is a fairly common disease — affecting 1 in 10,000 live births — and is associated with mutations in NIPBL and condensin. The disease is characterized by multi-organ deficiency in development.

These so-called structural maintenance chromosome complexes are older than histones and nucleosomes are, they are ancient chromosome organizers. In prokaryotes, those complexes have been implicated in control of gene expression.

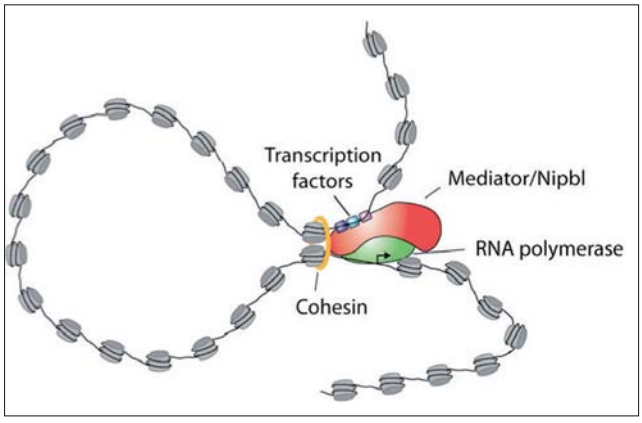

Figure 4
Mediator and cohesin link gene activity with chromosome structure.

Throughout the genome, cohesin is wherever mediator is. NIPBL loads the circuit or cohesin onto these sites. Cohesin and NIPBL are associated with mediator at enhancers and core promoters.

Cohesin occupies about 43,000 sites on the genome. The subset of genes co-occupied by mediator, NIPBL and polymerase II is usually exclusive with the set of sites bound by CTCF.

Knockdown of cohesin, mediator and NIPBL has similar effects on expression

of co-occupied genes. Using a DNA-binding activation domain as a means of purifying mediator is the only method to pull down cohesin and NIPBL. That's because the activation domain creates a conformational change in mediator, allowing binding of NIPBL, which then loads cohesin.

DNA loops:

Reduced levels of cohesin and mediator cause reduced DNA looping. In controls, in which these genes are not expressed, there is no such loop. Importantly, knockdown of cohesin or mediator eliminates the loop.

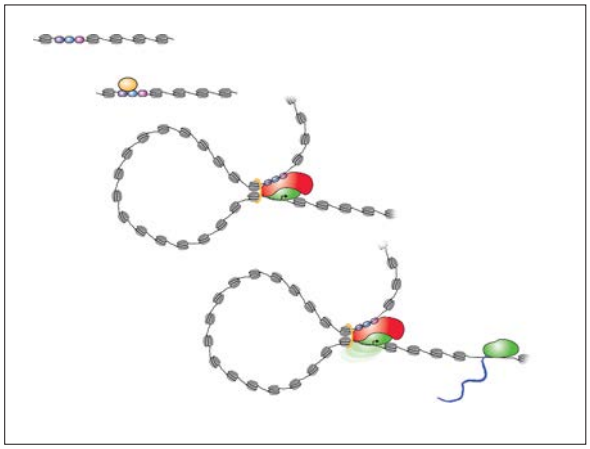

Figure 5
Model for transcription initiation.

Mediator, cohesin and NIPBL are found at the promoters of OCT4-regulated, actively transcribed genes in ES cells across the genome, and are essential for transcription. The three co-purify in the presence of an activation domain. Because each cell has a different expression program, each cell has cell-type specific chromosome morphology.

In this model, there are transcription factors bound on enhancers. They recruit chromosome regulators that help open up that region. They bind mediator, p300 is also at all these sites, and cohesin holds this together.

Most control operations are likely carried out at looped configuration of promoters. RNA polymerase II is in a paused state. Mediator is known to contribute to initiation as well as elongation. And there are many other regulators at this site, including CDK7/cycH, CDK8/cycC, CDK9/cycT and SEC, LSD1-NuRD complex (HDACs 1, 2) and SIN3A (TET1).

Finally, the downstream transcription factors for four key signaling pathways — STAT3, TCF3, SMAD2/3 and SMAD1 — all bind at sites bound by OCT4/SOX2 and Nanog. These downstream effectors co-occupy enhancers with master regulators of those cell types.

Master transcription factors are essential regulators of cell state. They control the selection of genes that will be activated. Some of them control initiation, some control elongation. Chromatin state and chromosome structure is downstream of DNA-binding transcription factors, which run the show.

Nucleosome regulators are critical, and play essential roles in coordination of transcription and genome packaging. The densely packed structure needs something to unpack portions of it to establish a new gene expression program. Transcriptional control involves looped chromosome structures at promoters, where most control operations are probably carried out.

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Role of polycomb repressors in stem cells, cancer and development

A report on a lecture by
Maarten van Lohuizen
The Netherlands Cancer Institute, Amsterdam, The Netherlands

Repressive polycomb-group protein complexes are involved in the dynamic maintenance of proper gene expression patterns during development, acting at the level of chromatin structure. As such, they are important controllers of cell fate. When deregulated, these master switches of gene expression are strongly implicated in formation of a diverse set of cancers. An example is the gene BMI1 which is over-expressed in many cancers. BMI1 is a critical regulator of stem cell fate in embryonic stem cells and many adult stem cells. The INK4a/ARF tumors suppressors are critical BMI1 target genes in stem cells and in cancer formation. A functional BMI1-RING1B heterodimer in the PRC1 polycomb complex also has an essential E3-ubiquitin ligase activity. This activity is required for maintenance of polycomb repression in normal and cancer stem cells, and offers potential ways to target cancer stem cells or tumor reforming cells in which the activity of the ligase is hyperactivated. The BMI1/RING1B PRC1 complex is also recruited to sites of DNA damage and is required for efficient double-strand break repair. ***Maarten van Lohuizen*** *addressed the implications of these findings for stem cell biology, development and cancer.*

Polycomb repressive complexes repress transcription or inhibit gene expression in diverse ways that are not known in detail.

For example, the PRC2 complex includes the EZH2 methyltransferase, which adds a repressive trimethyl group on H3K27. The PRC1 complex doesn't consist of one entity and has many different forms.

The PRC1 complex may lead to compaction of chromatin in larger domains. It can also add a mono-ubiquitin mark at lysine 119 on H2A. This mark can stall RNA polymerase II on target genes and may be one of the ways in which transcription is inhibited at target genes. There is new evidence that the ubiquitin mark can also independently influence DNA repair processes. The interplay between polycomb and DNA methylation also needs to be better understood.

Genome-wide profiling in embryonic stem (ES) cells shows that polycomb repressive marks are at many transcription factors involved in differentiation. This also holds true for *Drosophila*, suggesting that the polycomb complexes and their targets are both conserved through evolution.

When an ES cell differentiates, lineage-specific genes need to be expressed, and other genes, such as the pluripotency network, need to be tightly shut off. This is in part mediated by polycomb, followed by DNA methylation. But there may also be other mechanisms involved.

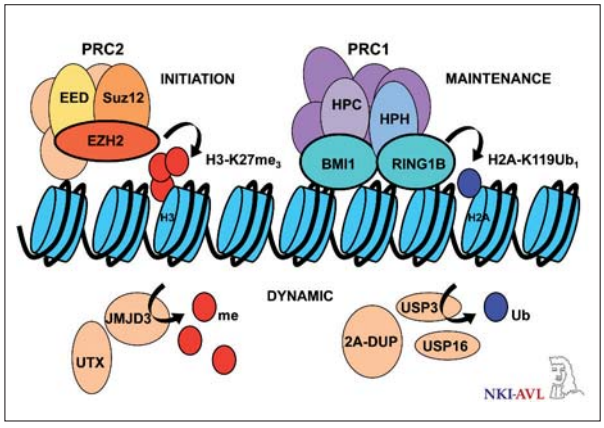

Figure 1
PcG proteins function in chromatin-modifying Polycomb Repressive Complexes.

Polycomb complexes can change in composition and can also be regulated by post-translational modifications. For instance, polycomb can be bound to chromatin, but be enzymatically inactive, allowing gene expression—and the repression can then later be activated by post-translational modification.

Like the PRC2 complex, the PRC1 complex also includes a co-enzymatic function. This is the E3 ligase, consisting of RING1B, also known as RNF2, and a regulatory component, BMI1. There are many diverse PRC1 complexes — for example, one complex exchanges BMI1 for MEL18.

BMI1 and MEL18 are 75% identical at the amino acid level and biochemically, both act as an E3 ligase and add a mono-ubiquitin mark on H2A. Yet, in development, they do very different things. And this is a recurring theme for these polycomb complexes, which are always subtly changed in one of their protein components. The E3 ligase is the only core component of this complex.

PRC1 complexes also contain CBX proteins, which contain chromo-boxes that recognize the trimethyl mark on chromatin. There are 8 of these proteins, and each is in a separate complex that also contains the E3 ligase.

Target specificity:

These polycomb complexes have been systematically analyzed both by mass spectrometric analysis and by following the changes during differentiation.

In ES cells, the MEL18-PRC1 complex purifies with only CBX7 but none of the other CBX proteins. However, when the ES cells differentiate into neural stem cells, there are three CBX proteins — CBX8, CBX4 and CBX6 — that associate with MEL18, and CBX7 is gone.

Using Chip-Seq, and another method called Dam-ID — a highly sensitive alternate to Chip-Seq — there are about 200 or so targets in ES cells. As ES cells differentiate into neural stem cells, there is a large increase in the number of these targets.

A major black box in polycomb biology is how these complexes are recruited to specific target genes. In *Drosophila*, there is some evidence of sequence-specific transcription factors that might recruit the complexes, but they cannot explain the whole phenotype.

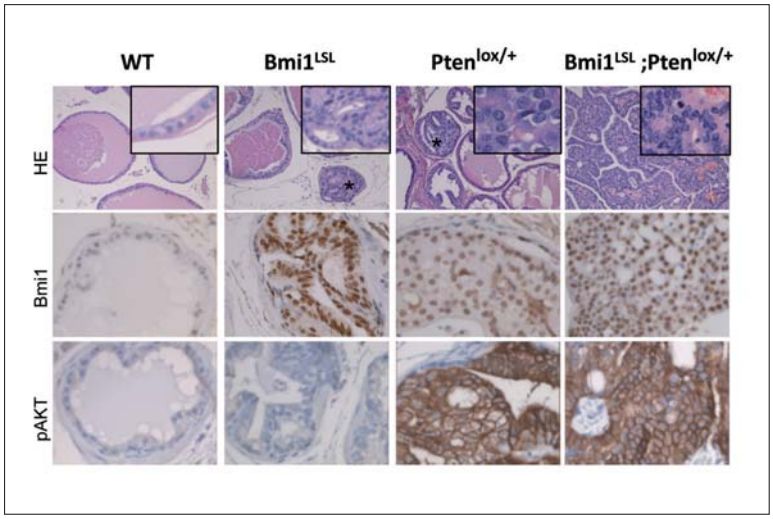

Figure 2
Role of BMI1 in prostate cancer progression.

JARID2, a non-active Jumonji protein, might also help recruit polycomb complexes to chromatin. There is also puzzling new data that long non-coding RNAs and small transcripts that occur at many promoters aid in attracting PRC2 complexes to genes. Neither of these explains the specificity for targets, however. The extreme view is that polycomb doesn't require specific targeting, but all the specificity lies with the transcriptional activator[1].

The BMI1-RING1B E3 ligase closely resembles the BRCA/BARD ubiquitin ligase (see Verma, page 103). Both complexes can add the ubiquitin mark on chromatin. BARD or BMI1 is the regulatory component of this complex, and their levels determine E3 ligase activity[2].

If RING1B is knocked out in ES cells in a conditional system, over time the cells look differentiated and can't proliferate. These cells seem to over-react to differentiation signals. The polycomb repression is required to maintain stable ES cell fate, depending on culture conditions.

PCR assays and genome-wide profiling show that all the lineage differentiation markers that are expressed during normal embryogenesis are turned on in these cells. Polycomb is required for the repression of lineage differentiation genes, but when cells start to differentiate, it is also required to at least help initiate repression of the pluripotency network, followed by DNA methylation.

This can be seen even at the single-cell level. In single normal ES cells, OCT4, Nanog and a pluripotency marker alkaline phosphatase are all expressed. If RING1B is floxed out, the expression of all three genes is retained, but all the lineage differentiation markers are also expressed. These cells essentially get conflicting signals, perhaps explaining why they ultimately can't survive.

By contrast, if the regulatory component BMI1 or MEL18 is knocked out, the mice are small, have neurological defects, a small cerebellum, and fear ataxia, which is progressive, and they die very young. They also have other interesting phenotypes. For example, they have skeletal transformations due to mis-expression of HOX genes, and hypoplasia in lymphoid organs. The latter is caused by an absolute requirement for BMI1 for the maintenance of adult stem cells of different origins.

Interestingly, although MEL18 is also required for HOX gene regulation, it is not needed for maintenance of stem cell phenotypes.

Cancer connections:

PRC1 and PRC2 complexes also set thresholds for differentiation decisions. For example, when the mammary cell line HC11 is exposed to a cocktail of pregnancy hormones, the hormones drive the cells to terminal differentiation, as measured by Q-PCR of the milk protein β-casein[3].

If BMI1 is knocked down about 90% in those cells, the expression of the differentiation marker spikes sharply in response to relatively low levels of pregnancy hormones. Conversely, if BMI1 is over-expressed in the presence of the hormones, differentiation is completely suppressed.

This suggests that BMI1 sets the thresholds for how cells in the mammary gland respond to differentiation signals delivered by hormones.

BMI1 — but not MEL18 — and EZH2, another enzyme that is part of the PRC2 complex, are often over-expressed in cancer. In fact, BMI1 was first isolated in a genetic screen for oncogenes that cooperate with c-MYC. BMI1 that has mutations that preserve the interactions of this protein, but disrupt its function as an E3 ligase, no longer functions as an oncogene when over-expressed.

Deregulation of these components is almost as, if not more, common than p53 mutations in cancer. One of its first identified targets is the tumor suppressor INK4a/ARF. Over-expression of BMI1 silences this tumor suppressor[4].

Transgenic mice that over-express BMI1, in this case in the lymphoid system, form B- and T- cell lymphomas. If this is combined with over-expression of c-MYC, the mice die very young of massive leukemia.

In the opposite experiment, if MYC transgenic mice — which normally develop B-cell lymphomas — are crossed on BMI1 +/- background, many of the mice no longer develop tumors within the time window.

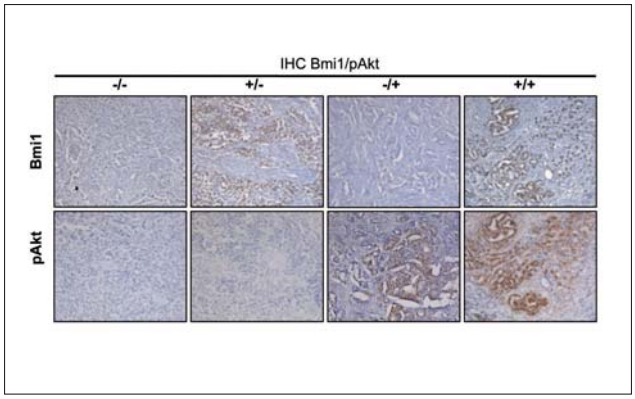

Figure 3
Association of BMI1 expression and PI3K/AKT activation in high-grade human prostate cancer.

These effects also hold true in epithelial-derived cancers. As in breast cancer, BMI1 or EZH2 over-expression are poor prognosis predictors for prostate cancer.

In prostate cancer development, the first stage is PIN, or prostate interepithelial neoplasias, characterized by lesions that remain confined to the ducts. Various events then drive the tumor to become invasive and even metastasize.

If BMI1 is over-expressed in this system, the mice get PIN lesions, but don't progress beyond that. This is reminiscent of another, well-known prostate cancer model, the loss of PTEN tumor suppressor. When over-expression of BMI1 is combined with deleting of one PTEN allele, the mice progress into full adenocarcinomas.

In human cancers, there's a clinically significant correlation between BMI1 over-expression and activation of the PI3K/AKT pathway in tumors with high histological grades and poor clinical outcome. Over-expression of BMI1 is associated with poor prognosis in many cancers, including leukemia, glioblastoma, medulloblastoma, prostate cancer and breast cancer.

Central activity:

BMI1 and other polycomb proteins are known to be phosphorylated, sometimes in a cell cycle-regulated manner. For example, BMI1 is phosphorylated in many different cancers. The primary phosphorylation sites are in its C-terminus, which is the least conserved area with differences between MEL18 and BMI1. The three serines that are the most predominantly phosphorylated are also the most highly conserved.

In normal conditions, BMI1 is phosphorylated and is active as an E3 ligase. When it's de-phosphorylated, it becomes inactive. AKT can phosphorylate BMI1 directly, indicating that phosphorylation induces the E3 ligase activity.

Phosphorylation is also required for BMI1's oncogenic potential. In human prostate cells that have lost PTEN, knocking down BMI1 in a dose-dependent manner shows a decrease in proliferation. Re-adding the wild type form of mouse BMI1 complements this defect, whereas the phospho-mutant does not.

The BMI1/RING1B E3 ubiquitin ligase activity is required for oncogenesis, and is regulated by phosphorylation. BMI1 phosphorylation control and its transcriptional repression may separately contribute to oncogenic transformation.

BMI1 is also recruited to sites of DNA breaks, and contributes to DNA damage-induced H2A ubiquitination and efficient DNA repair.

BMI1-deficient cells or BMI1-knockdown cells show a partial G2-M arrest, and the DNA damage response is activated, as measured by pCHK2 or pH2AX. This is interesting because BMI1-deficient cells in mice produce reactive oxygen species, which could lead to activation of these pathways.

Using UV laser stripe approach shows that BMI1 localizes to sites of UV damage. This response is not restricted to UV, and also happens with replication stress or radiation. BMI1, RING1B and CBX2 are all recruited to sites of DNA damage.

BMI1 can be placed in the DNA damage pathway using various knockout constructs. These experiments place BMI1 upstream of 53PB1/RAP80 and BRCA1, but downstream of phosphorylation to ATM/ATR and RNF8.

Importantly, BMI1 is required for mono-ubiquitination at sites of damage, but not for poly-ubiquitination, which also occurs at sites of DNA damage. BMI1 knockout abolishes ubiquitination of H2A at sites of DNA damage, which can be complemented by putting back wild type BMI1.

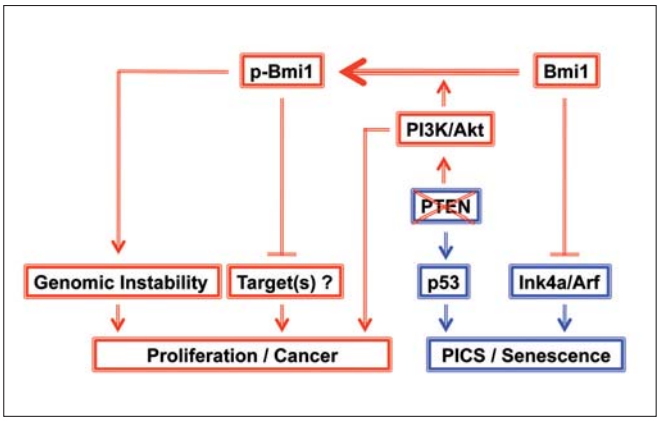

Figure 4
Model for cooperation between BMI1 and PI3K in prostate cancer.

The BMI1/RING1B E3 ligase mediates H2AX mono-ubiquitination at sites of DNA damage, and is required for efficient double-strand break repair. BMI1 3A phospho-mutant is deficient in DNA repair function but interestingly, retains transcriptional repression at many target genes.

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PART III: Epigenetic reprogramming in cancer

Long-range epigenetic deregulation in cancer

A report on a lecture by
Susan Clark
The Garvan Institute of Medical Research, Darlinghurst, Australia

In normal cells, the bulk of the genome is methylated, but CpG island-associated promoters of active or bivalent genes remain unmethylated. In cancer, tumor suppressor genes with CpG island-associated promoters are commonly hypermethylated and silenced, and repeat regions and oncogenes are reported to be demethylated and activated. Many histone modifications also exhibit a distorted regulatory pattern at gene promoters and in the body of genes. However, unlike in the case of epigenetic repression, there are few genome-wide studies to determine the extent of epigenetic activation in cancer. And it is unclear whether epigenetic deregulation contributes to cancer causation or is merely a bystander effect of transcriptional deregulation. ***Susan Clark*** *presented data showing that epigenetic reprogramming in cancer is not just limited to single genes, but can occur across large domains resulting in the repression or activation of domains of cancer genes, and is associated with concordant changes in gene expression, DNA methylation and chromatin landscapes in the cancer cell.*

There are two meters of DNA in every cell. One of the roles of chromatin in the nucleosome is to organize the DNA in such a way such that, during normal development and differentiation, active genes and associated control regions are in open or accessible states, whereas genes not essential for expression are in closed or repressive chromatin states.

Unmethylated CpG island promoters are commonly associated with active genes in open chromatin regions, whereas methylated CpG island promoters are repressed and embedded in closed chromatin regions.

In cancer, the methylation state of CpG island promoters is often perturbed with inactivation and hypermethylation of tumor suppressor genes. Conversely, oncogenes are thought to be activated by demethylation, but the process associated with aberrant methylation remodeling is unknown[1].

A full catalogue of all epigenetic changes that occur in cancer is required in order to understand the mechanisms that underpin these remodeling events. The biggest advance over the past 10 years is

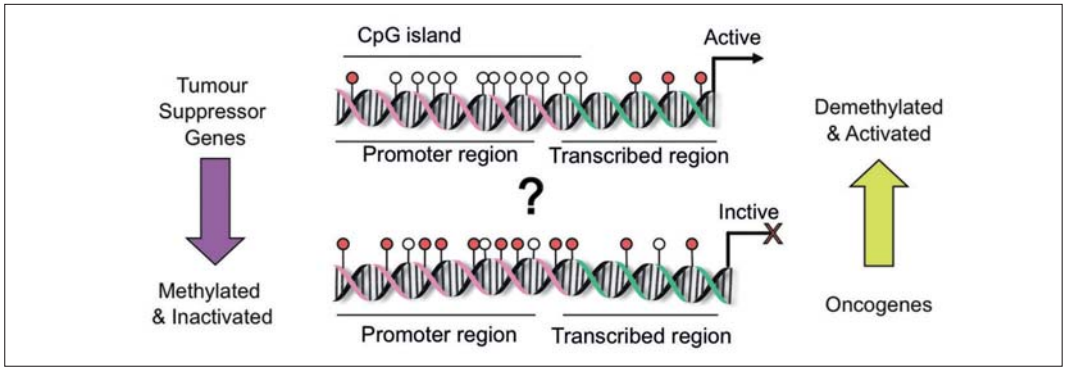

Figure 1
Single-gene loci in cancer.

the development of deep-sequencing tools that can 'mine' the cancer epigenome and dig deeper to discover the layers of information above the DNA sequence.

DNA is not naked, but is wrapped around histones, forming the 10nm-wide "beads on a string" as seen in the first electron microscope pictures in the 1960s. The challenge now is to use the new epigenomic tools of methylation and chromatin sequencing to unravel the relationship between the various layers of change that occur between a normal cell and cancer cell.

At the core of the epigenome is the DNA sequence, which can be modified by DNA methylation. Above the DNA sequence are the histones, which can be modified in various modes that lead to either gene expression or repression.

Interestingly, it is not just single genes that are deregulated in cancer: regions encompassing many genes can also be affected by both DNA methylation and histone modifications.

A study of colorectal cancer has revealed a 4 MB gene-rich and CpG island-rich region on chromosome 2q that is repressed[2]. Methylation sequencing of the CpG islands in this region show that this suppression is associated with DNA methylation. Surprisingly, multiple neighboring CpG islands are also methylated in the colorectal tumors and the entire region is embedded in repressive chromatin marked by histone H3K9me2.

This process, termed long-range epigenetic signaling (LRES), combines both DNA methylation and chromatin remodeling to repress large regions of the cancer genome[3].

Concordant repression:

One important question is why some of the CpG islands are methylated in cancer, but others are protected from methylation. Study results suggest that there is an inverse correlation between gene expression in the normal cell and the level of methylation found in the cancer cell. The level of gene expression in normal tissues may therefore protect some CpG islands from DNA methylation.

To determine whether LRES is common in other cancer types, Oncomine data sets were used to survey 380 prostate tumor samples and 215 normal prostate samples and find regions of contiguous repression.

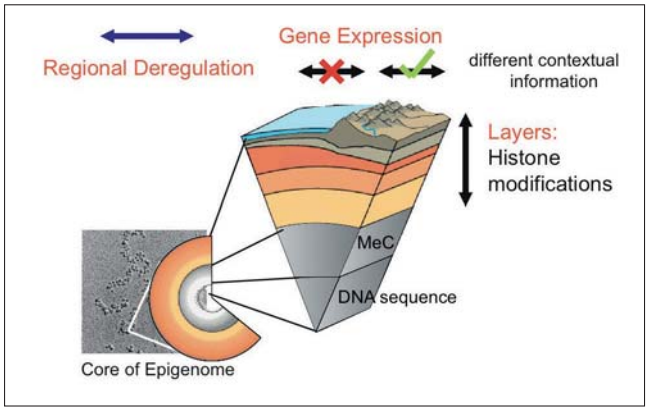

Figure 2
Mining the epigenome: Layers remodeled in cancer.

This study identified 47 regions that show concordant and significant repression of at least four neighboring genes in multiple data sets. Treatment with 5-Aza-dC of prostate cancer cell lines shows major reactivation of genes, including in these regions, across the genome. This includes the HOXA cluster, already reported to be epigenetically silenced in prostate cancer, as well as the 2 MB 7q31.2 region[4].

The 7q31.2 region typifies the diversity of epigenetic remodeling patterns found in the other LRES regions. Comparing normal prostate epithelium and prostate cancer LNCaP cells, three modes of epigenetic silencing have been identified.

The first mode (repression) or loss of expression in the cancer cells is typified by the overall loss of H3K9 acetylation. These regions also gain H3K9 dimethylation, a repressive mark and, unexpectedly, H3K27 trimethylation. In addition to gaining histone repressive modifications, the CpG islands are hypermethylated. These genes include a number of tumor related genes: TES, CAV1, CAV2 and c-MET.

The second mode (consolidation) or re-inforcement of repression in the cancer cell is typified by a further gain of H3K27 trimethylation.

The third mode (exchange) or replacement of repressive marks, is typified by an exchange of H3K27 trimethylation and DNA methylation (see Laird, page 57). So, in this example of cancer, a single region across 7q31.2 region harbors three different modes of epigenetic change that all result in repression or consolidation of repression.

Many other regions show similar blocks or modes of repressive marks, indicating that LRES frequently occurs in modules, and the role of LRES may be to reduce genome plasticity so there's less of the genome available for controlled regulation.

Concordant activation:

Because LRES is common in cancer and occurs at multiple regions across the genome, long-range epigenetic activation (LREA) may also occur in cancer and result in activation of contiguous genes[5].

Expression arrays and RNA.Seq were used to pinpoint regional changes in gene activation in prostate cancer, Chip on Chip and Chip.Seq to identify changes in active or inactive chromatin marks, MBD.Cap sequencing arrays to identify changes in DNA methylation, and SNP arrays and IP.seq to control for regions that are genetically changed versus epigenetically modified.

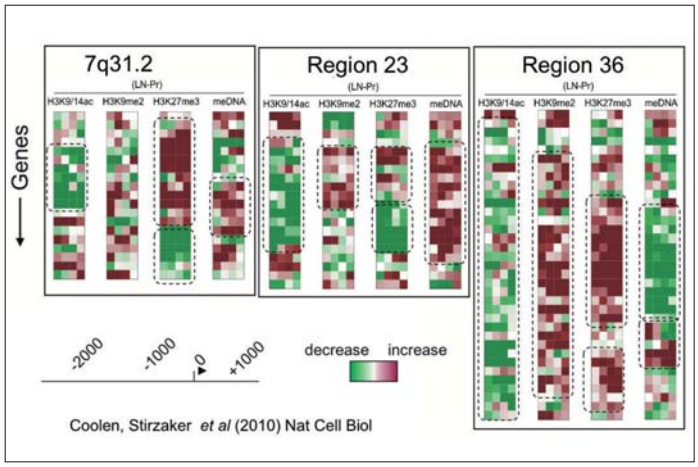

Figure 3
LRES frequently occurs in modules.

To faithfully interpret histone and DNA methylation marks using high-throughput sequencing, it's important to make sure that the interrogated regions haven't become amplified, thereby giving rise to a higher signal in the cancer samples.

Using Oncomine data and a number of prostate cell lines to identify 4 neighboring genes that are concordantly activated reveals 35 regions that have LREA potential in prostate cancer. These regions have high significance, and are distributed evenly among the chromosomes. They also show that concordant silencing and activation can occur in the same cell.

The regions have an average size of about 1 megabase. There are 251 genes in total, with the average size containing 7 genes. Interestingly, genes in this region include several interesting several prostate cancer biomarkers, such as KLK3 or Kallikrein 3, the prostate-specific antigen or PSA, which is currently used as a biomarker in prostate cancer.

In addition, one of the activated regions contains PCA3 (prostate cancer specific non-coding RNA), which is also over-expressed in 95% of prostate tumors. Many microRNAs in this region are also activated along with neighboring genes.

Comparing histone modifications across LREA and LRES regions, there's a general loss of H3K9 acetylation in LRES regions and a gain of H3K9 acetylation in LREA regions across transcription start sites (TSS). LREA regions also gain H3K4me3 and LRES regions lose it, and LREA regions show a loss of the H3K27me3 mark.

Modification modes:

There are two dominant modes of epigenetic histone modification. The first is an exchange of active marks with an increase in H3K9 acetylation and a decrease in H3K27me3. The second is an increase in H3K9 acetylation and an increase in H3K4 trimethylation.

There are 13,000 CpG islands in the genome near TSS (+/- 2.5 kb). Using MBDCap.Seq, most of these CpG islands have been shown to remain unchanged in methylation when the associated gene is activated.

Surprisingly, of those CpG islands that do show changes, most gain methylation. Across all CpG islands, 25% are hypermethylated and less than 1% are hypomethylated.

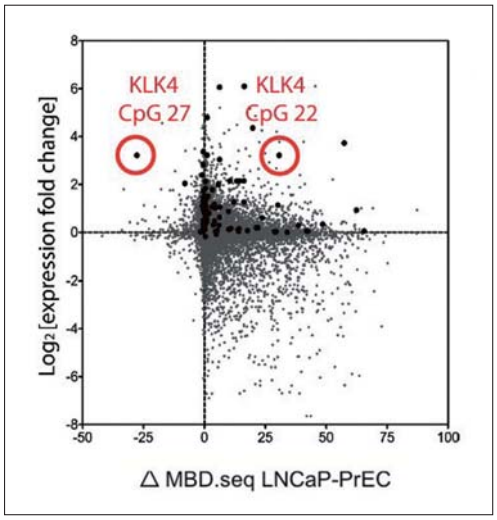

Figure 4
Changes in CpG island methylation and gene activation.

CpG islands in the LREA region show the same profile. Of the 135 islands in these regions, 23% gain methylation and less than 1% lose methylation. The islands that become hypermethylated and increase in expression are generally associated with poorly annotated promoters or alternate transcripts.

This pattern is unexpected, as the prediction would be that the islands would gain expression and lose methylation. Interestingly, one of the only hypomethylated promoters with an increase in expression is in an LREA region, that of KLK4.

However, further analysis shows that KLK4 has two CpG islands associated with the promoter and the other one gains methylation.

KLK4 is a serine protease and part of the KLK family. All the members of this family gain H3K9 acetylation, and lose H3K27 trimethylation. This hypomethylation has been validated using clonal bisulfite sequencing, the gold standard methylation detection technique.

Looking at the KLK4 structure, CpG27 is methylated in a normal cell and becomes demethylated in cancer. By contrast, CpG 22 is normally unmethylated and becomes methylated in cancer. RNA-Seq data combined with CAGE data shows that the change in methylation affects transcription.

The other class of hypermethylation identified is in those that gain methylation only at the borders, or flanks of a CpG island. This is associated not with a change in where the TSS is, but with an increase in H3K4me3 and ectopic activation of the transcript.

In some cases, the border is on both sides of the transcriptional unit, and it's mutually exclusive with H3K4me3 and the start of transcription.

Of all the CpG islands in this region, the majority falls into the class that gains border methylation. The group that shows a change in transcription comprises about a third.

Two models:

These observations suggest two models for gene activation and CpG methylation. The first is quite simple: methylating one promoter and activating an alternate promoter. In the second, methylation at the border of the CpG island doesn't affect the TSS.

One hypothesis for this second model is that the CpG site may harbor a repressive element and when that's methylated, the repressive element is no longer able to bind, allowing an increase in transcription or activating ectopic transcription.

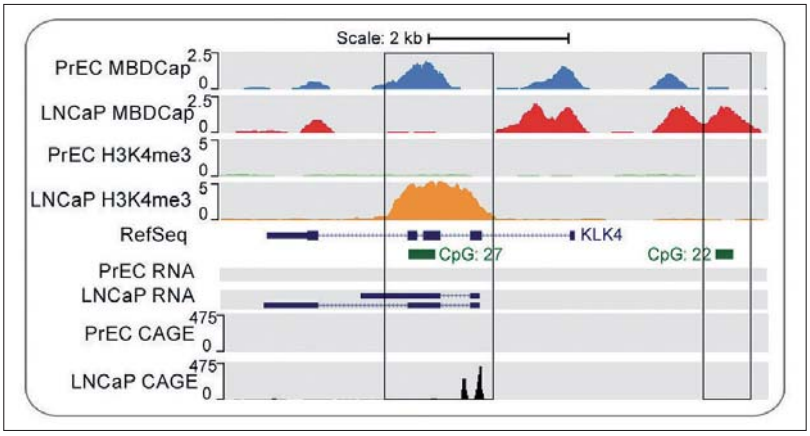

Figure 4
Activation of KLK4 at exon 2.

A bioinformatics analysis of these CpG sites shows that they harbor repressive elements. The next step is to find out whether this is a mechanism of gene activation by specific methylation of repressive elements.

In summary, CpG islands are inactivated by many different modes. In particular, there is gain, loss and exchange of repressive marks. In some cases, there is also a gain of DNA methylation.

For gene activation, there is very little evidence of demethylation that is associated with activation of CpG island genes and, in particular, oncogenes. This is not surprising because most CpG islands are normally already unmethylated.

The mechanism associated with aberrant changes in methylation is unknown, and the questions for the past 10 to 20 years have remained the same. Where are the loci that are epigenetically modified in cancer? And what triggers a change in DNA methylation and chromatin remodeling and gene expression in cancer?

The more the epigenome is mined, the more complicated will be the answers and the new questions that will arise. Recent work shows that in cancer, not just single genes but regions encompassing many genes can be deregulated. This raises questions about why some genes and not others are regulated in domains, what dictates the spread of chromatin remodeling and whether a change in gene expression dictates epigenetic remodeling or *vice versa*.

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Mining the cancer methylome

A report on a lecture by
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Epigenome Center, University of Southern California, Los Angeles, USA

Cancer develops not only as a result of genetic mutations and genomic rearrangements, but also as a consequence of numerous epigenetic alterations, including extensive changes in the distribution of DNA methylation throughout the genome. Much attention has focused on the phenotypic consequences of these epigenomic alterations, including the transcriptional silencing of tumor-suppressor genes through promoter CpG island hypermethylation. Comprehensive DNA methylation profiling of cancer genomes has shown that targets of the Polycomb Repressive Complex that are unmethylated in embryonic stem cells acquire DNA methylation in somatic cells as a function of aging. Over time, the acquisition of promoter hypermethylation at key developmental genes may render a stem cell unable to properly differentiate, thus giving rise to a self-renewing cell with an impaired capacity for differentiation, and a prime target for oncogenic transformation. This scenario suggests that an epigenetic block to cellular differentiation may sometimes be an initiating event in carcinogenesis. Even stronger concerted epigenetic change is seen in CpG Island Methylator Phenotypes (CIMP) that have been found in several types of cancer. There are strikingly strong associations between specific genetic mutations and CIMP. ***Peter Laird*** *presented two interesting examples of synergy between cancer genetics and epigenetics.*

Mining the cancer methylome or epigenome may unearth potential clues for understanding how a cell that starts off with a 'normal' epigenome ends up with one that's abnormal.

Across the genome, most normal cells have unmethylated promoters, except for those that show tissue-specific methylation in differentiated cell types. In cancer cells, some genes acquire promoter methylation whereas the gene body or intragenic regions — the areas between the genes — lose methylation.

In the 1990s, the approach to understanding this process was to dissect the molecular components — the enzymes responsible for laying down DNA methylation — and then try to understand what is defective in cancer cells. Over expression of DNA methyltransferases does not appear to be the underlying mechanism responsible for the epigenetic abnormalities observed in cancer, however.

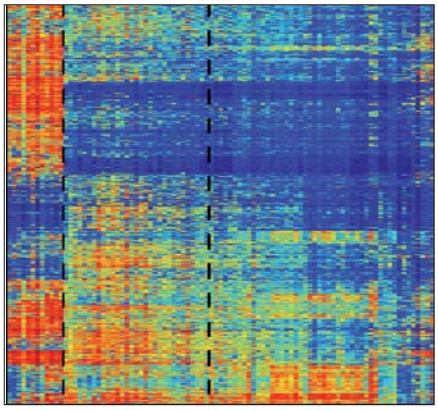

Figure 1
Glioma-CpG Island Methylator Phenotype (G-CIMP).

An alternative approach is to study the diversity of epigenetic alteration in cancer cells. Looking at different profiles and behaviors, between different cancer cells, proteins and genes, might yield some clues.

A 2010 paper characterized the DNA methylation component of glioblastoma. Infinium analysis of 91 glioblastomas and about 1,500 of the most variable DNA methylation loci identified a subgroup of glioblastomas that have a distinct DNA methylation profile. This subset has a large number of methylated genes that are unmethylated in other glioblastomas and in the normal brain[1].

This subset of glioblastomas doesn't just show a genome-wide uniform increase of DNA methylation. Rather, it shows methylation on a distinct set of at least several hundred CpG islands across the genome.

A 1999 study identified a CpG Island Methylator Phenotype, or CIMP, in a subtype of colorectal cancers. These gioblastomas can similarly be marked as G-CIMP tumors.

A paper from The Cancer Genome Atlas also looked at expression clusters of gliomas and identified four different expression subtypes. Comparing both sets of results, the G-CIMP tumors are subsets of proneural glioblastomas, although there are other proneural glioblastomas that do not show the G-CIMP pattern[2].

Survival edge:

These G-CIMP tumors tend to occur at a younger median age, roughly 35 years versus 60 years for other subtypes of gliomas. Proneural tumors have slightly better survival, although this is not statistically significant. But when proneural tumors are divided into G-CIMP non-G-CIMP tumors, the non-G-CIMP tumors have worse survival than the average non-proneural tumors, whereas the G-CIMP proneural tumors have a much better clinical outcome.

In fact, all of the survival benefit of proneural tumors is being driven by this epigenetic phenotype. This is a very interesting result because it suggests that epigenetic classification is identifying a more clinically relevant subtype than expression profiling, which is the norm for many types of cancers.

Non-TCGA samples validate this finding, showing that the G-CIMP phenomenon is present in low-grade gliomas more frequently than in high-grade gliomas. But importantly, the G-CIMP survival advantage is independent of grade and stage and remains a significant predictor of survival even when adjusted for those factors.

When IDH1 and IDH2 mutant tumors — which were not part of the TCGA — are sequenced, they all fall within this unique subtype. Each of the G-CIMP-negative tumors is wild type for IDH1, but there are also 5 G-CIMP-positives that are wild type for both IDH1 and IDH2.

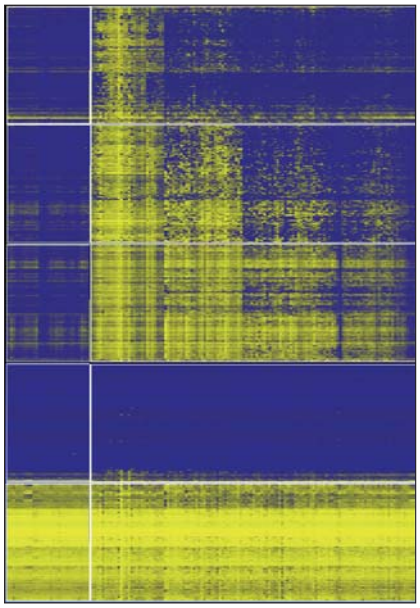

Figure 2
Epigenetic subtypes in colorectal cancer.

These results suggest several models for G-CIMP. The obvious model is that the IDH1 mutation causes aberrant CpG island methylation. This does not explain the discrepant G-CIMP cases without IDH1/2 mutations, however.

The subset of genes in G-CIMP is overlapping but distinct from colorectal CIMP, and from polycomb targets, suggesting that a specific histone demethylase is being affected.

G-CIMP Profile could alternatively reflect a different cell-of-origin with a distinct epigenetic profile. The cell-of-origin could be missed because of its low prevalence in the sample, but could have a methylation profile that gives rise to these cancers.

However, this is unlikely as CpG island hypermethylation tends to appear only in the context of abnormality, primarily cancer or inflammatory situations. This model also doesn't explain IDH1 mutation or the lack of G-CIMP methylation in the normal brain.

The third possibility is that G-CIMP is caused by an unknown defect — for example, a genetic defect in an epigenetic regulator, allowing encroachment of methylation at a large number of genes scattered throughout the genome, and silencing of genes that are antagonistic to IDH1 mutation.

Correlation vs. cause:

Work published a few years ago showed a tight association of CIMP with BRAF mutations. Among BRAF mutants in colorectal adenocarcinomas, each one falls within the so-called 'CIMP-high' epigenetic subtype, once again illustrating a close correlation between a genetic and an epigenetic event[3].

Is this association mere correlation or cause? When mutant BRAF is stably transfected into a CIMP cell line, cultured for many passages and checked for DNA methylation changes using arrays, there is no evidence of BRAF-associated DNA methylation changes[4].

A more likely explanation may be related to the observation that BRAFV600E induces cellular senescence in normal cells. There is some evidence that IGFBP7 mediates the BRAFV600E-induced senescence and apoptosis.

IGFBP7 is methylated in the overwhelming majority of CIMP tumors — including those with BRAF mutations — and transcriptionally silenced. This suggests that CIMP-associated transcriptional silencing of IGFBP7 is a precondition to acquiring BRAF mutation.

In summary, there is a clear association between genetics and epigenetics. There is some form of defect associated with CpG island methylator phenotype: either a gain-of-function defect involving misdirection of DNA methylation, or loss-of-function, involving defective protection.

Distinguishing between drivers and passengers is much more difficult with epigenetics than it is for mutations because the recurrence of independent mutations in independent tumors can't be used as a way to distinguish putative drivers from passengers.

In ES cells, polycomb target sites have a predisposition to acquiring abnormal DNA methylation in tumors. It's not surprising that tumor-specific methylation occurs at these polycomb sites, but it's worth noting because the vast majority of CpG islands in the genome do not acquire abnormal DNA methylation in tumors.

At the constitutively methylated sites, there are no K4 or K27 trimethylation marks. CpG islands that are methylation resistant have primarily the K4 activation mark. But in non-CIMP, CIMP-Lo and CIMP-Hi tumors,

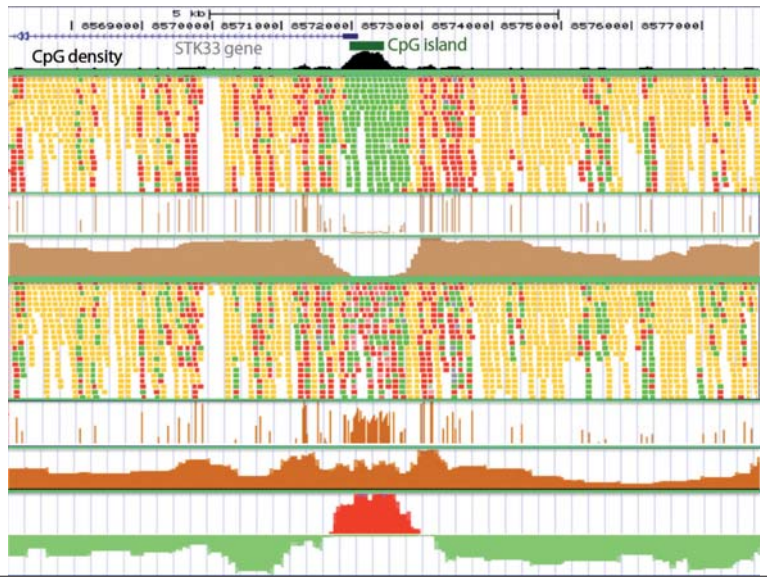

Figure 3
Methylation-prone CpG islands.

the majority of CpG islands in ES cells are targets of polycomb repression. The polycomb targets in ES cells have a 10-fold higher probability of acquiring tumor-associated DNA methylation compared with non-polycomb targets.

Plotting low to high DNA methylation in human ES cells and the normal colon shows a loss during the differentiation path from ES to normal somatic development. By contrast, the polycomb targets even in the normal colonic mucosa acquire methylation even though they remain unmethylated in ES cells. During tumorigenesis, there is a bigger shift in polycomb marks acquiring DNA methylation.

In this model, ES cells have bivalent sites that are polycomb occupied. Adult stem cells may also have similar patterns. When a key differentiating transcription factor acquires abnormal DNA methylation, the ES cell is unable to activate its differentiation program and becomes a prime target for transformation to cancer. For example, colorectal tumors may still show evidence of this crosstalk even though the polycombs are long gone at these sites.

This is a speculative, but attractive, model. It would explain the DNA methylation behavior for about half of cancer-specific methylated genes. One of the best colorectal methylation markers is MYOD1. This gene is never expressed in colonic lineage, yet it is exquisitely methylated in colorectal cancer.

The model is also consistent with the observation of epigenetic field effects adjacent to tumors, with the stem cell-like behavior of cancer cells, and with the evidence for tumor-initiating cells. It also suggests that therapeutic cloning strategies using human ES cells or iPS cells should incorporate screening for polycomb target DNA methylation abnormalities. Most provocatively, it suggests that, at least in some cases, the first steps of oncogenesis may be epigenetic in nature.

Cancer methylome:

The entire cancer methylome has been characterized for two human primary tissues.

In a normal colon, the CpG islands are unmethylated, but there is extensive methylation elsewhere. In the tumor, by contrast, there's no longer any distinction between the CpG islands and the surrounding regions. There's a gain of a lot of methylation within the island and in the region and loss of methylation outside the island.

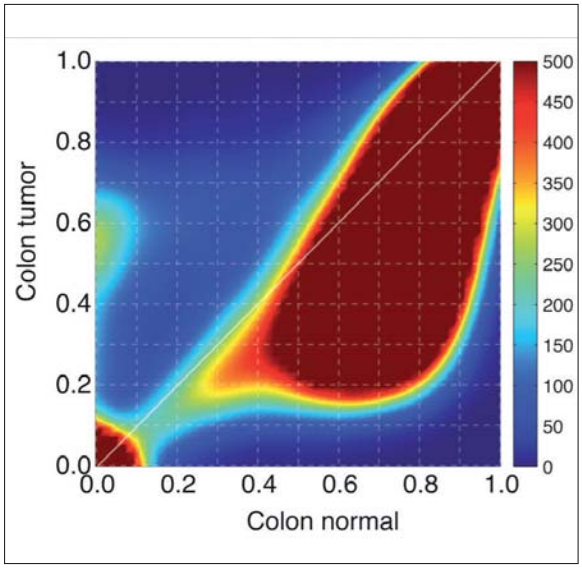

Figure 4
Cancer-associated DNA hypomethylation and hypermethylation

Whole-genome analysis using infinium array and whole-genome bisulfite sequencing both yield the same results. A large number of sites that were methylated in the normal become hypomethylated, a small number of CpG islands that are unmethylated in the normal acquire methylation. There are methylation-prone elements, methylation-resistant elements and constitutively methylated elements.

Repetitive elements are enriched in the methylation-resistant sites. Polycomb sites and some CpG islands tend to be methylation-prone, and these elements tend to be unexpressed or have low expression levels in both the normal and the tumor. Having robust expression in the

normal tissue is one of the preventive elements that reduces the probability of acquiring cancer-specific methylation and repression.

The methylation protection mechanism includes expression, the proximity to repetitive elements and passive demethylation through TET (see Helin, page 91).

On the log scale, the further sites are from the CpG islands, the more likely they are to be subject to hypomethylation. This suggests that the CpG islands prone to methylation are lying in oceans where the hypomethylation is more extreme. It's as if the two parts of the genome were segmented into a 60:40 ratio of epigenetically unstable and stable regions.

In IMR90 fibroblasts, hypomethylated oceans correspond to partially methylated domains (PMDs) and lamin attachment domains (LADs). These are the areas in the periphery of the nucleus that are kept in a repressed state and are epigenetically unstable in cancer.

In this model, epigenetic instability is associated with disruption of nuclear architecture. In the normal epithelium, there may be other repressors, but the nuclear architecture remains intact. In a cancer cell, there may be an association with the nuclear lamina, which then results in transition of DNA segments into the laminar attachment regions.

In summary, there are CIMP defects, methylation protection mechanisms, polycomb crosstalk and nuclear architecture changes. These are distinct mechanisms, with some overlap of targets. So, for each cancer type and each gene, there is going to be a different story that's quite complex and that will need to be teased out over the next few years.

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Linking the polycomb system to the evolution of epigenetic abnormalities in cancer

A report on a lecture by

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Epigenetic abnormalities in cancer present great potential for therapy. The best defined of these abnormalities is the silencing of hundreds of genes, often associated with DNA hypermethylation of promoter CpG islands. About 50% of the hypermethylated genes in colon cancer are marked by Polycomb in embryonic stem cells. PcG marking is almost solely in the context of the bivalent chromatin that maintains key developmental genes in a low, but poised, transcription state in order to maintain stem cell self-renewal capacity and prevent premature lineage commitment. An integrative genomic approach has shown that up to 80% of genes DNA hypermethylated in multiple cancer types have a history of promoter bivalency, not only in ES cells, but in adult stem cells. Abnormal DNA methylation provides the genes with tighter transcriptional repression, and this could help evolve a stem-like state for key sub-populations that initiate and perpetuate tumors. Exposure of cells to reactive oxygen species, a key component of cancer risk and tumor progression, rapidly induces a large complex of proteins, which targets away from the CpG-poor regions of DNA methylation loss to gene promoter CpG islands. This complex contains DNA methyltranferases, SIRT1 and PcG proteins and is tightly bound to chromatin. ***Stephen B. Baylin*** *reported that chronic exposure of cells during tumorigenesis to the formation of this complex may be a key step in rendering genes vulnerable to abnormal promoter DNA methylation in cancer cells.*

Several epigenetic abnormalities have been linked to cancer in addition to the many mutations in cancer-related genes. Epigenetic abnormalities could contribute heavily to the derivation and phenotypes of stem-like, self-renewing cell populations in cancer. And if so, they may present a therapeutic opportunity.

Aside from oncogenic addiction to either tumor suppressors or oncogenes, there could also be an epigenetic addiction. For example, oncogenes or some other mutations might be able to drive signal transduction to influence the epigenome.

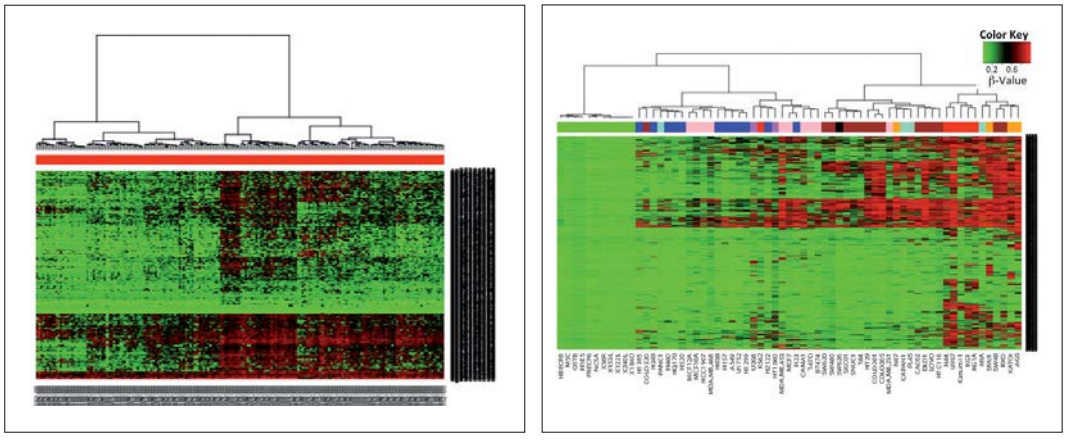

Figure 1
There are clear differences in methylation patterns between cancer samples (right) and normal samples (left).

They might be able to create or inherit the survival of a relatively primitive cell population. And if that terrain is pulled away from them with therapeutic maneuvers, despite the mutations, it might engender an anti-tumor effect.

There is an imbalance of the epigenome in cancer. Sequencing shows regionality with simultaneous losses and gains of methylation in the body of the gene or in the promoter.

Some of the genes that are known to be epigenetically altered in cancer are the familial tumor suppressor genes, such as RB, p16, APC and BRCA1. In an individual who has inherited the mutation in one allele, DNA hypermethylation is an alternative mechanism for inactivating the gene. In some cancers, more than one of these genes might be hyper-methylated at the same time, resulting in a powerful effect.

The Illumina Infinium methylation array is a platform to screen across the genome for the DNA methylation status of annotated CpG islands in the promoter region, and other regions. Such an analysis of 55 cell lines of human cancer, including colon, lung and breast cancers, reveals that there are hundreds of hyper-methylated genes. Embryonic stem (ES) cells, osteoblasts, mesenchymal stem cells, non-immortalized breast epithelial cells and prostate epithelial cells are all unmethylated in this region for these genes.

Among samples of primary breast cancers, colon and lung cancer samples from the Cancer Genome Atlas, even when normal samples adjacent to tumors show a field effect of changes, there is a clear demarcation of the cancers from the normal samples.

About 50% of the genes that are hypermethylated in cancer overlap with 10% of genes that are marked by Polycomb in ES cells, suggesting that Polycomb marks make the genes somehow vulnerable. The epigenetic marks in precursor stem cells may be instructive in defining aberrant methylation.

Bivalent marks:

But human cancers in adults don't start in ES cells, they start in adult cells. In comparing a human bone cancer to adult bone marrow derived mesenchymal stem cells, and cells differentiated from these, a ChIP-Seq analysis for the bivalent marks — the active mark H3K4Me2 and the inactive repressive mark H3K27Me3 — matched to arrays for gene expression and methylation, 400 genes hypermethylated for gene promoter CpG islands in the cancer aren't methylated at all in the progenitor cells.

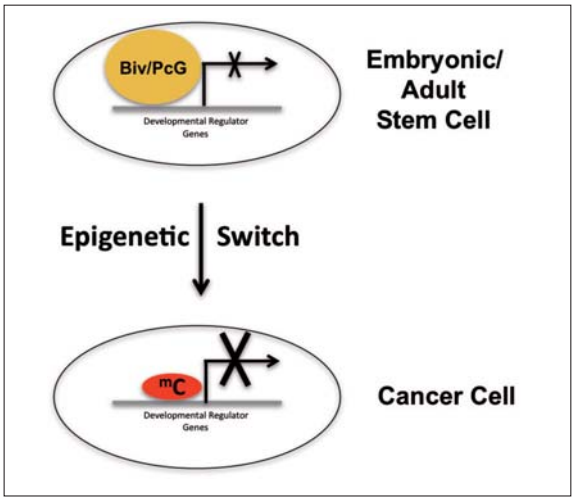

Figure 2
Model for molecular progression to DNA hypermethylation of many genes in cancer.

Looking closely gene by gene, there is an important region along chromosome 9p, with three tumor suppressor genes in a relatively short space: p16, p14 and p15. Where the progenitor cells have bivalency of marks, mesenchymal stem cells and osteoblasts are remodeled and have a remnant of bivalency, but predominantly have the active mark. All three of these genes are hypermethylated in osteosarcoma cells, and they lose both marks[1].

About 80% of genes in osteosarcoma and every other tumor track back to the bivalency, and not only in human ES cells. About 70% to 80% of those hypermethylated genes in the cancers also have bivalency in hematopoietic stem cells. Even in a slightly later progenitor, those marks have all remodeled, and are mostly Polycomb marks.

Interestingly, the genes that are normally bivalently marked in stem cells but abnormally methylated in cancer are predominantly developmental regulators, including DNA-binding genes, transcription regulatory proteins, and transcription factors. About 300 of these genes are hypermethylated in one or more human cancers.

The genes that are methylated but marked only by H3K4, the more active mark in ES cells, are also developmental regulators, but most are cell-surface receptors and cell signaling genes. These genes are important for maintaining the stemness of ES cells or hematopoietic stem cells.

The PRC module includes a group of genes that have low expression in stem cells and in cancer cells, but are activated in differentiated cells. In osteosarcoma cells, about 15% of these genes are hypermethylated, and have a lower level of expression compared with the unmethylated genes.

DNA methylation, thought to be the most stable repressive mark, may prevent those genes from being accessed. Although all cancers have some number of these genes, the proportions vary. Lung cancer has the least found so far, whereas leukemia and gastric cancer have many, for example.

In this model, during tumor progression, the bivalency of an important subset of genes switches to a methylation mark. If this mechanism locks in the survival of a key subpopulation of 'relatively primitive' cells, it could be the target for therapy.

Driving cancer:

The key mutations that drive colon cancer are in APC and β-catenin. Every colon cancer also has one or more anti-WNT genes that are hypermethylated, blocking WNT-FZD interaction at the cell surface receptor. This is a mechanism for increasing β-catenin in these cells.

One anti-WNT nuclear gene, SOX17, is methylated in nearly 100% of colon polyps and human colon cancers. That gene blocks the interaction of β-catenin and TCF. So genes that would otherwise block the WNT pathway are epigenetically altered, in combination with a mutation that would also help drive the pathway. What's more, in about 10% of cases of human colon cancer, APC itself can be methylated.

The HIC1 gene, for hypermethylated in cancer 1, was cloned several years ago by randomly scanning chromosomes for hypermethylated genes in cancer. HIC1 is located close to p53 on chromosome 17p. Although it's methylated at one of two upstream exons, no meaningful mutations have been described in the gene.

HIC1 is methylated in every cancer and often even in the precancerous state. It's a transcription factor with a conserved so-called POZ domain. HIC1's downstream repressive targets include the survival genes SIRT1, ATONAL, EPHA and SOX9.

If HIC1 is knocked out homozygously, the embryos develop many mesenchymal and epithelial defects, so the gene is critically important for development. Heterozygous mutants live but, as they age, they develop tumors.

In these tumors in heterozygous mutant animals, the wild type allele is almost always hypermethylated, similar to what's seen in human cancers. Combining that knockout with p53 augments it, and combining it with hedgehog activation in the cerebellum augments medulloblastoma formation.

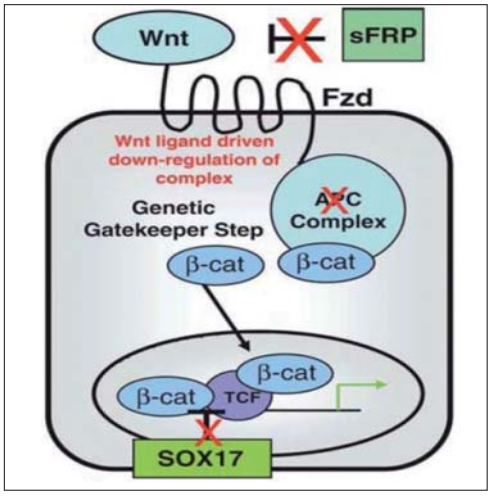

Figure 3
WNT activation in colon cancer.

In the gut, HIC1 heterozygotes have about 25% more crypts per villus compared with controls, and the crypts look dysplastic by pathology. APC mice don't have this effect, but when the HIC1 heterozygotes are crossed with APC mice, the double heterozygotes show the crypt effect.

They also have enhanced tumorigenesis, with about four- to five-fold increase in colon polyps[2]. The phenotype is increased in stem cell-like crypts in the gut.

In the colon, HIC1 is in the terminal, more differentiated cells, primarily in the nucleus, and the heterozygote is haplo-insufficient. In the APC heterozygous mice, the colon is not normal even outside the polyps. When APC is knocked out, the animals also lose a lot of HIC1.

In the wild type animal, the transcriptional targets for HIC1, such as SIRT1, are mostly in the crypt cells, but in the HIC1 knockout, the amount of SIRT1 is intensified, and is seen throughout the crypt region and up through the tip of the villi. The APC mice have exactly the same phenotype even without knocking out HIC1.

Molecular progression:

The relationship between Polycomb marks and DNA methylation is not completely understood, but the working model describes it as a molecular progression. During tumorigenesis, a gene that's marked bivalently in ES cells or adult stem cells or in progenitor cells somehow switches bivalency more toward DNA methylation, resulting in stable repression of transcription and abnormal gene silencing[3].

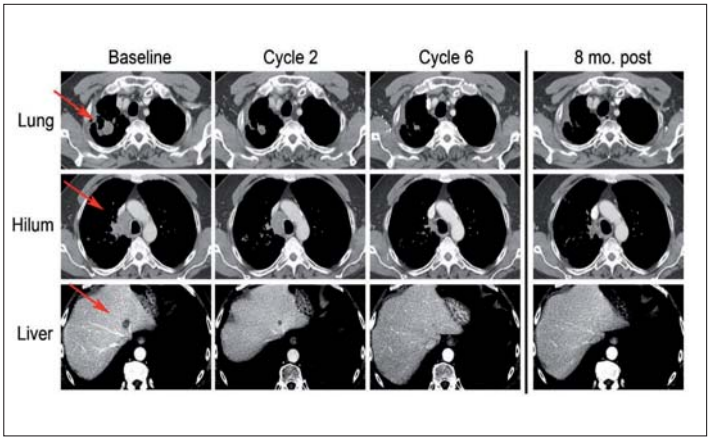

Figure 4
Images of patient with partial response to vidaza+etinostat: Hepatic metastases.

DNA damage of double-strand breaks recruits some of these same chromatin aspects, such as Polycomb and DNA methyltransferases. One factor that underlies common risk factors of chronic inflammation is the stress that aging puts on cell renewal systems.

When embryonic carcinoma cells and human colon cancer cells are insulted with hydrogen peroxide to raise levels of reactive oxygen species, within 30 minutes there is a dramatic change in all the complexes.

Before damage, there are many smaller molecular weight complexes of direct or indirect interaction between DNMT3B and DNMT1, which bind to HDAC. The demethylase LSD1 can also interact with one or more of these proteins. But after exposure to ROS, within 30 minutes, all of these players, which also show interaction with Polycomb constituents, form a large complex that is at least 1.5 megabases in size.

This stress-induced silencing complex, dubbed PRC4, also has a special isoform of EED2, which is a partner for EZH2, and SIRT1. PRC4 is present in cancer cells, embryonic stem cells and adult stem cells. DNMT1 is no longer salt labile to the chromatin, and binds tightly to chromatin.

Along the chromosome, the key constituents of this complex — EZH2, DNMT1, H2AX and DNMT3B — move away from GC-poor to GC-rich areas, and are often right over CpG islands. During chronic inflammation, complexes like this may keep certain genes under pressure over years of development of the cancer.

Epigenetic therapy raises the possibility of reprogramming these events. Existing agents such as 5-azacytidine and HDAC have myriad effects, but it may be possible to distill those down to epigenetic effects.

The drugs are so toxic that no patients can get through them, much less show efficacy. But since the doses have been lowered, drugs like azacytidine have had real therapeutic efficacy in myelodysplasia and AML (see Issa, page 69), and the U.S. Food and Drug Administration has approved them for that use.

Most solid-tumor trials so far have been with high doses, so trials need to be revisited. Also, the tumor does not respond immediately and it often takes months to see clinical improvement.

At the lowest doses against leukemia cell lines or solid tumor cell lines, the drugs do not cause much apoptosis, DNA damage or kill the cells in the first few days. When the drug gets into cells, however, DNMT1 is gone within 3 days.

When a leukemia cell line is exposed to the drugs, the cells fail to clone in long-term cell renewal assays. But more important, when they are put back into an animal that hasn't been treated with drugs, the cells don't generate tumors in the periphery or bone marrow, suggesting that the drugs have a 'memory' effect[4].

Timed expression and methylation arrays show a drop in methylation at the low doses. There are anti-tumor effects by the reactivation of hypermethylated genes, driving of the cells into the cell cycle, a loss of enzymes that would maintain an alternative energy source, or increased immune antigens on the cell surface.

In a preliminary trial of non-small cell lung cancer, low doses of azacytidine and an HDAC inhibitor were given to 32 individuals who had failed chemotherapy. The trial generated several robust responses.

In one typical response, that of a smoker who had been through three attempts with chemotherapy and still had big masses, the drugs didn't have much effect two months after the dose. The tumor responded slowly over time, however, and was gone at 8 months after treatment. More than two years later, the patient is still alive. However, it's important to understand which sub-group of patients might respond to this treatment, and whether it really is working predominantly through an epigenetic mechanism.

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Epigenetic therapy

A report on a lecture by
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Epigenetic reprogramming reverses key aspects of the malignant phenotype. Drugs that target epigenetic modifiers have proved effective in subsets of patients with cancer. There are two classes of epigenetic drugs currently in the clinic: DNA methylation inhibitors and histone deacetylase inhibitors. The first clinical trials with DNA methylation inhibitors have produced spectacular responses in some patients, but the nature and pace of responses is peculiar. The delayed nature of some responses suggests either cancer stem cell exhaustion or an immune mechanism. A strategy targeting genetic changes and epigenetic changes combined or in sequence might be most effective at eradicating neoplasia — a concept being tested in the clinic. Using a new screening method, an unbiased screen of FDA-approved drugs at different doses and different conditions reveals that up to 5% of all drugs in use in the clinic may have epigenetic effects, suggesting that epigenetic modulation could be part of the mechanism of action of drugs developed to treat non-malignant diseases. ***Jean-Pierre Issa*** *made a case for epigenetic drugs for treating and preventing cancer.*

Cancer cells have been found to harbor a number of epigenetic abnormalities, including global hypomethylation, CpG island promoter hypermethylation, histone code alterations and genetic changes affecting epigenetic modifiers.

One of the first genes found to be methylated in any cancer is the estrogen receptor (ER-α). In colon cancer, the tumor and the normal mucosa adjacent to it are both methylated. However, plotting the methylation against age shows that ER methylation is age-dependent.

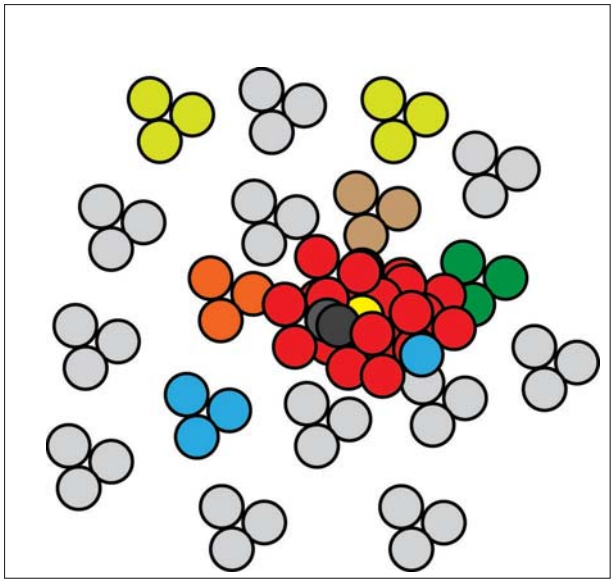


Figure 1
Epigenetic mosaicism in aging stem cells.

Essentially, most of what is thought of as a cancer phenomenon is not cancer-specific, but is an aging phenomenon. Cancer accelerates and extends this process, but it starts in normal aging.

Interestingly, this doesn't apply to all genes. Some oncogenes such as EGFR gain methylation with age, but then lose it in cancer. Most of the genes are Polycomb targets, but about 10% show age-related loss of gene expression.

About 20% of the mouse genome also gains or loses methylation with age. In three years, mice gain about the same degree of methylation as humans do in 90 years. This suggests that the change in methylation patterns is a clock for physiological aging.

Every time a stem cell is brought to replicate, it has a finite chance of accumulating these defects.

As a mouse progresses from pre-neoplastic stages to full-blown acute myelogenous leukemia, there is an increase in methylation. This process is accelerated in cancer, but at the root of it is a default program that happens in normal stem cell replication.

At birth, all stem cells look the same. As we age, stem cells slightly drift from each other, and the stem cells in older people don't function as well as those in young people. What this creates is variability, which is the source of Darwinian evolution and perhaps the root of evolution of cancer.

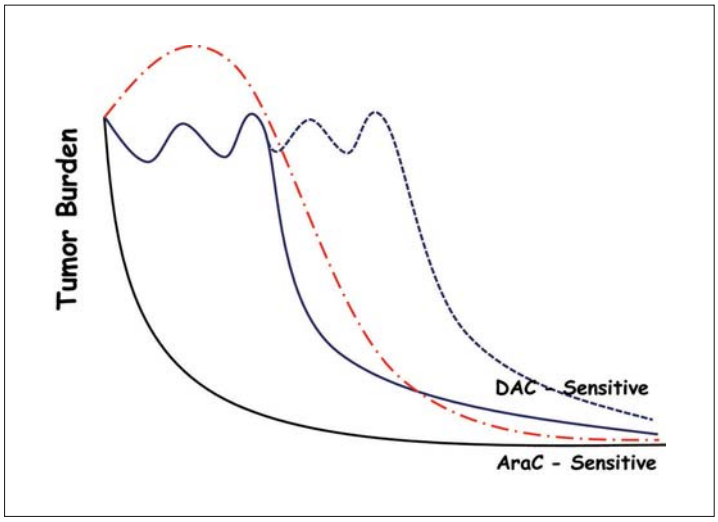
This model argues that the beginning of cancer is epigenetic, and that the reason we get cancer as we age is the accumulation of epigenetic defects in cancer. This is an interesting model, but it flies in the face of dogma, which holds that cancer results from the accumulation of mutations with age.

Modified model:

The problem with the mutations model is that the data do not support it. For example, comparing mutations in APRT in T cells among newborns and 80-year-olds, most of the increase in mutations happens in the first decade of life.

This makes sense because mutations occur during replication, and the most replicative period in life is the first decade of life. There is a slight accumulation of mutations between ages 20 and 40, and between ages 60 and 80 it is virtually non-existent.

Figure 2
Cytotoxic vs. epigenetic responses.



If the mutational theory of cancer were enough to explain age-related cancers, most people should develop cancer in their 20s. Perhaps accumulation of mutations alone is not enough to explain cancer. Cancer may in fact occur when genetics meets epigenetics.

If an oncogene is activated in a fully normal cell, then that cell does not transform into cancer but senesces or dies. However, as people age, some of the cells become primed for transformation. If RAS is activated in one of these primed cells, it would lead to a tumor.

So, the explanation for the age effect in carcinogenesis is that mutations happen in tissue that is primed by epigenetic variability associated with age. This has important implications for prevention and public health.

Hypermethylation with age explains about 80% of aberrant methylation in cancer. The remaining 20% of events are explained by CpG island methylation (see Laird, page 57). This is not related to aging, but to epigenetic instability. It happens very rapidly, with the cells accumulating about 6 to 10 times more aberrant methylation.

If global hypomethylation is the driver in carcinogenesis, it may be through genetic instability that makes cells more sensitive to chemotherapy. If it's through activation of oncogenes, for which there's no data, drugs

could potentially target oncogenes. CpG island/promoter hypermethylation, histone code alteration and epigenetic modifiers could all also be sensitive to drugs.

Clinical success:

Clinical data for the methylation inhibitors decitabine (DAC) and azacitidine (AZA) are very clear. In myeloid lekeumias, they show a success rate of 10-70% depending on dose and the population of patients treated. They prolong survival in MDS compared with supportive care or chemotherapy. There are also anecdotal reports of responses in solid tumors, although the response rate is not yet well defined.

In MDS, AML and CML, the difference in the rate of 10-70% is whether the drugs are used as front-line therapy or used in multiply-relapsed refractory patients. In the latter group, which is the group being tested for colon cancers and other solid tumors, the response rate is 5-10%. But this 5% response rate in a solid tumor might represent a 70% response rate in a previously untreated patient[1].

In vitro, when leukemia cells are treated with DAC, the level of methylation decreases and then plateaus, and then at high doses of the drug, the hypomethylation effect disappears. This explains in part the dose effect with these drugs, underscoring the importance of choosing the right dose. At high doses, these drugs become cytotoxic.

In a clinical trial in which DAC levels increase starting from 50 mg/m2 —about 40 times lower than the maximally tolerated dose of the drug — the response seen at low doses disappears at higher doses. Other trials that followed have led to approval of the drugs by the U.S. Food and Drug Administration, in part because the drugs are better than cytotoxic chemotherapy.

The mechanism appears to be epigenetic, rather than chemotherapeutic, because a cytotoxic drug typically reduces the tumor burden right away. If the patient responds to the therapy, the tumor burden stays low and normal cells come back.

Typical AZA response in leukemia is very different. The tumor burden typically stays the same for about 3 months, and then abruptly disappears. Some patients show no activity for 5-6 months, and then the tumor disappears. Some patients even show an increase in tumor burden, and then a paradoxical disappearance[2].

One remarkable patient, a 75-year-old man who had a mantle cell lymphoma 4 that was effectively treated with chemotherapy, developed a secondary AML in the bone marrow. There was also evidence, by cytogenetics, of a lymphoma relapse. Because of his age, he was treated with AZA or DAC as a single agent for 3 months.

In this patient, AML blasts started at 2-3%, and after one course of therapy, they went up to 60%. This looked like marked acceleration of disease. In the early days, the patient might have been taken off therapy because this looked like progression of disease. But when therapy was continued, the blasts disappeared. And 4 years later, this patient is in remission from secondary AML.

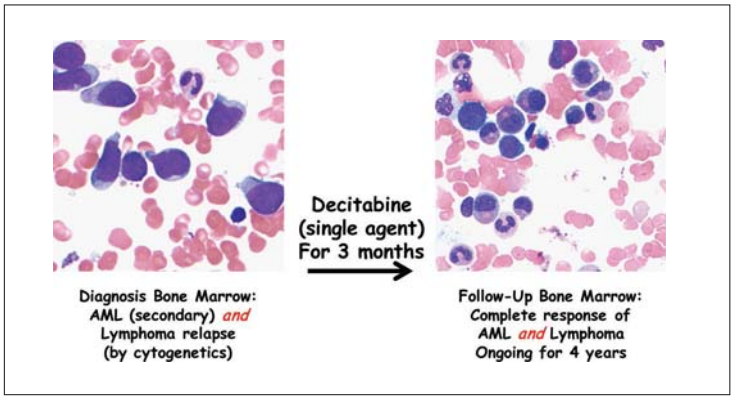


Figure 3
A 75-year-old man treated for mantle cell lymphoma 4 years earlier now presents with cytopenias.

Delayed response:

One likely explanation for this sort of remarkable response is that the therapy affects the tumor cell niche. When these cells can no longer home to the bone marrow, they are released into the bloodstream where they eventually die, whether by apoptosis or by other mechanisms.

The patient lived long enough to get a third malignancy, and developed an isolated plasmocytoma 4 years into his treatment with the hypomethylation drug. It's unusual at that age to have an isolated plasmacytoma, as patients usually develop multiple myeloma, so maybe the drug has some effect.

In some patients, the levels of p15 methylation before treatment are high. The levels decrease in every treated patient within 5 days of therapy, but their tumors don't respond until about 3 months after starting therapy, suggesting that the modulation of the epigenome occurs well before clinical response.

Interestingly, in some patients, the methylation levels decrease and stay low whereas in others, they rebound. Those patients tend to be non-responders. This pattern also holds true for methylation status of other genes.

Gene expression is also a marker of response. For example, by real-time PCR, p15 expression in non-responders is relatively flat. But in responders, expression level goes up and stays up, and this happens starting on day 5, months before clonal replacement happens in these patients.

HDAC inhibitors such as vorinostat and romidepsin are about 30-40% effective in lymphoid disease, but not in myeloid diseases or solid tumors, perhaps because of the nature of the mutations in lymphoid neoplasms[3]. Almost every lymphoma seems to have a mutation in some sort of epigenetic modifier.

To investigate the effect of these drugs, an inducible model was developed in which GFP driven by a hypermethylated CMV promoter is transfected into the YB5 colon cancer cell line. This derives a fully methylated CpG clone at 18/20 CpG sites, with silent chromatin (low H3K4me3, high H3K27me3, no Pol-II occupancy)[4].

When this model is treated with the drugs, demethylation is surprisingly equal in the GFP+ and GFP- cells. Many cells with fully demethylated alleles have no GFP expression. The key to gene reactivation is resetting of chromatin.

The reactivated cells show a decrease in H3K27me3 and H3K9 methylation. IP sequencing of the active alleles shows that about 30% of them are still highly methylated. In this system, it appears that even with DNA methylation inhibitors, the key to their mechanism of action is resetting of chromatin, and not an effect on DNA methylation.

Epigenetic therapy:

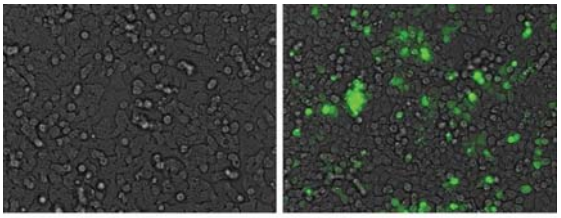


Figure 4
Arsenic trioxide as epigenetic therapy.

All HDAC inhibitors can activate GFP in this system to different degrees. The key difference between HDAC inhibitors and DNA methylation inhibitors is in the stability of the reactivation. DNA methylation provides a memory of the gene expression state. Ultimately, the methylation inhibitors would be better at

achieving long-term epigenetic therapy that is more stable than chromatin resetting alone.

Using a high-throughput 96-well flow cytometry system to look for drugs that activate GFP in this system, in an FDA-approved library of 1,270 drugs, up to 5% have epigenetic effects.

The top 2 hits are DAC and AZA, and the next two are commonly used non-oncology drugs. One of them, surprisingly, is arsenic trioxide, the single most active agent against a form of leukemia called APL. Epigenetic effects of AsO3 could be part of the mechanism of action of this drug in APL. Other drugs might similarly have unidentified epigenetic effects.

Epigenetics is also important for other species. For example, the difference between queen and worker bees — which are genetically identical — is entirely epigenetic. Queen bees live 7 to 8 times longer, a big difference that is purely epigenetic. Queen bee larvae grow up feeding on 'royal jelly', whereas worker bees are exposed to royal jelly once and then never see it again. So the main difference is the environment: the bees' diet.

When NIH 3T3 cells are transfected with an activated RAS, it results in epigenetic silencing of many genes including FAS. Treatment with royal jelly or 10HDA, a component of royal jelly, brings back FAS. By weight, 10HDA is about 10% of royal jelly.

10HDA has chemical similarity to vorinostat and to sodium butyrate and is, essentially, an HDAC inhibitor. *In vitro*, royal jelly and 10HDA both inhibit HDACs and activate histones. Interestingly, broccoli contains an HDAC inhibitor, and sodium butyrate is made by the body.

Nutritional approaches to cancer need solid data but there appears to be pretty solid evidence that people are surrounded by things that modify the epigenome. If it appears to be important for human physiology, it could also modify aging and perhaps modify or treat cancer.

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The dinucleotide CpG as a genomic signaling module

A report on a lecture by
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Throughout most of the mammalian genome, CpG is rare and highly methylated, but so-called 'CpG islands' are often non-methylated and include the transcription start sites and promoters of genes. A subset of CpG islands becomes methylated during development and in cancer. Contrary to previous estimates, CpG islands are equally abundant in humans and mice. More than half in each species are remote from annotated promoters, being either intra- or inter-genic. Despite this, orphan CpG islands, like promoters, are associated with H3K4me3 and co-localize with sites where RNA polymerase II is concentrated. Orphan CpG islands may mark promoters of as yet uncharacterised transcripts, including perhaps numerous non-coding RNAs. In colorectal tumors, methylated CpG islands include both orphan and annotated promoter islands equally, suggesting that cancer does not necessarily recapitulate a developmental program. In both mouse and human genomes, CpG density correlates positively with the degree of H3K4 trimethylation, supporting the hypothesis that these two are mechanistically interdependent. CFP1 associates with the SETD1 H3K4 methyltransferase complex and localizes to the vast majority of CpG islands in the mouse genome. Depletion of CFP1 reduces H3K4me3 at many CpG islands. Insertion of an artificial CpG island-like sequence into the genome results in recruitment of CFP1 and creates a novel peak of H3K4me3 in the absence of RNA polymerase II. ***Adrian Bird*** *proposed that the ability of CpG density to influence chromatin modification directly via CFP1 suggests that this is an important function common to CpG islands.*

CpG islands (CGIs) are vertebrate genomic landmarks that encompass the promoters of most genes, and often lack DNA methylation. CpG islands are located in various places in the genome, but they have similar functions throughout cells[1].

Invertebrates have long stretches of both methylated and unmethylated DNA. In vertebrates, however, DNA is apparently recalcitrant to the methylation-sensitive restriction enzyme, but closer examination reveals a small non-methylated CpG fraction due to CpG islands.

CpG islands are derived from DNA sequences with homogeneous general properties, but differ from one another in DNA sequence. They are usually about 65% GC, whereas the bulk of the genome is 40% GC, and are usually unmethylated. Just under 60% of human genes have a CpG island at the promoter.

Using a DNA-binding domain (CXXC) that has an affinity for non-methylated CpG, CXXC affinity purification plus deep sequencing (CAP-Seq) generates clear, almost background-free profiles showing that clusters of non-methylated CpG are at the 5' ends of most genes.

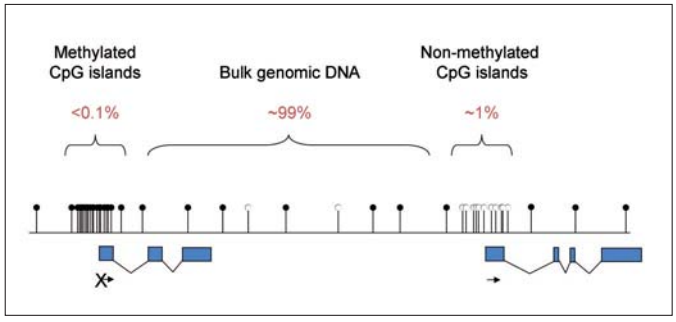


Figure 1
Distribution of CpG in the mammalian genome.

Bioinformatic methods have predicted that the number of CpG islands in mice is considerably less than the predicted number for humans. Contrary to previous estimates, however, CAP-seq shows that the numbers in mice and humans are quite similar, and most of the CpGs, including those at non-promoter regions, are conserved.

About half of the mammalian CpG islands found using this assay are at the promoters of protein-coding genes. Another half are 'orphan' CpG islands that are not associated with annotated promoters but are found at the 3' ends of genes or at random locations in between genes.

These orphan CpG islands are associated with a signature mark, trimethylation of the histone H3 lysine 4, supporting the hypothesis that these two properties are mechanistically interdependent. Many orphan CGIs show evidence of transcriptional initiation and dynamic expression during development.

In humans, about half of these islands are at promoters and the other half are distributed between intragenic and intergenic CpG islands. Intergenic orphan CpG islands are much more dynamic with respect to expression and methylation than are those located at promoters. Monitoring methylation at CpG islands in the mouse hematopoietic lineage confirms dynamic methylation at intragenic CpG islands.

In conclusion, mice and humans have similar numbers of CpG islands. Roughly half of all CpG islands are remote from annotated promoters but have promoter-like properties. Intragenic orphan CpG island promoters are dynamically active or methylated during development. The function of most orphan CpG islands, however, is unknown. They may mark promoters of as yet uncharacterised transcripts, including perhaps numerous non-coding RNAs.

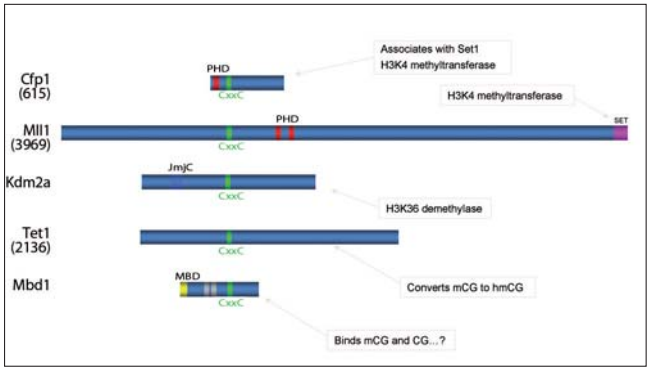
Colon cancer:

Unlike CpG islands at known promoters, orphan CGIs are frequently subject to DNA methylation during development, and this is accompanied by loss of their active promoter features.

Based on five colorectal tumors with matching normal mucosa, however, orphan CGIs are not preferentially methylated[1]. Thus, unlike that in normal tissues, tumor-specific CpG island methylation affects annotated promoters and orphan CGIs equally, suggesting that tumor-specific CpG island methylation does not recapitulate development.

Otherwise, there is a considerable overlap between methylation of CpG islands in normal colon and the neighbouring tumor that shows the typical enrichment for methylated intragenic orphan CpG islands. The study also confirms that CpG islands methylated specifically in the tumors are often marked by histone H3K27 methylation in human embryonic stem (ES) cells.

Figure 2
Proteins with CxxC domains.



When CpG islands are binned by base composition, H3K4me3 tracks the density of CpG, meaning that more CpG correlates with higher levels of H3K4me3. This and other findings suggest a causal link, that CpG richness encourages trimethylation of H3K4. An obvious possibility is that CXXC proteins that specifically bind to CpG are involved.

The CXXC protein CFP1 was discovered a decade ago, and is associated with an H3K4 methyltransferase in the SETD1 complex. In yeast, the ortholog of CFP1

is SPP1, which is part of the SET1 complex; the only H3K4 methyltransferase in yeast. It can be recruited to chromatin by RNA polymerase II. Using ChIP-Seq with a CFP1 antibody shows genome-wide binding to CpG islands in the brain, and a strong overlap of H3K4me3 with CFP1.

This suggests that it could be recruiting H3K4 trimethylation through the CXXC domain[2]. This idea is supported by depleting CFP1, which dramatically lowers H3K4 trimethylation at CpG islands. Reduced CFP1 in NIH3T3 somatic cells also results in altered morphology and slowed growth.

Most convincingly, insertion of a promoterless CpG cluster of about the same length and base composition as a CpG island into the genome using eGFP creates novel peaks of H3K4me3 in the absence of polymerase II. This suggests that CFP1 recruits H3K4me3 to CpG-dense regions[3].

In summary, CFP1 can bind to non-methylated CpG islands *in vivo*. Depletion of CFP1 causes a dramatic reduction of H3K4me3 at selected CpG islands and insertion of an artificial CpG cluster creates a novel focus of H3K4me3 and CFP1 binding in the absence of RNA polymerase II.

CFP1 significance:

In this model, the DNA sequence at non-methylated CpG islands appears to influence chromatin structure via the CFP1/SET1 complex, regardless of transcriptional activity. The function of CpG islands would in this case be to create a promoter-friendly chromatin structure. CFP1 assists this by recruiting this presumed promoter-friendly structure.

In ES cells lacking CFP1, the SET1 complex remains apparently intact. The loss of CFP1 from this complex does not have any significant effect on the ES cells. By contrast, somatic cells are viable with CFP1 loss, but they don't fare well[4].

In ES cells lacking CFP1, there is a significant loss of H3K4 methylation at GAPDH and ActinB promoters compared with controls, although it is not eliminated. At other genes such as BDNF, however, H3K4 trimethylation is completely unaffected by the loss of CFP1.

If these genes are characterized as active or inactive in ES cells, about 8,000 active genes are the ones most affected by CFP1 loss and show loss of H3K4 trimethylation. These active genes are identified by the presence of both RNA polymerase II and H3K79me2, and lose H3K4me3 at promoters. Most of the inactive genes remain unaffected.

Using nascent RNA sequencing (NRO-Seq) to reveal polymerase activity, surprisingly, at least in ES cells, transcription is largely unaffected by the absence of CFP1 and H3K4me3. Even in cases with a 90% reduction in H3K4me3, there is no difference in the amount of transcriptional activity. The results may be different in somatic cells, however.

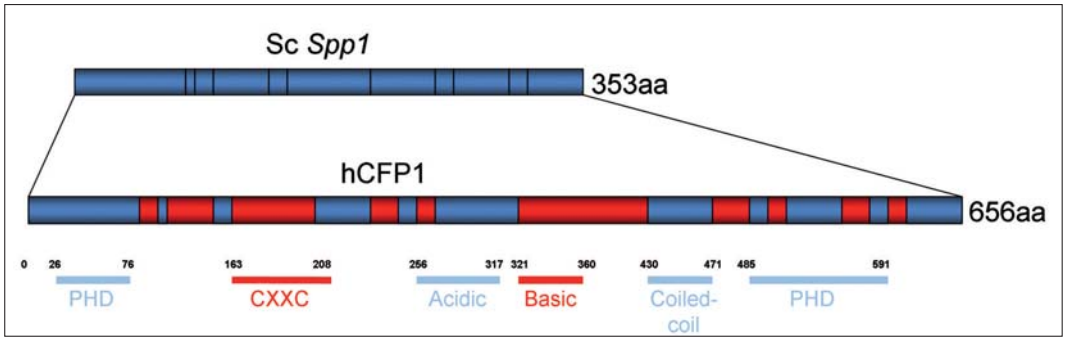


Figure 3
Mammalian CFP1 resembles yeast SPP1.

Wild type CFP1 can rescue the H3K4me3 loss in CFP1-/- ES cells. The C169A mutation in CFP1 disrupts one of the protein's two CXXC domains, which is essential for the structure. The mutation drastically affects the protein's ability to bind non-methylated CpG.

Still, the GAPDH and ActinB genes are almost completely rescued, both by wild type CFP1 and by the CFP1 that doesn't selectively bind non-methylated CpG. The mutated protein rescues H3K4me3 to a lesser extent than the wild type, but it still suggests that CFP1 is not the only functional interpreter of CpG islands.

Ectopic peaks:

There are thousands of H3K4me3 peaks in the CFP1-null ES cells, but not in wild type cells. Rescuing the CFP1-null ES cells with wild type CFP1, but not mutant CFP1, gets rid of the peaks.

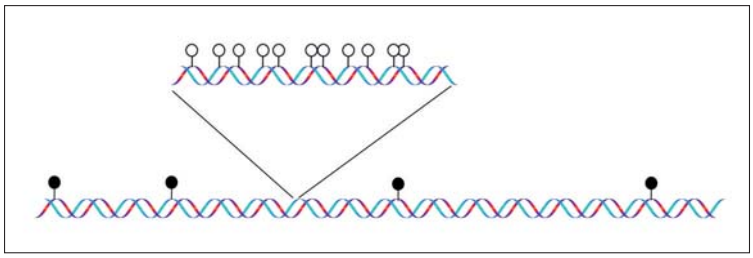


Figure 4
Are CpG clusters sufficient to create a focus of H3K4me3?

An emerging view is that CpG islands represent genomic platforms for transcriptional regulation. In general, CpG islands encompass about 5 nucleosomes. There is evidence that a subset of non-methylated CpG islands are reluctant to form nucleosomes, so that, when there is an inducible promoter, it can be induced instantly without the need for chromatin remodelers.

CpG islands of this kind are deficient in nucleosomes and accessible to transcription factors. They are rich in non-methylated CpG, which attracts proteins like CFP1, part of the SET1b complex, and KDM2a, a K36 demethylase. Transcription may reinforce the active chromatin state.

But CpG islands are also adapted to silence transcription. Being rich in CpG, they are a substrate for DNA methyltransferases. CpG methylation prevents transcription by repelling transcription factors, or by attracting proteins that impose a silent chromatin structure.

A third scenario also links CGIs to repression by polycomb, as there is evidence that G+C-rich DNA recruits H3K27 trimethylation which attracts polycomb group proteins. The mechanistic nature of the link between the base composition of CpG islands and polycomb is unknown.

Finally, there are the so-called bivalent promoters, which are prominent in stem cells, and show features of both active (H3K4me3) and inactive (H3K27me3) CpG islands simultaneously.

It has been proposed that bivalent promoters are repressed, but poised to become active during development. These scenarios raise the possibility that CpG islands are platforms for stable transcriptional switching, with predispositions to reinforce both active and repressive promoter function.

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PART IV: The role of cancer genes

The role of DNA methylation in intestinal and lung carcinogenesis

A report on a lecture by

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Whitehead Institute for Biomedical Research,
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Cancer develops as a consequence of genetic and epigenetic changes, and much evidence indicates that tumor-specific increase or loss of genomic DNA methylation may play a prominent role in both. Using conditional alleles as well as DOX-inducible transgenes of de novo methyltransferases has shown that DNMT3B silences tumor suppressor genes. DNMT1 has dual roles. Hypomethylation induced by inhibition of DNMT1 enhances genome instability and mitotic recombination leading to loss of heterozygosity of APC and an increased incidence of microadenomas. However, inhibiting DNMT1 strongly decreases the incidence of intestinal macroscopic adenomas because it leads to less efficient maintenance of the silenced state established by DNMT3B-mediated de novo methylation. Deletion of DNMT3A has no effect on intestinal tumor formation. When the Lox-K-RAS G12D transgenic mice are exposed to an Ad-Cre vector, it initiates tumor formation by activating the K-RAS tumor gene and inactivates the DNMT3A or 3B conditional alleles. DNMT3B deficiency has no effect on lung tumor formation. However, deletion of DNMT3A significantly accelerates tumor growth and grade of malignancy but does not increase the number of tumors formed. ***Rudolf Jaenisch*** *proposed that DNMT3A, counter to expectation, acts like a tumor suppressor gene in lung carcinogenesis.*

The *de novo* methyltransferases DNMT1, DNMT3A and DNMT3B have distinct but equally important roles in methylation. DNMT3A and DNMT3B accomplish *de novo* DNA methylation, whereas DNMT1 maintains DNA methylation. Passive demethylation involves inhibiting of DNMT1; what active demtehylation involves is controversial.

Total methylation hits off a wave of global demethylation during cleavage, global *de novo* methylation after implantation, and then maintenance of methylation.

DNMT1 is expressed in every phase when cells replicate DNA. DNMT3A and DNMT3B are activated after implantation. When DNMT1 or DNMT3B are deleted, embryos are not viable. When DNMT3A is lost, they stay viable a bit longer, but eventually also die.

The role of methylation and demethylation in cancer is complex. It has been long known that genome-wide hypomethylation is an early event in tumorigenesis. Regional hypermethylation and silencing of tumor suppressor genes is also well understood.

It's important that in principle, epigenetic alterations are reversible by therapeutic intervention, and there are several drugs in trials as treatment for cancer.

In the Vogelstein model, the progression of tumorigenesis involves APC mutations. The DNA of adenomas shows that APC is hypomethylated, an early and important event.

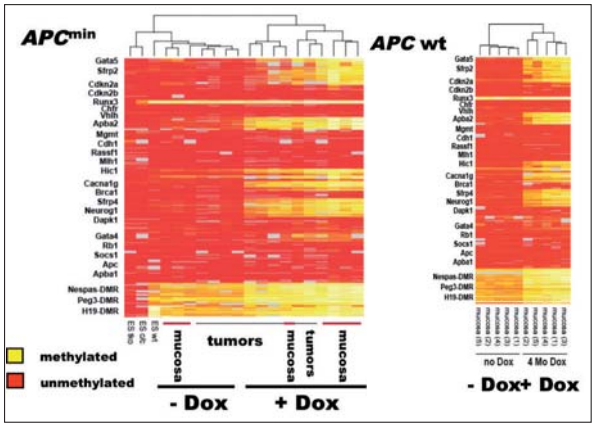


Figure 1
DNMT3B mediated hypermethylation in APC^{min} and wild type APC mice.

In APCmin mice, having heterozygous DNMT1 reduces the number of polyps significantly. Decreasing the levels of DNMT1 further by 5-aza-deoxycytidine confers a protection of two orders of magnitude against tumors. These results suggest that DNMT1 acts like an oncogene. Higher expression correlates with increased tumor incidence.

In the intestine, hypomethylation protects against macroscopic adenomas. In other tissues, it enhances tumors. In fibroblasts and embryonic stem (ES) cells, it results in increased mitotic recombination and loss of heterozygosity. This suggests that methylation has different roles in different tissues.

Multiple models:

Using hypomorphic DNMT1, conditional alleles of DNMT1, DNMT3A and DNMT3B, and drug-inducible expression of DNMT3A and DNMT3B, these genes can be over- or under-expressed in any cell to study the different effects.

In intestinal tumorigenesis, when the normal crypt loses AP1 it forms a microadenoma that is β-catenin positive and then, in a second step, makes a microscopic tumor. In a DNMT1 hypomorphic mouse, the LOH rises, but there is decreased formation of a macroscopic tumor, presumably by an epigenetic event[1]. DNMT3B inhibits the formation of macroscopic tumors, and its over-expression increases it[2].

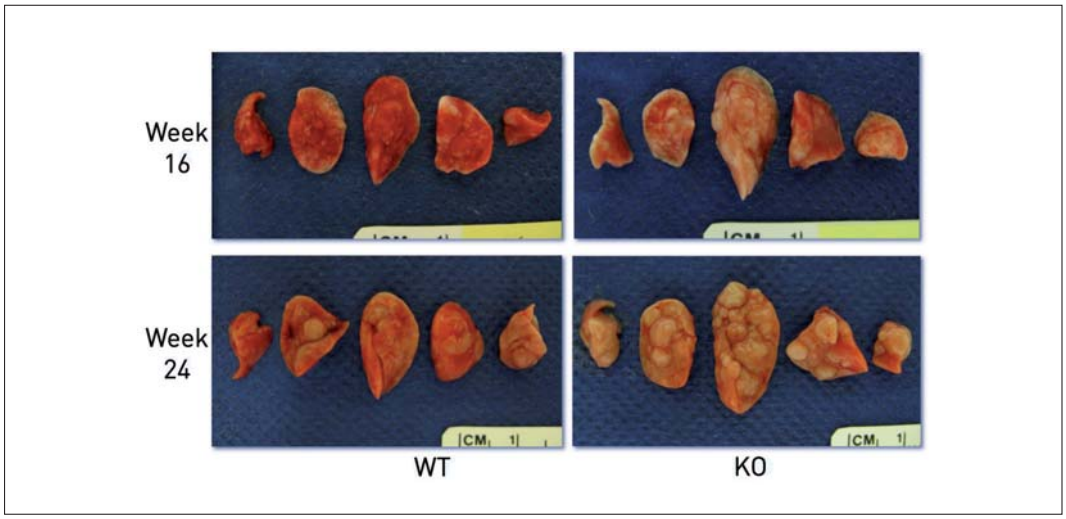
The role of DNMT1 is to maintain methylation, which is important for genome integrity. DNMT3B collaborates with DNMT1 to silence tumor suppressor genes. DNMT3A has no effect in the intestine.

DNMT1 and DNMT3B act like oncogenes: their increased expression enhances intestinal tumor formation. A conditional over-expression of DNMT3B can help find its target genes that are relevant for cancer.

Over expression of DNMT3B results in the widespread hypermethylation of many genes, both in APCmin mice and in wild type APC mice. DNMT3B targets the same genes in normal mucosa as in tumors[2].

Comparing RRBS data for mice with DNMT3B over-expression to human colon cancer, 28 of 30 of the genes that are methylated in the mice are similarly methylated in human colon cancers, and the 11 genes unmethylated in mice are all unmethylated in the human samples as well[2]. So there is a good correlation between what happens in human cancer and DNMT3B over expression[3].

Figure 2
DNMT3A deletion causes larger and more advanced tumors.



To summarize, some regions of the genome are inherently susceptible to *de novo* methylation by DNMT3B. Over-expression of DNMT3B induces a methylation pattern that resembles that of human colon cancer. The tissue of origin shows the same methylation patterns as tumors, indicating that it has the same regulation as in tumors. DNMT3B and cancer-relevant genes that are susceptible to DNMT3B may offer therapeutic targets.

In this model, DNMT1 and DNMT3B cooperate in enhancing intestinal cancer, but there is no role for DNMT3A. It serves instead as a useful negative control.

Unexpected effects:

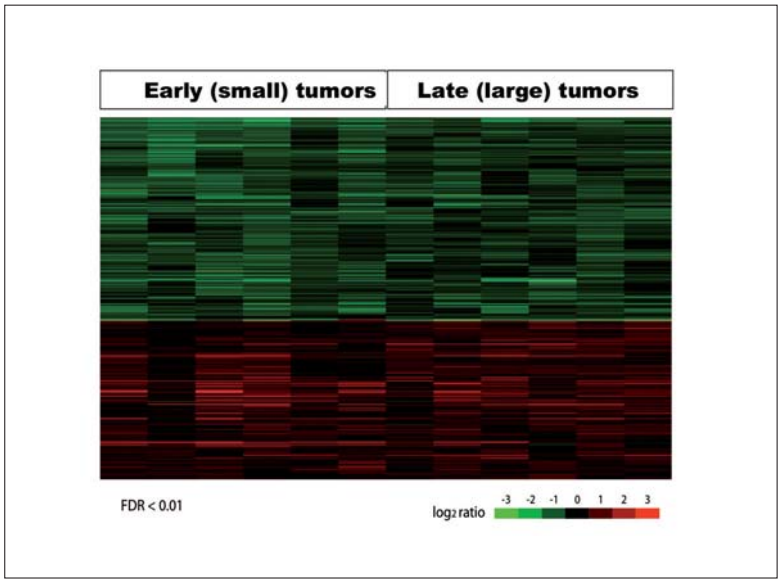


Figure 3
DNMT3A-deficient tumors have significant expression changes in about 1,000 genes.

In the conditional mouse model called LSL-K-RAS G12D developed by Tyler Jacks' lab, mutational activation of K-RAS is regulated by a removable silencing element. Activating the oncogene induces multi-stage lung cancer, which develops in 16 weeks from hyperplasia to full blown adeno-carcinoma. Activating mutations in K-RAS occur in 25%-50% of human lung adenocarcinomas[4].

The strength of this model lies in its ability to control tumor initiation and follow tumor progression. Using this model, when K-RAS is activated and crossed with DNMT3A or DNMT3B conditional knockouts, activation of K-RAS and inactivation of DNMT3A or 3B occurs in same cell.

In the wild type tumors, DNMT3A localizes to the nucleus. But in the DNMT3A knockout, there is no nuclear staining, only some faint cytoplasmic staining, indicating that it's a true knockout.

There's no difference between these DNMT3A knockouts and the wild type mice in terms of the number of tumors. But the appearance of the tumors is unexpected.

Compared with the wild type, the DNMT3A knockouts show an incredible growth of tumors, with larger and more advanced tumors. At 24 weeks, 50% of the lung is covered with tumor. There is no difference between wild type and DNMT3A knockouts at 8 weeks, but the tumor size is four-fold higher at 16 weeks and six-fold higher at 24 weeks.

Staining for Ki67 shows a higher density of Ki67-positive cells, suggesting that DNMT3A-deficient tumors are bigger because they have a higher proliferation rate. There is no change in apoptosis. The DNMT3A knockout mice also have significantly higher-grade tumors compared with wild type. At 16 weeks, for example, very few tumors in the wild type are grade 2, most are grade 1 tumors. In the DNMT3A knockouts, by contrast, there are many more grade 3s and grade 2s. At 24 weeks, the knockouts show a 10-fold expansion of grade 3 tumors, but there are few of those in the wild type mice.

So the DNMT3A knockout mice have significantly higher-grade tumors, they're more malignant, and those tumors also show local invasion, which the wild type animals do not show. Mice with the DNMT3A deletion also have a shorter life span.

In the DNMT3A knockouts, about 1,000 genes are significantly changed in their expression levels when compared with the wild type. The genes that are most significantly changed are those involved in angiogenesis, cell adhesion, cell motion, extracellular matrix and Wnt signaling. These are generally consistent with a role in tumor progression.

So in summary, DNMT3A deletion does not increase tumor number, so its effect has nothing to do with tumor initiation. But DNMT3A deficiency accelerates tumor growth by enhanced proliferation. So at face value, DNMT3A functions as a tumor suppressor gene.

Cancer candidate:

Using exactly the same approach, the role of DNMT3B can also be studied. When the K-RAS mutation is crossed with DNMT3B conditional knockout, there's no effect of the DNMT3B deletion on the number or the size and progression of the tumors. In this case, DNMT3B seems not to have an effect on cancer.

The current understanding of the players in *de novo* methylation and gene silencing in cancer is that DNMT3B induces promoter methylation and gene silencing in the intestine, and its deletion is protective. DNMT1 cooperates with DNMT3B in maintaining the silent state so that its inhibition is protective. DNMT3B and DNMT1 are therapeutic targets, but DNMT3A has no established role in cancer.

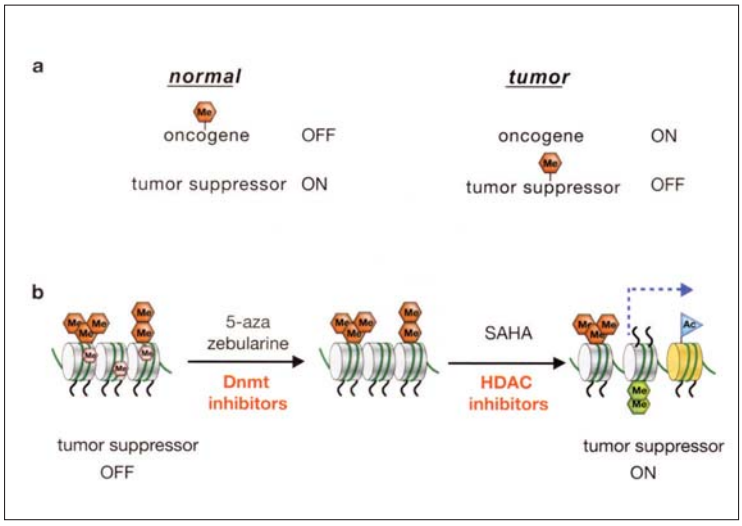


Figure 4
Epigenetic modifications in cancer are reversible: Relevance for therapy.

A paper published last year showed an association of DNMT3A mutations with acute myelogenous leukemia. More recently, most of the AML mutations in DNMT3A have been shown to be missense mutations, and are associated with poor prognosis of AML.

Most of the mutations seem to cluster at the carboxyl end in the methyltransferase domain, with a few others upstream. Some of the mutations decrease enzyme activity but others do not, so it's unclear what the function of these mutations is. They must be dominant negative mutations, however, because in all the patients, only one allele is mutated.

DNMT3A protects against cancer by acting like a tumor suppressor in lung cancer. This was an unanticipated result, and it's unclear whether its effects depend on DNA methylation or another mechanism. It's possible, for example, that it silences oncogenes, affects genome stability, or that its activity is important for suppressor genes.

Using the DNMT3A conditional knockout in an *in vitro* system of neural precursor differentiation shows that its

loss is important for transcription. DNMT3A deficiency inhibits neural differentiation and correlates with reduced gene body methylation as seen by MeDip.Seq, but not promoter methylation.

The genes that are down-regulated in DNMT3A knockout tumors are hypomethylated in the gene body, and those that are more expressed tend to be hypermethylated. There is no correlation with methylation changes in promoters.

It's completely unexpected that a *de novo* methyl transferase would act as a tumor suppressor. This is a new paradigm for methylation and cancer. It may have therapeutic and prognostic implications.

Methylation-based therapies are aimed at inhibiting DNMT1 or DNMT3B. What effect would inhibiting DNMT3A have? There are ongoing clinical trials in which HDAC analogs are being used in combination with DNMT1 inhibitors. These scenarios might be more complicated and need further study.

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The sorcerer's apprentice: nuclear and cytoplasmic functions of the MYC oncoprotein

A report on a lecture by
Robert Eisenman
Fred Hutchinson Cancer Research Center, University of Washington, Seattle, USA

The proteins encoded by the MYC gene family (c-MYC, N-MYC, and L-MYC) function in multiple normal physiological processes including cell growth, proliferation, differentiation and death. They are also profoundly involved in the etiology of a wide range of cancers. MYC forms obligate heterodimers with the bHLHZ protein MAX in order to bind DNA and regulate a large number of target genes. MYC is also implicated in transcriptional activities mediated by all three RNA polymerases. In addition to MAX, MYC proteins associate with a number of factors including histone acetyltransferases such as GCN5, elongation factors such as P-TEFb, chromatin remodeling factors and other DNA binding transcription factors. The number and diversity of these interactions suggests that MYC exists in multiple complexes capable of exerting a range of transcriptional activities. ***Robert Eisenman*** *presented research on two different aspects of MYC function: the role of MYC in large-scale changes in chromatin, and, the function of a cytoplasmic MYC cleavage product (MYC-NICK) that increases the rate of muscle differentiation and that may be associated with a more differentiated, highly aggressive form of rhabdomyosarcoma.*

MYC is the focus of much research because it is abnormally expressed in many cancers, including about 30% of neuroblastomas, 30% of liver cancers and 20% of lung cancers. MYC deregulations take many forms: amplification, chromosomal translocation and promoter mutation, for example. MYC's widespread deregulation in cancer and its involvement in the proliferation and growth of nearly every cell type suggest that it plays a fundamental role in cell biology[1].

The N-terminal region of MYC contains several highly conserved domains. MYC Box I is a phospho-degron, MYC Box II interacts with transcriptional co-activators. The most conserved domain is the C-terminal basic-helix-loop-helix-zipper (bHLHZ), which interacts with the MAX protein. This interaction allows MYC to heterodimerize and bind the canonical E-box sequence CACGTG.

Beyond the MYC-MAX interaction, however, MYC is part of a network of interacting proteins. In mammalian cells, there's a family of MYC proteins, c-MYC, L-MYC and N-MYC, all of which form heterodimers with MAX and bind the E-box sequence. All of them regulate a set of target genes.

In addition, the MXD (formerly MAD) family of proteins, which are also bHLHZ proteins, heterodimerize with MAX, and bind E-box containing target genes.

MXD proteins are dedicated repressors: they interact with SIN3 co-repressor to suppress gene expression at target sites and act as MYC antagonists.

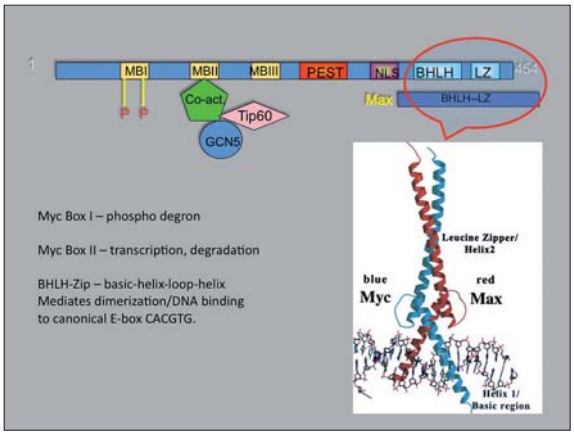

Figure 1
Simplified organization of MYC family proteins.

In addition, there is another MAX-like protein called MLX, which interacts with a subset of MXD proteins as well as two other MYC-like proteins called MondoA and MondoB. These proteins also heterodimerize with MLX and bind to E-box target genes and they can, depending on the context, activate or repress the genes. They are involved in metabolism and glycolysis, and may somehow modulate MYC function at promoters.

Complex interactions:

MYC-MAX dimers recruit higher order complexes to their binding sites. The stoichiometry of complex association with MYC-MAX is unclear.

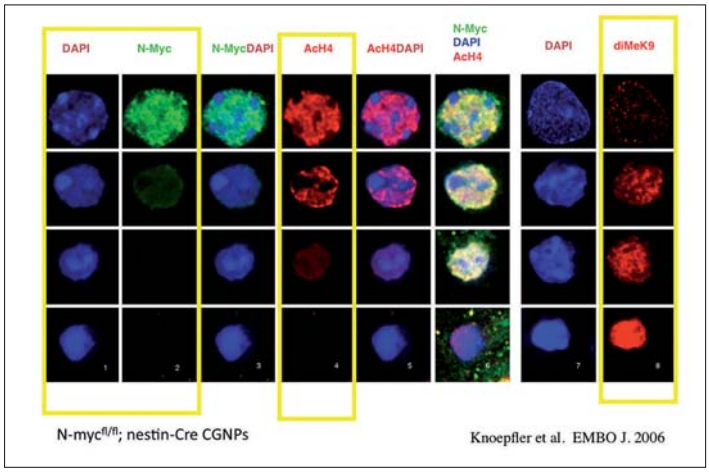

Figure 2
Widespread effects of MYC LOF on histone modifications.

The best-characterized interactions of MYC are those with the co-activator TRRAP, which in turn interacts with the GCN5 histone acetyl transferase. TRRAP also interacts with the TIP60 chaperone, which is involved in damage response and in acetylation. MYC interacts with p-TEFb which, along with the GCN5 interaction, is probably the best characterized. p-TEFb can function in RNA polymerase II phosphorylation and anti-pausing.

To make things even more complicated, MYC-MAX has been found to interact with the MIZ1 protein. MIZ1 is a transcriptional activator but, strangely enough, MYC-MAX interactions with MIZ1 turn it into a repressor. MIZ1 normally activates a number of cyclin-dependent kinase inhibitors, but MYC is able to turn those off by inactivating MIZ1.

The MXD-MAX dimers also associate with complexes including the SIN3 co-repressor and histone deacetylases.

MYC binds about 11% of genomic loci in mammalian cells. Many of these genes are involved in cell growth and metabolism and in ribosome biogenesis. In ES cells, MYC also regulates a number of chromatin modifiers and genes such as HIRA that are involved in chromatin assembly. MYC also controls a number of cell-cycle inhibitors and microRNAs, several of which inhibit differentiation, metabolism and proliferation.

MYC's wide-ranging binding and its broader effects on chromatin modification were unexpected. In N-MYC knockouts, neuronal cells are very small and dense compared with wild type littermate controls. The nuclei of those cells display decreased histone H3 acetylation and increased H3K9 methylation.

Decrease in MYC levels also affects DNA sensitivity to micrococcal nuclease, suggesting that chromatin goes into a more closed conformation[2]. These changes are likely to be an indirect effect of MYC target genes that affect chromatin structure.

H2A.Z deposition:

The essential histone H2A variant H2A.Z is assembled with H2B into nucleosomes, mediated by SWR1 complex. Although the functions of H2A.Z are still not fully known, it has been linked to chromatin instability. Nucleosomes assembled with H3.3 and H2A.Z are unstable and highly soluble. H2A.Z is also associated with polycomb

binding in ES cells, for example, where it's been shown to be important in differentiation. H2A.Z is also important in mitosis, during which H2A.Z nucleosomes are located at transcription start sites (TSS) of repressed genes that are poised to be activated in G1.

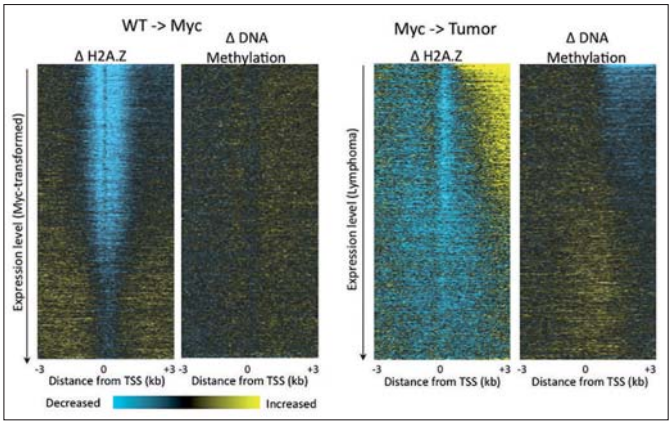

Figure 3
Epigenomic changes during B-cell lymphomagenesis.

Interestingly, in *Arabidopsis*, H2A.Z deposition and CpG methylation are quantitatively anti-correlated. This is established by the fact that mutation of the SWR1 ortholog results in increased CpG methylation, and mutation of DNA methylase results in increased H2A.Z deposition.

Translocations of MYC occur in Burkitt's lymphomas. In mice, introducing the MYC transgene containing the immunoglobulin enhancer promoter into lymphoid progenitor cells results in monoclonal B cell lymphomas. During this process, there is selection for suppression of apoptosis and of tumor suppressors, and activation of other oncogenes.

The lymphomas from Eμ-MYC mice have metastases that are more 1 cm in size. Chromatin from pre-malignant Eμ-MYC B cells and tumors shows a substantial decrease of H2A.Z at and around TSS compared with wild type cells. These changes in Eμ-MYC cells correspond to decreased DNA methylation at promoters and increased DNA methylation in gene bodies.

There are also changes in gene expression between the wild type pre-B cells and the Eμ-MYC cells, and these changes increase as the cancer progresses. The highest levels of H2A.Z correspond to the lowest levels of DNA methylation and highest levels of the gene expression[3]. Unbiased clustering of these genes shows that 30% of gene bodies show H2A.Z gain and a loss of methylation. About 35% show a gain in H2A.Z at the TSS, without changes in methylation. Another 35% show a loss in H2A.Z and an increase in methylation.

Based on micrococcal nuclease digestion and chromatin extraction at low salt, the Eμ-MYC lymphomas show an increase in solubility at all promoters compared with the wild type. This may correspond to the generalized increase seen in neuronal cells when MYC levels are altered, suggesting that there's a change in DNA accessibility.

One possible explanation for these dramatic changes is that the loss of H2A.Z at the promoters in Eμ-MYC pre-B cells somehow frees those promoters, allowing more polymerase binding and more transit of polymerase. The redistribution of H2A.Z to gene bodies may suppress methylation and perhaps synergize with the increase in solubility, the changes in chromatin dynamics and the anti-pausing effects of MYC.

However, not all the genes that show these changes are MYC target genes. These broad changes may reflect the fact that as Eμ-MYC B cells progress to tumor, there is an enormous amount of selection that occurs for suppression of apoptosis and differentiation, and perhaps for activation of other oncogenes.

Cytoplasmic form:

MYC-NICK is a cytoplasmic form of MYC that promotes differentiation. In cells that are either confluent and shut down their growth or cells that are differentiated, there are two pools of MYC: full-length MYC, located in the nuclear fraction, and a smaller form of MYC that migrates at about 42 KDa.

This form, called MYC-NICK, is generated by calpain cleavage at a site 298 amino acids from the N-terminus of MYC. This cleavage removes the whole C-terminal region (including the nuclear localization signal and the bHLHZ domain) and gives rise to this N-terminal protein that contains all the conserved domains involved in turnover and interaction with transcriptional activating complexes[4].

MYC-null rat fibroblasts grow and proliferate slowly. If wild type MYC is introduced into them, they start to grow at a normal rate and also undergo apoptosis. But MYC-NICK doesn't show the same effects. Instead, it triggers a dramatic change in the structure of the cells. The cells have long filaments, which turn out to contain acetylated alpha-tubulin and are resistant to the drug nocodozole.

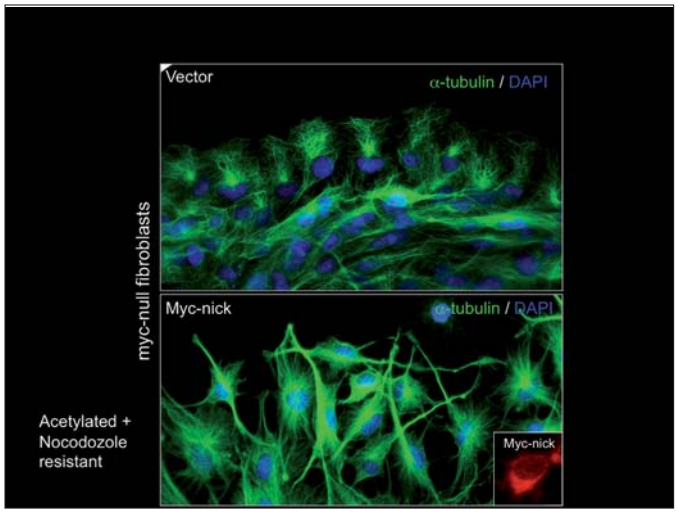

Figure 4
Ectopic expression of MYC-NICK alters cell morphology.

In vitro binding experiments reveal that MYC-NICK directly binds to microtubules and recruits the histone acetyl transferase GCN5 to them.

Acetylation and calpain activation are important in the differentiation of many cell types. A good example of this is myoblast differentiation. When primary mouse myoblasts or C2C12 cells differentiate, they produce MYC-NICK, and MYC levels decrease. This correlates with an increase in acetylated alpha-tubulin and increases in differentiation-specific proteins such as troponin C.

When MYC-NICK is introduced into C2C12 myoblasts, it promotes differentiation and myofiber formation. MYC-NICK renders RAT1 MYC-null fibroblasts sensitive to MYO-D induction of trans-differentiation into muscle.

One of the reasons MYC-NICK levels persist is that this protein becomes stabilized. MYC is turned over due to phosphorylation at the MYC box1 phospho-degron. MYC-NICK is initially highly phosphorylated, but that phosphorylation greatly decreases during differentiation when the protein becomes more stabilized.

The current model for MYC-NICK dynamics is that MYC is normally produced in the cytoplasm. Most of it is rapidly translocated into the nucleus, binds to MAX and turns on genes involved in growth and proliferation. However, a small subset of MYC is retained in the cytoplasm where, upon calcium signaling, calpains are activated and cleave off the C-terminus. The resulting MYC-NICK can interact with GCN5 and acetylate microtubules.

MYC controls protein acetylation at multiple levels. For example, one of the targets of full-length MYC is GCN5, which is itself recruited by MYC and is required for MYC transcriptional activity, representing a potential feed-forward mechanism.

It also stimulates acetyl-CoA production by regulation of cell metabolic pathways. Therefore MYC drives cellular acetylation at several distinct levels.

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Functional roles of TET proteins and hydroxymethylation in stem cells and cancer

A report on a lecture by
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DNA methylation at transcriptional start sites is believed to mediate transcriptional silencing through the recruitment of methyl-binding proteins and changes in chromatin structure. The high frequency of abnormal DNA methylation in cancer might be due to the inactivation of a proofreading or fidelity system regulating it. TET1 catalyzes the conversion of methyl cytosine to hydroxymethylated cytosine. TET2, which belongs to the same family, is one of the most frequently mutated genes in hematological diseases. TET1 binds throughout the genome, with the majority located at transcriptional start sites and within genes. More than 80% of its targets are highly enriched for CpG. In contrast, hydroxymethyl cytosine is significantly enriched on about 1,000 promoters, and the majority of these have low and intermediary CpG content. Because the majority of CpG-rich promoters are rarely methylated in ES cells, the major function of TET1 may be to prevent aberrant DNA methylation of CpG-rich promoters. Surprisingly, TET1 also seems to have a role in transcriptional repression, and binds to a significant proportion of target genes that are positive for the Polycomb repressive histone mark H3K27me3. TET1 also associates with the SIN3A co-repressor complex, which co-localizes with TET1 throughout the genome. ***Kristian Helin*** *proposed that the major function of TET1 is to regulate the fidelity of DNA methylation.*

Cytosine methylation is a major DNA modification in most eukaryotic genomes, including mammals and plants. In mammals, methyl cytosine is present throughout the genome. However, it is specifically DNA methylation of CpG islands close to transcription start sites that has attracted attention.

There are many unanswered questions about DNA methylation. For example, what protects CpG islands from DNA methylation, and how is DNA methylation deregulated in cancer?

DNA methylation is known to be important for the regulation of normal development, and methylation of promoters is also a key mechanism in tumorigenesis.

In normal cells, CpG island-rich promoters are free of DNA methylation, whereas gene bodies are heavily methylated. During normal differentiation, a few of those promoters become methylated, and some of the gene bodies lose methylation, shutting off transcription. Likewise in cancer, some promoters gain DNA methylation on CpG islands, shutting down expression.

There are enzymes involved in the maintenance of DNA methylation. In mammals, the enzymes DNMT3A and DNMT3B are responsible for *de novo* DNA methylation, whereas DNMT1 is mainly involved in catalyzing the maintenance of DNA methylation.

When DNA replicates, it loses methylation on one strand and then these enzymes are recruited. DNMT1 is recruited to hemimethylated DNA by binding to the UHRF1 protein, which allows DNMT1 to copy the methylation status from the old DNA strand to the newly synthesized one.

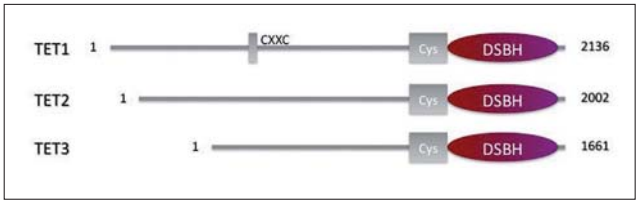

Figure 1
The TET family.

DNA methylation is not always maintained, and demethylation may occur through passive or active processes. Passive demethylation could be achieved by blocking the maintenance DNA methylation during DNA replication. However, the observation that DNA methylation is rapidly lost during fertilization and primordial germ cell migration suggests that active DNA methylation exists.

The high frequency of abnormal DNA methylation in cancer might be due to the inactivation of a proofreading or fidelity systems regulating the correct patterns of DNA methylation.

There is much to discover to about the specifics of DNA methylation including recruitment, proofreading, maintenance and fidelity, about which close to nothing is known. One key question is why DNA methylation patterns are changed in cancer.

DNA demethylating enzymes could potentially provide a fidelity system for DNA methylation. However, enzymes contributing to DNA demethylation were not known until recently.

The family of histone demethylases, which includes UTX, UTY and JMJD3, all contain the JmjC or Jumonji domain, which can catalyze the demethylation of H3K27. JMJD3 and UTX have strong demethylase activity, but UTY doesn't seem to have any activity at all.

Looking for proteins that associate with UTY and may be required for its activity reveals the MLL complex, which is known to bind to UTX.

Other proteins called TET1, TET2 and TET3 were also found about three years ago but at that time, these proteins were not characterized. In a groundbreaking paper, TET1 (Ten-Eleven Translocation 1) was shown to catalyze the hydroxylation of the methyl group of methyl cytosine, converting it to hydroxymethylated cytosine. TET1 is translocated in some acute myeloid leukemias (AML), where it is a partner of MLL[1].

Family motif:

TET1 belongs to a family of three proteins. All three have an α-ketoglutarate iron-depending catalytic domain, which leads to hydroxylation of its substrate. There is a similar domain in homologous yeast proteins, which helped elucidate the structure.

TET1 contains a CXXC motif (see Bird, page 75). This motif has been shown to have affinity for CpG-dinucleotides and might be part of what is required for its recruitment to DNA. However, TET2 and TET3 do not have this domain. They may be recruited to DNA through binding to other proteins containing a CXXC motif. Some of those candidate proteins are also mutated in AML.

TET1 was initially thought to be specific for embryonic stem (ES) cells, but it is also expressed in other stem cells. TET2 is expressed in hematopoietic stem cells and in hematopoietic cells in general, but it also seems to be expressed in other tissues. TET3 is found in oocytes and fertilized eggs. So the different family members are not as specific to certain cells as they were initially thought to be. There might be redundancy, which could be important.

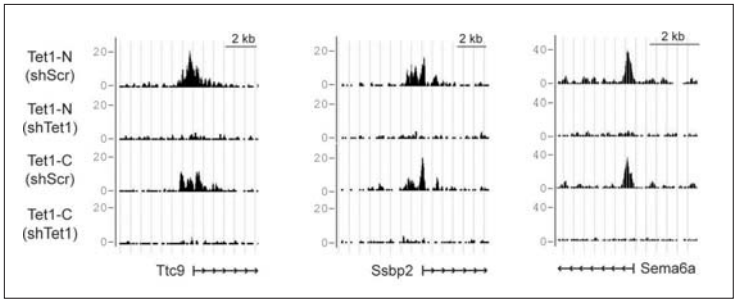

Figure 2
TET1 target genes.

These proteins have been associated with cancer. As mentioned above, TET1 is expressed as part of a MLL-TET1 in some AMLs. This is relatively rare. By contrast, TET2 is the most frequently mutated gene in hematopoietic tumors so far. It is found in up

to 20% of patients with AML, up to 30% of those with myeloproliferative neoplasms and nearly half of those with chronic myelomonocytic leukemia[2].

Interestingly, both have mono- and bi-allelic mutations. The loss of one allele is sufficient to contribute to tumorigenesis, suggesting that TET1 is haploinsufficient.

Interestingly, isocitrate dehydrogenase genes, or IDH, are mutated in about 70% of glioblastomas, but they're also mutated in about 15-30% of AML. Those mutations are mutually exclusive with TET2 mutations. Neomorphic dominant mutations in IDH1/2 produce a metabolite called 2-hydroxyglutarate, which is a competitive inhibitor of α-ketoglutarate-dependent enzymes.

Neomorphic IDH1 and IDH2 mutants inactivate TET2, which by one group has been reported to lead to a global and specific hypermethylation phenotype seen in those cases of AML[3]. However, contradictory to these findings, another team has reported that loss of TET2 decreases levels of 5-hydroxymethylcytosine and of methylation at CpG sites[4].

IDH1/2 neomorphic mutants may also inhibit other α-ketoglutarate-dependent enzymes, including JmjC-containing histone demethylases.

TET1 is strongly expressed in ES cells. When TET1 is knocked down in mouse ES cells, they express normal levels of OCT4 and Nanog and the ES cells proliferate normally. This suggests that TET1 is not required for ES cell self-renewal.

Diverse targets:

To identify genes regulated by TET1, two different antibodies recognizing the N- and C-terminus of TET1 have been generated[5]. These antibodies were used for ChIP-sequencing and led to the identification of many target genes, including TTC9 and SSBP2.

At a genome-wide level, TET1 binds to discrete sites throughout the genome, to transcription start sites, promoters, intergenic regions, introns and exons. By density of tags, TET1 clusters on transcription start sites. Even with stringent screens, however, it has 6,572 target genes.

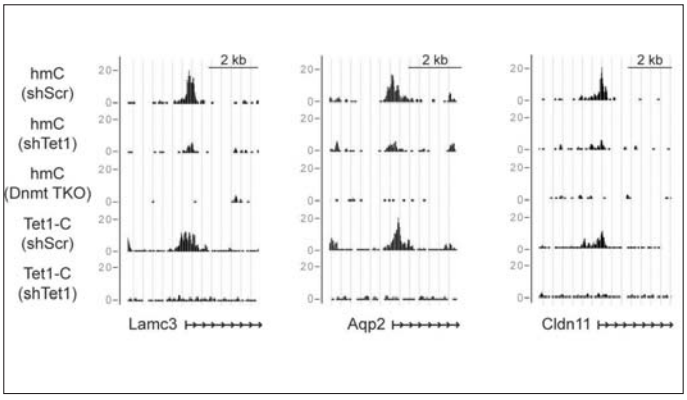

Figure 3
Examples of genes associated with hydroxymethyl cytosine.

These DNA- and chromatin-modifying enzymes bind to a lot of target genes because part of their function is to ensure that transcription can occur. Interestingly and in agreement with having a CXXC motif that has affinity for CpG sites, the majority of TET1 targets have high CpG content, which is not DNA methylated. So TET1 may be keeping unmethylated DNA unmethylated.

Nearly all of the TET1 target genes are trimethylated on lysine 4 on histone H3 (H3K4me3), and a significant number are also H3K27me3 positive. However, it is unclear whether this is important and whether TET1 target genes are transcriptionally active. Still, there is a significant overlap with Polycomb target genes, which is expected because they're CpG-rich and Polycomb binds to CpG-rich promoters. The genes regulated or bound by TET1 include those involved in all types of cellular processes. They are genes required to grow

and ones that should not be methylated. However, the list also includes genes involved in cell cycle and development. Those are the Polycomb target genes, and a few of them gain DNA methylation during differentiation.

In summary, TET1 is predominantly associated with gene bodies and transcription start sites. TET1 binds to high content CpG-rich promoters, but its binding does not predict promoter activity.

TET2 and TET3 can also lead to hydroxylation of 5-methylcytosine. This could result in active demethylation, facilitate passive DNA demethylation, and it could itself be a mark.

Antibodies to hydroxymethyl cytosine have been made and tested in two assays. They can be used for DNA immunoprecipitation assays without cross-reacting to methylcytosine.

Using the antibody for genome-wide analysis in ES cells shows the distribution of hydroxymethyl cytosine throughout the genome. In contrast to methyl cytosine, there is some accumulation of hydroxymethyl cytosine on transcription start sites. Between 35 and 50% of the genes are TET1 targets. If anything, this selects for genes that are inactive so, in this process, TET1 is not a transcriptional activator.

Interestingly, in the distribution of hydroxymethylation, less than 50% has higher content CpG, much less than for TET1.

When TET1 is knocked down, there are minor changes in global methyl cytosine in these cells. Of the few genes that acquire methyl cytosine, some are methylated, and about a third of those are methylated later during differentiation. Those are primarily the ones that have intermediate to high CpG content.

If TET1 is knocked down in ES cells using two different shRNAs about 550 genes are down regulated. Of those, about 200 are direct targets of TET1. There are also about 850 genes that are up regulated, including about 500 that are direct targets of TET1. This suggests that TET1 may not work very well in transcriptional activation, but could have a role in transcriptional repression.

Repression role:

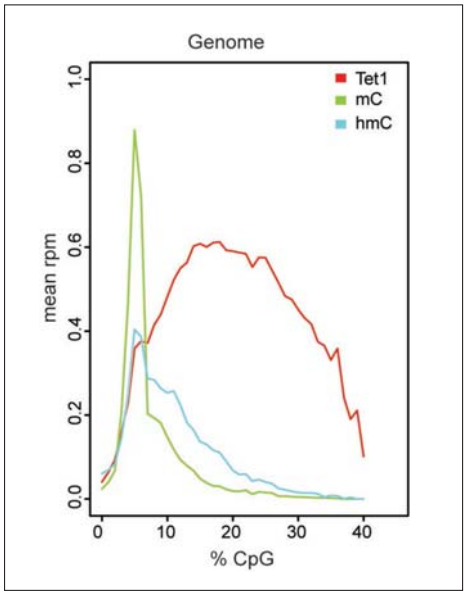

Figure 4
Distribution of marks.

Purified TET1 and TET2 complexes in ES cells show that TET1 associates with the SIN3A transcriptional co-repressor complex. All the proteins in this complex, including HDAC1, HDAC2 and ARID4B, bind to TET1.

Using ChIP sequencing with SIN3A antibodies, SIN3A and TET1 co-localize on target genes, perhaps explaining the repression effect.

The recruitment of SIN3A is in part dependent on TET1. When TET1 is down regulated, there is a reduction in SIN3A on target genes, including SSBP2 and TTC9. When either TET1 or SIN3A is down regulated, these target genes are transcriptionally activated, or at least de-repressed.

In summary, TET1 could work as a transcriptional regulator. It binds to more than 6,500 genes in ES cells. Down regulation of TET1 leads to transcriptional changes of about 600 genes. TET1 binds to and co-localizes with the SIN3A transcriptional co-repressor complex. TET1 also contributes to transcriptional repression and perhaps also activation by putting on the hydroxylmethyl cytosine mark.

TET1 might have another function that is not well described: regulating DNA methylation fidelity. The distribution of methyl cytosine in the genome is mainly on low to intermediate CpG content promoters. By contrast, hydroxymethyl cytosine is also distributed on some higher concentration CpG sites.

More than 80% of its targets are highly enriched for CpG. In contrast, hydroxymethyl cytosine is significantly enriched on about 1,000 promoters, and the majority of these have low and intermediary CpG content.

Approximately 5,000 high CpG density promoters bound by TET1 are not methyl cytosine or hydroxymethyl cytosine positive.

The major function of the TET proteins may be to remove aberrant DNA methylation on the promoters.

In cancer cells, tumors with TET2 mutations are more likely to have DNA methylation of those promoters. But many cells will die on the way because promoters required for normal proliferation will also be hypermethylated. The role of TET2 is still being worked out, but through a mechanism that involves demethylation, it might function by erasing aberrant DNA methylation.

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Metabolic inputs into cancer epigenetics

A report on a lecture by
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Memorial Sloan-Kettering Cancer Center, New York, USA

Aerobic glycolysis exhibited in many tumor cells is accompanied by elevations in cytoplasmic acetyl-CoA levels. The rate of glycolytic flux could contribute to a 1-2 log increase in cytosolic acetyl-CoA levels. This has a generalized influence on histone acetylation and the expression of a wide variety of genes. The activity of the GNAT-family of protein acetyl transferases is regulated in part by variations in cytoplasmic acetyl-CoA levels. Altered DNA methylation has been established as a hallmark of acute leukemia and yet very little is known concerning the mechanisms through which this occurs. Neomorphic mutations of the citrate metabolism genes IDH1 and IDH2 induce DNA hypermethylation and impair differentiation in hematopoietic cells. IDH mutations create a block to DNA and histone demethylation as a result of the production of 2-hydroxyglutarate (2HG). 2HG acts as a competitive inhibitor of α-ketoglutarate-dependent enzymes. The epigenetic effects of 2HG are caused in part though inhibition of TET2, a DNA demethylase enzyme also mutated in leukemia. ***Craig Thompson*** *presented IDH1/2- and TET2-mutant leukemias as a biologically distinct disease subtype, linking cancer metabolism with epigenetic control of gene expression.*

Mammalian species determine their nutrient uptake through tyrosine kinase receptor-mediated signal transduction, not in a cell-autonomous fashion.

There are two critical pathways: the first involves receptor tyrosine kinase activation of PI3K/AKT/TOR pathway to reprogram glucose and amino acid metabolism. In response, glucose becomes the primary substrate and allows cells to net lipid synthesis by reprogramming mitochondria from catabolic to anabolic organelles.

Citrate produced at the first step of the TCA cycle is exported and used to build primarily lipids. And through TOR activation, amino acid uptake is reprogrammed into tRNA charging and increased protein translation. Glucose becomes a primary substrate driven in a dose-dependent fashion by the activation of this pathway.

Second, cancer cells also have to be able to proliferate, and that requires a source of nitrogen. The source of nitrogen in the body is through metabolism of glutamine. In proliferating cells, glutamine is taken up through a separate signal transduction pathway with MYC as the key transcriptional effector and influenced by RAS and hedgehog signaling. Glutamine replaces glucose as the bioenergetic substrate to maintain the TCA cycle, but also becomes the major source of NADPH for anabolic synthesis.

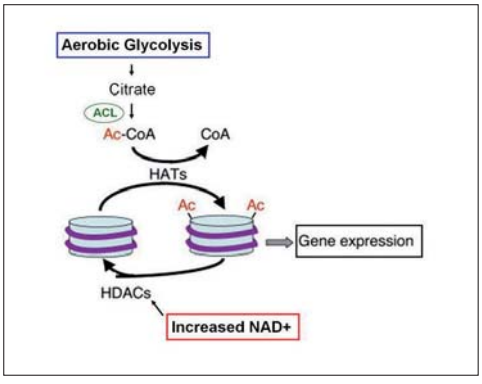


Figure 1
Histone modifications are responsive to metabolic input.

This is the way cells get their two primary nutrients to maintain both their viability and cell survival: one, through a signaling pathway that informs glucose and amino acid metabolism, and two, through glutamine metabolism.

The citrate that's produced through excess glycolysis is exported into mitochondria and broken down into acetyl-CoA and oxaloacetate. This acetyl-CoA is the primary

source of all the lipid synthesis and lipid modifications of proteins and glycolipids that are involved in the growth of proliferating cells.

Acetyl-CoA generated by glucose metabolism regulates histone acetylation. There are profound variations in H3 and H4 signal transduction as the extracellular glucose is modified or if ACL is eliminated. The primary class of acetyl transferases that seem to correlate with that are the GNAT subfamily.

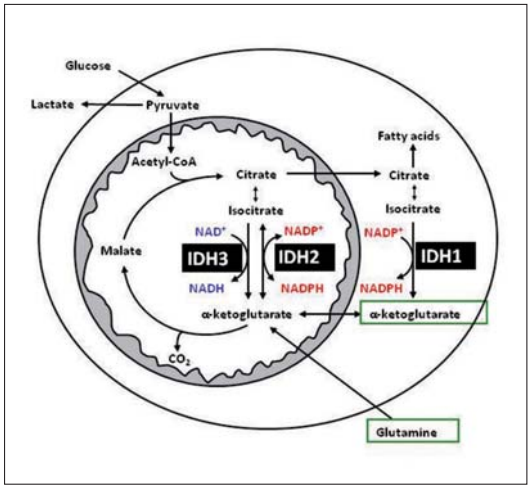
In this intriguing model, there are two different metabolic inputs for histone modification of chromatin. Caloric restriction, in which there is not enough glucose to maintain bioenergetics, leading to an increase in the NAD/NADH ratio in the cytosol and nucleus. That activates the sirtuin family of histone deacetylates to lead to histone deacetylation.

A high level of glucose availability through the production of acetyl-CoA drives the activation of the GNAT family of acetyl transferases to shift acetylation in the opposite direction. So the level of intracellular metabolites of carbon metabolism in the cell affects global acetylation in both directions.

Histone modifications:

Histone acetylation increases during adipocyte differentiation in an ACL-dependent manner. When fibroblasts go through an ordered differentiation to become adipocytes, at the end of the differentiation process, there are dense lipid dropules and huge quantities of triglycerides. There is also a profound change of histone acetylation.

Figure 2
IDH1 and IDH2 interconvert isocitrate and α-ketoglutarate.



Blocking ACL by RNAi completely eliminates the ability to induce acetylation; adding super-physiologic levels of acetate reverses the effect[1]. Silencing of ACL does not affect adipocyte differentiation, however.

By contrast, when ACL is blocked, the cells are incapable of up-regulating their ability to increase glucose uptake. They are unable to engage GLUT4, the specific glucose transporter that allows cells to take up glucose in bulk and turn it into fatty acids.

There is specificity in the system — at the uptake of glucose, its metabolism to acetyl-CoA and its ability to be modified for lipid biosynthesis. Adding acetate to the system rescues the suppression or loss of gene induction. This is an entirely specific regulation of glycolytic enzyme metabolism based on acetyl-CoA as a cofactor for acetylation.

Glucose availability regulates histone acetylation and gene expression in a dose-dependent fashion. It increases the expression of GLUT1, hexokinase 2 and PFK1 — the genes that control glucose capture and metabolism through the glycolytic pathway, and the ability to get rid of excess glucose as LDHA. This process is dependent on ACL, and knocking down ACL blocks the effect.

Acetylation appears to play a profound role in the specificity of this process. Once receptor tyrosine kinase initiates glucose uptake, its metabolism to produce acetyl-CoA drives histone acetylation. An unknown component targets histone modification and increased transcription of a set of genes and allows for a feed-forward loop. The cell detects that glucose is available to it and shifts its metabolism to using glucose as a substrate for both bioenergetics and net synthesis.

If there is an increase in NAD levels — an alternative sensing method for bioenergetic limitation — it leads to activation of histone deacetylation, shutting down genes and blocking that consumption of energy because the cell is bioenergetically compromised.

So this toggles the rate at which genes are transcribed based on the energetic status. Histone acetylation generally leads to an increase in gene expression — metabolism has an input into this, and levels of both NAD and acetyl-CoA affect it. Acetyl-coA might affect the process at multiple levels, from the net output of transcription all the way to protein.

When the glucose available to cells is restricted, not only do sirtuins become activated in an NAD-dependent ability to deactylate, but there are also specific marks on histones involving phosphorylation. This is through AMPK, which moves to the nucleus, directly phosphorylates specific residues on H2B, and modifies the ability to transcribe genes — particularly the stress response gene, p532.

Alternate metabolism:

MYC-transformed cells metabolize glutamine in a manner that optimizes NADPH production rather than ATP. When MYC is up regulated, it has a profound effect on glutamine uptake and metabolism.

Glutamine is consumed, through mitochondrial metabolism and ultimately through cytosol. About 75% of the carbon gets thrown away in this process, providing the cell with two essential things for growth: nitrogen and NADPH.

The step that produces NADPH depends on malic enzyme, but the malic enzyme is unexpectedly in the mitochondria and not in the cytosol.

The IDH3 enzyme in the Krebs cycle takes electrons off isocitrate and makes α-ketoglutarate. There are two other enzymes, IDH1 and IDH2: IDH2 has a duplicated mitochondrial targeting sequence, IDH1 does not, and the two are in an exchange cycle.

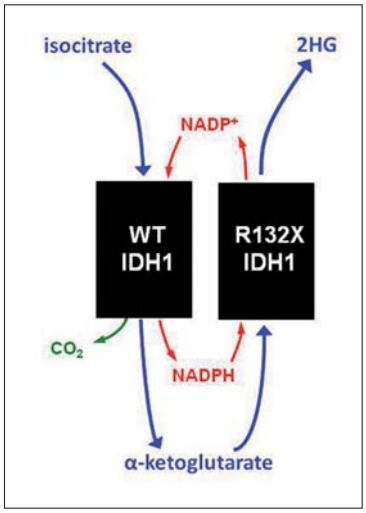


Figure 3
A heterodimer between wild type and mutant IDH1 potentiates 2HG production.

IDH1 and IDH2 interconvert isocitrate and α-ketoglutarate. When cells take up a high amount of glutamine, it's converted in the mitochondria to α-ketoglutarate, and IDH2 can drive it in the reverse reaction using NADPH to produce citrate. The citrate is then exported into the cytosol, and IDH1 does the reverse reaction to produce NADPH and a pool of α-ketoglutarate.

The signature of this form of metabolism is high levels of α-ketoglutarate in the cytosol and high levels of NADPH. Wild type IDH1 and IDH2 are both critical for the proliferation of glutamine dependent-cells.

Complete exome sequencing of cancer cells has shown mutations in several enzymes known to be involved in glioblastoma formation. The most frequent one of these not previously described is in IDH1; about 12% of glioblastomas have that mutation. Also, 100% of patients who have a secondary glioblastoma that arose 6-8 years after an astrocytoma or oligodendroma have the mutation.

There are two cancers associated with frequent mutations of IDH1 or IDH2: gliomas and acute myelogenous leukemia (AML). Among gliomas or secondary glioblastomas, in almost every data set, about 80% of tumors have mutations in either IDH1 or IDH2. In AML, mutations in IDH1, IDH2 and TET mutations account for 75% of tumors. There are also isolated cases in colorectal cancer, prostate cancer and melanoma.

Most mutations occur in a single arginine in either IDH1 or IDH2. These mutant enzymes can't bind isocitrate and convert it to α-ketoglutarate. About 50% of leukemias have mutations in another arginine (Arg140) in IDH2.

Organic metabolite:

If a mutant IDH1 is introduced into tumor cell lines, of 612 metabolites, all but 3 are identical in the wild type and tumor cells. These 3 spots correspond to different derivatives of the same organic metabolite, 2-OH glutarate (2HG). The mutant fails to carboxylate α-ketoglutarate, so it accepts electrons, resulting in the production of 2HG[3].

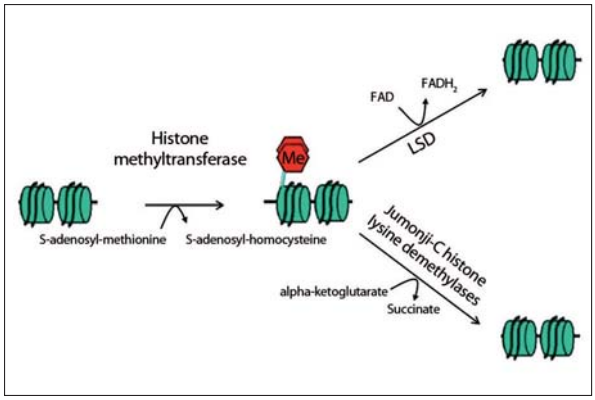


Figure 4
Dynamics of histone lysine methylation.

The production of 2HG is about 800-fold slower than the normal forward reaction. The oncogenic enzyme is a heterodimer between wild type and mutant IDH1. The wild type partner takes isocitrate, produces NADPH and α-ketoglutarate, and the high local concentration of these two enzymes drives the reverse reaction of the mutant to produce 2HG[4].

Essentially, in this process isocitrate is converted into 2HG. The slower-than-normal rate doesn't allow any other perturbation of metabolism other than net accumulation over time of 2HG.

Analyzing intermediate metabolites in glioblastomas and leukemias shows no difference between tumors that have wild type or mutant IDH except in 2HG production. Tumors with IDH1 mutations show about a 100-fold difference increase in 2HG over wild type levels.

In leukemia data sets, essentially every other gene known to be involved in pathogenesis of adult AML is found to be mutated in association with IDH1 or IDH2 — with one exception. TET2 is the most commonly lost tumor suppressor in leukemia, but these loss-of-function TET2 mutations are mutually exclusive with IDH1/2 neomorphic mutations.

This suggests that TET2 is an α-ketoglutarate dependent dioxygenase that hydroxylates 5-methylcytosine. Cells transfected with TET2 acquire large levels of hydroxymethylated cytosine. Transfecting a mutant IDH into these cells completely blocks TET2 hydroxylation of 5-methylcytosine.

When IDH mutations are introduced exogenously using retroviruses, AML cells also become highly methylated, with about a 20% increase over 3 days[5]. This correlates with a 10-fold change in hydroxylation, which happens almost immediately.

The timing of these effects suggests the IDH mutations affect the ability to pass on the methylation during replication, rather than having a direct effect on DNA methylation. In direct proportion to that, there is also an accumulation of cells with a stem cell-like phenotype.

IDH mutation is tightly associated with DNA hypermethylation. Gliomas also have a subset of cells that are hypermethylated, and this is dubbed the glioma-CpG island methylator phenotype, or G-CIMP. Hydroxylation is the most profoundly affected, and there's usually a log change of loss in hydroxylation with loss-of-function mutations in IDH1 or TET2.

Blocked differentiation:

Histones can also be methylated. In wild type cells, there's an intermediate stage of methylation for the various marks — di- and tri-methylation of H3K9, H3K36, H3K27, H3K4, H3K27.

IDH mutant alleles that produce 2HG result in a profound increase in histone methylation in a wide variety of marks. 2HG is a competitive inhibitor of α-ketoglutarate in histone demethylation.

For example, adding α-ketoglutarate or 2HG to cell extracts has no effect on H3K9 trimethylation. But when KDM4C is added, there is a profound demethylation in the presence of α-ketoglutarate, and that's completely reversed by adding 2-HG. Dosing back in more α-ketoglutarate reverses that effect.

Adding a cell-permeable α-ketoglutarate to intact cells has the same kind of effect on H3K9 methylation. The IDH1 mutation R132H increases the methylation of these genes, and adding α-ketoglutarate reverses the methylation.

2HG-producing IDH mutations result in a block to differentiation. The mutations block all the transcription factors involved in allowing the cells to go from fibroblasts to fat cells.

Looking at individual promoters using chromatin immunoprecipitation, this correlates with a highly reproducible effect of H3K9 methylation of C/EBP-α and adiponectin. There is some effect on K27 but no difference in H3K4. It's unclear where the specificity comes from.

The hyper-methylation of H3K9 lasts throughout the course of differentiation, which takes four days. The same is true of the H3K27 effect. Strikingly, histone acetylation at H3 is profoundly blocked under the same conditions.

So, there seems to be a reciprocal relationship between methylation and acetylation, at least during differentiation.

In summary, α-ketoglutarate levels — and particularly how much cells take up or metabolize glutamine — has a profound influence on the ability to use methylation codes, both at histones and at DNA, to modify gene expression and differentiation. In cancer, this blocks the ability to engage in methylation and DNA acetylation cycles that are required for cellular differentiation.

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BRCA1 tumor suppression occurs via maintenance of heterochromatin mediated silencing

A report on a lecture by
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The Salk Institute for Biological Studies, La Jolla, USA

BRCA1 is implicated in a multitude of cellular functions, including DNA damage repair, cell cycle checkpoint activation, DNA replication and centrosome function, among others. BRCA1 is highly expressed in proliferative cells and its loss leads most prominently to genetic instability and growth arrest. Mutations in the tumor suppressor gene BRCA1 lead to breast and ovarian cancer. BRCA1 loss in mice results in transcriptional derepression of tandemly repeated satellite DNA and loss of ubiquitination of histone H2A. Ectopic expression of an H2A fused to ubiquitin reverses the effects of BRCA1 loss on gene silencing at satellite repeat regions, cellular proliferation and survival, suggesting that BRCA1 maintains heterochromatin structure via ubiquitination of histone H2A. This function requires the RING finger domain of BRCA1 in which many pathogenic mutations leading to breast cancer reside. Satellite DNA derepression has also been seen in BRCA1-deficient mouse and human breast cancers. Ectopic expression of satellite DNA can phenocopy BRCA1 loss in centrosome amplification, cell cycle checkpoint defects, DNA damage and genomic instability, all hallmarks of cancer. ***Inder Verma*** *proposed that the role of BRCA1 in maintaining global heterochromatin integrity accounts for a major part of its tumor suppressor function.*

Each year in the United States, about 220,000 women are diagnosed with some form of breast cancer. Of these cases, 90% are sporadic, and between 5-10% have familial genes that are highly penetrant. These last are primarily because of mutations in BRCA1 and BRCA2, but there may also be other genes involved.

The penetrance of mutations in BRCA1 is very high. By the age of 70, those with a BRCA1 mutation have 95% chance of getting breast cancer[1].

Breast cancer manifests in many different forms of tumors. BRCA1 tumors are generally medullary carcinomas of the basal epithelium. More importantly, BRCA-related breast cancer is negative for estrogen and progesterone receptors. In most cases, it's also negative for HER2 amplification, so it has all the worst features, and is a very aggressive form of breast cancer.

The BRCA1 protein was identified in 1994. Since then, it has been found to be a multifunctional nuclear protein. Based on its structure, it seems to have a role in DNA damage signaling. It has a DNA-binding domain with poorly defined binding to AT-rich domains.

It also has a transcriptional activation domain with tandem BRCT repeats and a RING domain, meaning that it has the ability to act as a ubiquityl ligase. It is involved in centrosome function and in X-chromosome inactivation, although this latter function remains controversial. Although BRCA1 appears to have many functions, it's unclear which its main function is.

BRCA1 forms a heterodimer with the BARD1 protein, which also has a RING domain. BRCA1 almost always functions together with BARD1. In fact, like the knockout of BRCA1, a knockout of

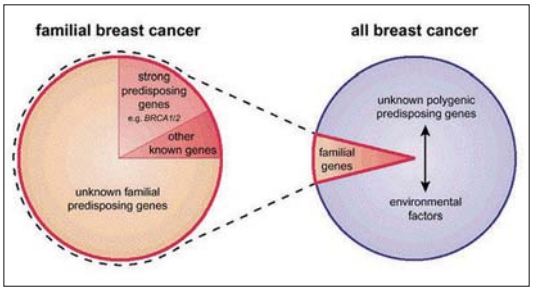

Figure 1
Genetic risk of breast cancer.

BARD1 is also early embryonic lethal around gastrulation E6.5.

Surprisingly, BRCA1 is highly expressed in the embryonic neuroectoderm. BRCA1 expression is up regulated in neurogenic areas, especially the dentate gyrus, in the adult brain[2].

Using a complicated breeding scheme, conditional BRCA1 knockout mice have been generated. These Nestin-CRE conditional knockouts generally survive for less than three weeks, and they have a body weight that is one-third that of control mice.

The mice also have a very interesting behavioral phenotype. They are highly ataxic and have poor motor coordination. They also have tremors, and have much become more agitated compared with wild type mice, when separated from their mothers.

Size matters:

The knockout causes severe disorganization of the brain structures. For example, the cortex of these mice is much thinner than in the wild type mice. Sectioning of the brain shows that the wild type cortex has 6 layers. By contrast, the knockouts have a much thinner cortex, and they loose some of the upper layers II/II.

The brains of these knockout mice are smaller in size because of a high degree of apoptosis. The ganglionic eminences, which are the origin of the tangentially migrating interneurons, are highly apoptotic compared with controls and almost completely absent by embryonic day E15.5.

The cerebellum in the knockouts has almost no foliation. This might explain the tremors and the lack of coordination because the cerebellum, which regulates motor control, is essentially gone. The olfactory bulb also doesn't have much lamination in the knockouts compared with the wild type. In some ways, this complete dysregulation is reminiscent of cancer.

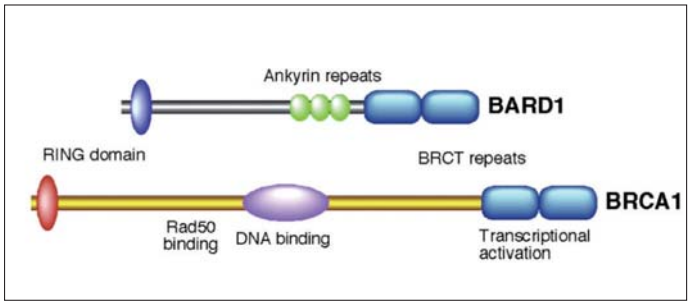

Figure 2
BRCA1 is a multifunctional nuclear protein.

Deletion of BRCA1 also reduces the number of heterochromatic centers (see Alzmouzni, page 9) in the mouse brain. The median number of heterochromatic centers in cortical sections decreases from 5 in controls to 1 upon deletion of BRCA1.

Minor and major satellite repeats are located in the pericentromeric and subtelomeric regions. Pericentric constitutive heterochromatin is modified with SUV39h and G9b methyltransferases, which methylate histone H3 at lysine 9. This methylated H3K9 then binds and thus recruits the HP1 protein through the chromodomain, which in turn suppresses transcription of the minor and major satellite DNA. Fewer heterochromatic centers might therefore be expected to lead to more expression of satellite DNA.

In fact, there's an almost 20-fold increase in the expression of satellite DNA from the knockout mice compared with wild type. However, no other interspersed repetitive DNA, such as LINE elements or IAPs increase in transcription. However there is a dysregulation of imprinting genes such as H19 and IGF2, which are up-regulated two-fold as would be expected from an imprinting failure.

ChIP assays from P7 mouse cerebellum demonstrate that BRCA1 binds mouse major and minor satellite

DNA *in vivo*. It binds to the same satellite DNA as HP1α and HP1γ in wild type mouse brains, indicating that there might be some interaction between BRCA1 and HP1.

HP1 is involved in heterochromatin formation and transcriptional silencing. It also has a role in centromeric stability, DNA replication, DNA damage response and cancer, many of the same functions described for BRCA1.

Fewer foci:

There are far fewer HP1 foci in the BRCA1-deficient brains compared with controls. The reductions of HP1 parallel decreases in HP1 DNA binding. Compared with controls, the BRCA1 knockouts also have substantially lower amounts of all three isoforms of the HP1 protein in the cerebellum.

Interestingly, this effect is only at the level of protein; HP1 messenger RNA is not affected, again hinting that there's an interaction between HP1 and BRCA1. Blocking BRCA1 expression with Lenti siBRCA1 in HeLa cells also destabilizes HP1 proteins, especially HP1α. Here again, mRNAs of all three isoforms of HP1 are not altered.

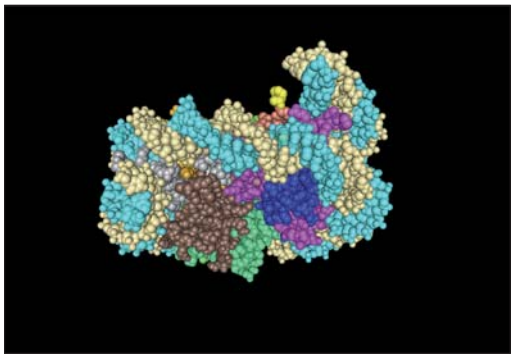

Figure 3
The histone H2A ubiquitylation site lysine 119 is exposed on the nucleosomal surface.

BRCA1 knockout brains also show reduced numbers of foci containing ubiquitinated histone H2A. This effect parallels the reduction of HP1 foci and DAPI foci.

There are several papers showing the role of H2A ubiquitination in Polycomb silencing. Polycomb group proteins Ring1A/B link ubiquitination of histone H2A to heritable gene silencing and X inactivation. BRCA1 also has the ability to ubiquitinate because it has ubiquitin ligase activity in its Ring domain.

One of the substrates for BRCA1 ubiquitin ligase activity is H2A, but only in the presence of BARD1[3]. Many of the BRCA1 pathological mutations, including one in cysteine 39, are in its RING domain.

The HCC1937 cell line has a truncated and highly unstable form of the BRCA1 protein that is functionally inactive. This cell line has a high level of expression of satellite DNA. If BRCA1 is reintroduced using lentiviruses, the satellite DNA levels return to normal. BRCA1 with Ring V11A substitution, a Ring-domain polymorphism, behaves like the wild type. But Ring finger mutants T37R, C39Y and I26A show effects that mimic the loss of BRCA1.

In the ChIP assay, ubiquitination of histone H2A in the wild type is enriched in both the major and minor satellite DNA, and the ubiquitinated histone H2A accumulates at pericentric satellite regions compared with Histone H3. In the knockout, however, there is little to no binding and no accumulation of ubiquitin-H2A adducts at satellite regions.

The histone H2A ubiquitination site lysine 119 is exposed on the nucleosomal surface. A surrogate for ubiquitination made by fusion of a single ubiquitin domain to the C-terminus of histone H2A mimics the natural ubiquitinated histone H2A. Introducing this surrogate mono-ubiquitinated H2A to BRCA1-deficient primary neural cells restores silencing of satellite DNA to a normal state.

This offers genetic proof that H2A ubiquitination can substitute for BRCA1 function in terms of the ability to suppress transcription of satellite DNA. The exogenously expressed ubiquitin-H2A fusion proteins are

incorporated into the chromatin and nuclear matrix fractions.

BRCA1 is a suppressor gene, so its loss would be predicted to increase cell proliferation. Surprisingly, however, BRCA1-deficient cells show a defect in cell proliferation. The ubiquitin-H2A mimic also corrects this proliferation defect in BRCA1 knockout cells, suggesting that it is the target for BRCA1 and can substitute for multiple BRCA1 functions.

Clinical validation:

As in the brain, mammary tissue that lacks BRCA1 also has very high levels of satellite DNA transcription, both major and minor. In agreement with the mouse brain tissue, there is also a dysregulation of imprinted H19 expression, but not LINE1.

When the breast tumors that develop in these mice are examined, the satellite DNA expression is elevated in comparison with expression in a control mammary gland, or in the kidney where BRCA1 is intact.

In collaboration with the Netherlands Cancer Institute, when 9 breast cancer samples from BRCA1 patients were analyzed, almost each case has much higher levels of satellite band DNA compared with cultured primary normal mammary epithelial cells or normal human breast tissue. This validates the results from the mouse mammary tumors.

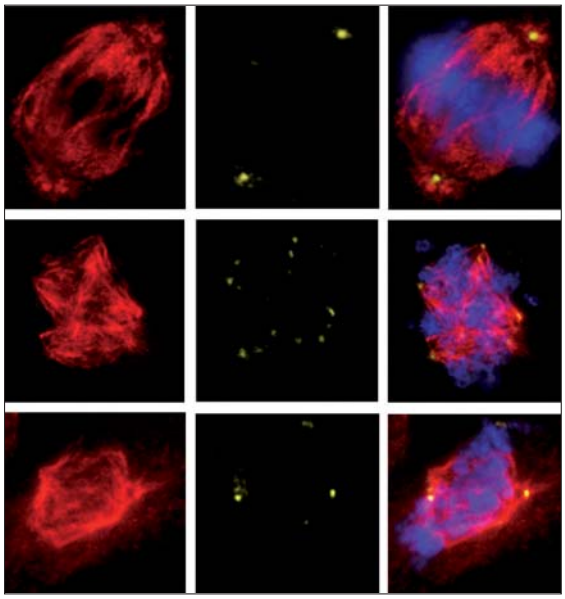

Figure 4
Ectopic satellite DNA expression or BRCA1 deficiency induces centrosome amplification

To investigate how the high expression of satellite DNA contributes to the pathology of loss of BRCA1, a lentiviral vector was made with an H1 promoter to the expression of the major and minor satellite DNA. Consistent with the H2A-ubiquityl results, ectopic satellite DNA expression induces genomic instability and mitotic catastrophes though centrosome amplification. The cells can't segregate their chromosomes because they are fused at their ends, a common occurrence in cancer.

Ectopic satellite DNA expression or BRCA1 deficiency also induces centrosome amplification. Controls have two centrosomes but in the case of the satellite DNA over expression, there are multiple centrosomes. BRCA1 knockout also has the same effect. In essence, satellite DNA over expression largely recapitulates BRCA1 loss BRCA1 in causing genomic instability.

Satellite DNA transcripts in fact cooperate with the loss of p53 in the induction of mitotic abnormalities. Satellite over expression induces chromosome instability, and serious problems with chromosome multiplication such as aneuploidy and polyploidy.

Satellite DNA over expression also has a role in cancer. At least in pancreatic and lung epithelial cancer, there is aberrant over expression of alpha satellite DNA transcripts.

In summary, BRCA1 plays an important role in maintaining heterochromatin integrity. The loss of BRCA1 leads to the loss of a silencing mark, ubiquitinated H2A at constitutive heterochromatin. This in turn leads

to the transcribing of satellite DNA regions. Over expression of satellite DNA transcript can cause checkpoint failure, genomic instability and presumably eventual tumorigenesis.

The functions of heterochromatin correspond to BRCA1 functions. BRCA1's role, like HP1's role, may be to maintain heterochromatin. BRCA1 may also have a physical association with HP1, although whether it directly binds HP1 is as yet unproven.

BRCA1 is also known to have a role in homologous recombination but the effects of BRCA1 loss on homologous recombination are relatively marginal. This could be an indication that the defect of homologous recombination occurs through a failure in heterochromatin integrity, which affects homologous recombination.

To return to the ataxic phenotype, there were recent reports of a role for BRCA1 in brain morphogenesis in patients. In one case of twins with BRCA1 mutations, one of the twins when she was 9 years old also had epilepsy, as a result of which she got anti-estrogens and many other drugs. Axial proton density weighted magnetic resonance imaging scan showed a nodular heterotopia on one side of the hemisphere, which is reminiscent of what was seen in the mouse[4]. This interesting parallel warrants further investigation.

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Licensed to elongate: a molecular mechanism for MLL rearranged leukemia

A report on a lecture by
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Stowers Institute for Medical Research, Kansas City, USA

Chromosomal translocations that fuse mixed lineage leukemia 1 (MLL1) to any one of a large number of translocation partners are indicative of a poor clinical outcome in acute leukemias. Most MLL translocation partners share little sequence or functional similarities, and the mechanism by which they induce leukemia is unclear. The RNA polymerase II elongation factor ELL was the first translocation partner of MLL for which a function was determined. Based on this finding, the regulation of the rate of transcription elongation by Pol II has been proposed to play a central role in MLL-based leukemogenesis. Some of the most frequently occurring translocation partners of MLL are components of two newly discovered complexes: the ELL containing Super transcription Elongation Complex or SEC, and DotCom, a histone methyltransferase complex. These findings suggest that MLL fusion proteins are recruited to MLL target genes and license polymerase II to elongate without the appropriate checkpoints, leading to an unregulated expression of MLL targets. ***Ali Shilatifard*** *discussed the molecular role of MLL-chimeras in leukemic pathogenesis.*

Model systems such as *Drosophila melanogaster* and *Saccharomyces cerevisiae* can help us understand the molecular mechanism of pathogenesis of human malignancies through the role of epigenetic regulation in gene expression.

For example, the mixed lineage leukemia (MLL) protein contains about 4,000 amino acids and was characterized about 20 years ago. MLL translocations result in hematological malignancies, but very little is known about the biochemistry and molecular biology involved.

The mammalian homolog of MLL is clipped by a protease and held together by hydrogen bonding. The SET domain of SET1 in *S. cerevisiae* is homologous to MLL and its counterpart Trithorax protein in *Drosophila.*

SET1 is part of the yeast complex called COMPASS, which stands for complex proteins associated with SET1. COMPASS also includes 7 polypeptides, CPS60 through CPS25. COMPASS is the first histone H3K4 methylase identified in *S. cerevisiae*.

COMPASS was the first histone H3K4 methylase identified and can mono- di- and tri-methylate H3K4 with varying kinetic parameters: monomethylation is rapid, whereas trimethylation takes much longer; all the components of the complex are required for its enzymatic activity.

Drosophila has three COMPASS-related complexes, including one that is the direct descendant of the yeast complex. Each complex has a non-redundant function as a H3K4 methylase, with SETD1A, Trithorax and TRR representing the enzymatic components. Deleting any of the complexes results in lethality.

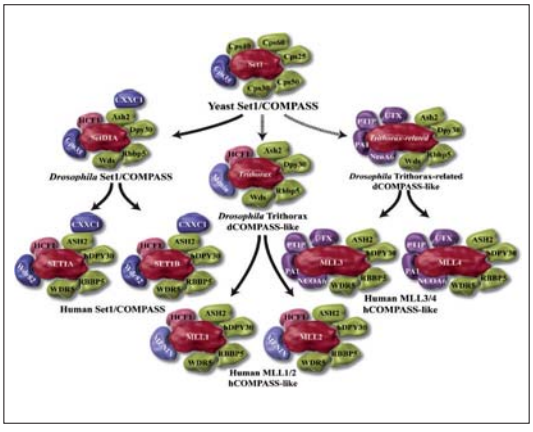

Figure 1
COMPASS-like complexes exist in several species

In humans, SET1/COMPASS is the direct descendant of the yeast complex, with SET1A/1B as the enzymatic component. MLL1 and MLL2, and MLL3 and MLL4 are also present in COMPASS-like complexes. The difference is that the MLL3/4 class of enzyme carries UTX, and the MLL1/2 complex does not have UTX but associates with Menin[1].

Yeast is a great model system because there is a deletion collection library available for the yeast genome. Using these mutants, a GPS (global proteomic analysis in *S. cerevisiae*) method showed that mono-ubiquitination of histone H2B by RAD6/BRE1 is required for H3K4 methylation by COMPASS and DOT1.

This mono-ubiquitination/K4 methylation crosstalk is highly conserved from yeast to human. It plays a very important role in Wnt signaling, stem cell pluripotency, DNA damage repair and stability and many other pathways.

Conserved processes:

RAD6/BRE1 mono-ubiquitination of histone H2B results in the recruitment of the PAF1 complex, which recruits COMPASS and leads to H3K4 methylation. The same process also results in K79 methylation through DOT1. These processes are conserved from yeast to mammalian systems.

A robotic screen with more than 780 point mutations in histones H3, H4, H2A and H2B reveals 6 residues important for K4 methylation. Of the 6 identified residues, 4 land in a single patch of the nucleosome that regulates K4 methylation independently of ubiquitination. This patch is also highly conserved from yeast to human.

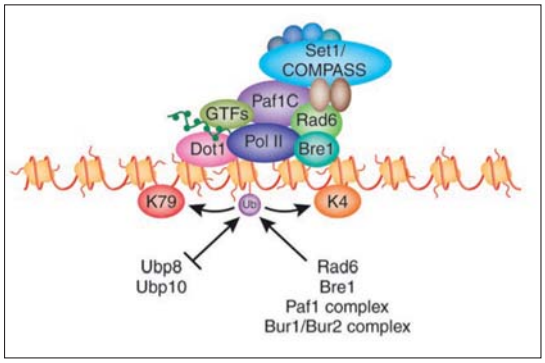

Figure 2
Histone monoubiquitination/methylation crosstalk.

In wild type yeast, all the components of the COMPASS can be purified. But with a single point mutation at the site of ubiquitination or with RAD6 deletion, the level of CPS35 within COMPASS is reduced by several fold. Chromosome immunoprecipitation shows that CPS35 interacts with chromatin in a H2B mono-ubiquitination dependent manner.

In humans, WDR82 is the homolog of CPS35. But WDR82 is specific to the hSET1 complex, and does not purify with MLL1, 2, 3 or 4. Knocking out WDR82 only affects the hSET1 complex, but not the MLL complexes.

Surprisingly, in five different human cell lines tested and in mouse cells, reducing the level of WDR82 results in a global loss of H3K4 trimethylation, but has very little effect on mono- or dimethylation.

When MLL1 is deleted, of 20,000 genes screened, only 299 genes lose their K4 methylation, indicating that MLL1 targets only about 2% of the mammalian genome. But these genes are all important in development.

For example, a large number of genes within the HOX cluster require MLL1 and MLL2 for their function. A5 through A9 show a loss of trimethylation in the absence of MLL1. However, A1 and A3 have a normal pattern of methylation even without MLL. If MLL1 and MLL2 are deleted together, K4 methylation is almost abolished over the entire HOX cluster.

In summary, H3K4 methylation is a complicated process. It's important to understand why there are six K4 methylases in humans with non-redundant function, how they obtain their target specificity, and why MLL translocations cause pathogenesis of hematological malignancies.

Elongation factors:

A large number of genes translocate into MLL, resulting in leukemia. However, with multiple partners and models, the field is far from clear. Almost 16 years ago, ELL, one of the MLL translocation partners, was shown to be an RNA polymerase II elongation factor, suggesting that the regulation of transcriptional elongation is central for the pathogenesis of hematological malignancies[2].

Since then, human ELL1, ELL2 and ELL3 have all been cloned. These proteins contain the elongation activation domain at the N-terminus, and the C-terminal domain is required for pathogenesis of leukemia through the translocations.

In early embryos of *Drosophila*, ELL is a nuclear protein. The elongation activation domain of ELL is required for its localization to chromatin. For transcriptional regulation to occur, basal factors are recruited, which then recruit RNA polymerase II. The polymerase lingers around the template, a term known as a Pause Pol II or Poised Pol II.

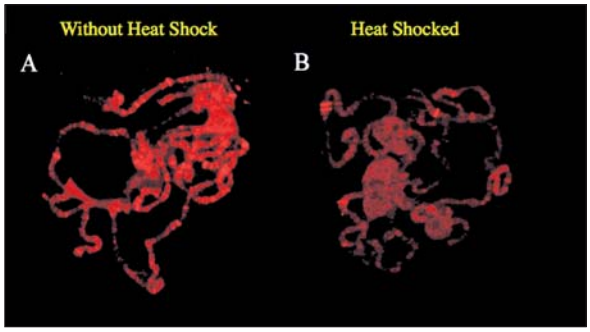

Figure 3
dELL localizes to 87 & C loci upon heat shock.

Poised Pol II is found on many developmentally regulated genes in both humans and *Drosophila*. Identifying the elongation factor that can release Poised Pol II could be central for the process of development and perhaps disease pathogenesis.

In *Drosophila*, ELL is at the polytene chromosome, but upon heat shock, localizes to 87A and C. In the presence of ELL, there is 100% expression of heat shock gene HSP70; in its absence, expression is reduced by about 90%, indicating that ELL is involved in the process of poise release.

Biochemical isolation of mammalian ELLs 1, 2 and 3 has shown that they associate with many of the MLL translocation partners found in leukemia. All of these unrelated proteins seem to be in the same complex. Most importantly, they carry an elongation factor named CDK9 or pTEF-b.

AFF4 is a central component of this complex. Purification of AFF4, also a known MLL translocation partner, results in the purification of all the polymerase II elongation factors, many of the MLL translocation partners, and pTEF-b. Given that there are 6 elongation factors in this one complex, it has been called the super elongation complex, or SEC.

Size-exclusion chromatography shows that when AFF4 is reduced, CDK9 does not associate with this large SEC complex, and loses its kinase activity within this large complex. This indicates that SEC seems to be the CDK9-containing large complex that is active in the phosphorylation of CTD of RNA Pol II.

Licensed to elongate:

The *Drosophila* homolog of AFF4, a gene named Lilly, is required for embryogenesis. Like human ELLs, it also localizes to heat shock loci upon heat shock. In humans, reducing the levels of AFF4 by RNAi also creates defects in heat shock gene expression.

So, many of these MLL partners are found in the SEC. Purifying the MLL-ELL, MLL-ENL, MLL-AFF4 and MLL-AF9 complexes and analyzing by mass spectrometry shows that AFF4 is a core component of the MLL chimeras found in leukemia.

Figure 4
The super elongation complex.

This model, called licensed to elongate, is a molecular mechanism of MLL translocation. AFF4 is at the center of the complex and holds it together. There could be translocations such as MLL-ELL translocation, MLL-ENL, AF1 and AF9. Regardless of which the translocation is, reducing the levels of AFF4 in those cells should show defects in HOX gene expression.

AFF4 is required for the expression of MLL-chimera target genes. It is recruited to MLL target genes HOX 19 and HOX a10 in leukemic cells carrying MLL-chimeras. Although it's involved in leukemia, SEC is a general transcription elongation factor, with the most active form of p-TEF-b[3].

H3K79 dimethylation plays an important role in cell cycle regulation. It is implemented by an enzyme called DOT1, for disruptor of telomeric signaling 1. DOT1 is thought to play an important role in telomeric silencing in *S. cerevisiae*, and to be the histone mark that's involved in epigenetic processes.

ChIP sequencing analysis of the yeast genome shows that genome-wide, the patterns of H3K79 di- and trimethylation do not overlap.

Mono-ubiquitination of H2B is required H3K79 methylation by DOT1. Using an antibody for mono-ubiquitinated histone H2B shows that mono-ubiquitination is associated with H3K79 trimethylation, but not dimethylation. This suggests that mono-ubiquitination regulates mono-, di- and trimethylation by DOT1.

Position effect variegation (PEV) is a process discovered in the 1930s. Essentially, if a gene is inserted near heterochromatin, it can be silenced by heterochromatin. This was shown with eye color experiments in flies, but is not a fly-specific phenomenon. It has also been seen in agouti mice, petunias and many different organisms.

Using a URA3 reporter assay for disruption of telomeric silencing, DOT1 has been shown to be the H3K79 methylase, and K79 methylation is required for telomeric, HM, HMR and rDNA silencing.

Telomeric silencing:

In this model, SIR proteins are held at the telomeres because the euchromatin is methylated on H3K79. In the absence of H3K79 methylation, the SIR proteins rush in and disrupt telomeric silencing.

DOT1 can mono- di- and trimethylate H3K79. But which one of these marks is involved in telomeric silencing?

SWI4 and SWI6 are involved in dimethylation, but not trimethylation; these two actually form a complex in yeast. ARD1 is required for trimethylation, but not dimethylation. A large number of mutants that can distinguish between mono-, di- and trimethylation have also been identified through screens.

Microarrays can help analyze the natural pattern of gene expression and define naturally what happens to telomeric de-repression in the absence of H3K79 mono-, di- or trimethylation. The only gene that popped out as affected is COS12, which is on chromosome VII-L.

Genome wide, there is not much difference in the localization of SIR1, SIR2 and SIR3 in the presence or absence of DOT1 or H3K79 methylation. That suggests that this mechanism of regulation is SIR-independent.

Lastly, K79 was also thought to be involved in HML and HMR silencing, but when DOT1 is deleted, there is

no difference in the natural pattern of gene expression, indicating that H3K79 methylation is not involved in the maintenance of HML/HMR silencing.

Common telomeric gene silencing assay is affected by nucleotide metabolism. URA3 mostly reports on the ribonucleotide reductase level in these cells, and is not a good reporter for studying telomeric PEV. In fact, it might be better to screen for natural patterns of gene expression rather than looking at reporter patterns.

Biochemical purification of human DOT1 complex shows that DOT1 exists in a 2 MDa complex capable of K79 methylation. Most importantly, it also identified TRRA1 and SKP1 as co-existing with DOT1. β-catenin also associates with DOT1, indicating that K79 methylation could play important role in the Wnt signaling pathway.

The wing imaginal disc in *Drosophila melanogaster* can be used as a model system to study Wnt signaling. The wing imaginal disc is a layer of cells called dorsal-ventral boundary cells. These require wingless in the Wnt signaling pathway for the expression of senseless, so it can be an indicator of Wnt signaling.

Engrailed GAL4 RNAi reduces the level of Wnt signaling machinery in the posterior half, leaving the anterior half as control. If the level of DOT1 complexes, or DotCom, is reduced in this site, there is very nice expression of senseless. But in the absence of K79 methylation, and more specifically K79 trimethylation, the Wnt signaling pathway is defective[4]. This suggests that H3K79 trimethylation regulated via H2B monoubiquitination is required for Wnt signaling.

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Abbreviations and glossary

1. ABBREVIATIONS

AML	acute myelogenous leukemia	**iPS cells**	induced pluripotent stem cells
AZA	azacitidine	**LPS**	lipopolysaccharide
bHLHZ	basic-helix-loop-helix-zipper	**LREA**	long-range epigenetic activation
CGI	CpG island	**LRES**	long-range epigenetic silencing
CIMP	CpG island methylator phenotype	**PcG**	Polycomb group proteins
DAC	decitabine	**PMD**	partially methylated domain
DMR	differentially methylated regions	**PRC**	polycomb repressive complex
DNMT	DNA methyl transferase	**PSA**	prostate-specific antigen
ES cells	embryonic stem cells	**ROS**	reactive oxygen species
HDAC	histone deactylase	**SNP**	single nucleotide polymorphism
HIC	hypermethylated in cancer	**TCGA**	the cancer genome atlas project
IL-1	interleukin-1	**TSS**	transcription start site

2. GLOSSARY

Terms in *italics* are defined elsewhere in the Glossary.

acetyl-CoA an important molecule in *metabolism*, mainly to convey the carbon atoms within the acetyl group to the citric acid cycle to be oxidized for energy production

centromere a region of DNA typically found near the middle of a chromosome; involved in cell division

chromatin the combination of DNA and other proteins that make up the contents of the nucleus

cistrome the set of DNA binding sites of a transcription factor on a *genome* scale

CpG islands *genomic* regions that contain a high frequency of CpG sites

epigenetic changes in gene expression caused without altering the underlying DNA sequence

epigenome the *epigenetic* equivalent of the *genome*

genome the complete genetic information (either DNA or, in some viruses, RNA) of an organism

glioblastoma the most common and aggressive malignant primary brain tumor in humans

glycolysis the sequence of reactions that converts glucose into pyruvate with the concomitant production of a small amount of ATP

heterochromatin	a tightly packed form of DNA
histone	highly alkaline proteins that package and order the DNA into structural units called *nucleosomes*
histone chaperone	factors that associate with *histones* and help regulate their storage or transfer from one place to another
hypermethylation	an increase in the *epigenetic methylation* of DNA residues
Induced pluripotent stem cell	commonly abbreviated as iPS cells or iPSCs, a type of pluripotent stem cell artificially derived from a non-pluripotent cell by inducing a forced expression of specific genes
macrophage	a white blood cell that phagocytizes necrotic cell debris and foreign material, including viruses and bacteria
metabolism	the set of chemical reactions that occur in living organisms in order to maintain life
methylation	the addition of a methyl group to a substrate or the substitution of an atom or group by a methyl group
methylome	The set of nucleic acid *methylation* modifications in an organism's *genome* or in a particular cell
myelogenous leukemia	leukemia resulting from a malignant transformation occurring in the bone marrow
neoplasia	an uncontrolled and disorderly proliferation of cells to form a tumour, which may be either benign or malignant
nucleosome	any of the subunits that repeat in *chromatin*; a coil of DNA surrounding a *histone* core
pluripotency	ability to develop into or effect any (or most) cell types i.e. not restricted to a specific system
polycomb	a protein (or one of a larger group) whose function is to modify gene expression via changes to *chromatin*
promoter	the section of DNA that controls the initiation of RNA transcription as a product of a gene
stem cells	cells characterized by the ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types
tumorigenesis	the process by which normal cells are transformed into cancer cells
transcriptome	the complete set of messenger RNA molecules (transcripts) produced in a cell or a population of cells
ubiquitination	the modification of a protein by the covalent attachment of one or more ubiquitin molecules
wild type	gene of interest with no known *mutations*; animal carrying such a gene; often designated as +/+ if both alleles are wild type, or +/- if one allele is wild type, the other mutated (-/- indicates that both genes are mutated

Participants in the group picture

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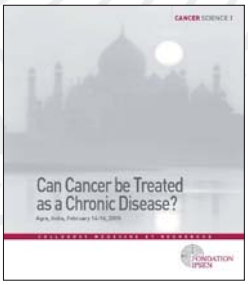
Cancer Science meeting series



CAN CANCER BE TREATED AS A CHRONIC DISEASE? • Agra, February 14-16, 2005

ORGANIZING COMMITTEE

Inder M.Verma (*Salk Institute, La Jolla, USA*), Yves Christen (*Fondation IPSEN, Paris, France*), Jacqueline Mervaillie (*Fondation IPSEN, Paris, France*)

SPEAKERS AND DISCUSSANTS

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ARE INFLAMMATION AND CANCER LINKED ? • Cape Town, Frebruary 12-15, 2006

ORGANIZING COMMITTEE

Inder M.Verma (*Salk Institute, La Jolla, USA*), Yves Christen (*Fondation IPSEN, Paris, France*), Jacqueline Mervaillie (*Fondation IPSEN, Paris, France*)

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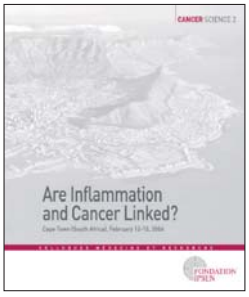

METASTASIS AND INVASION • Spineto, May 20-23, 2007

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METABOLISM AND CANCER • Villa Caletas, March 9-11, 2008

ORGANIZING COMMITEE

Inder M.Verma (*Salk Institute, La Jolla, USA*), Yves Christen (*Fondation IPSEN, Paris, France*), Jacqueline Mervaillie (*Fondation IPSEN, Paris, France*)

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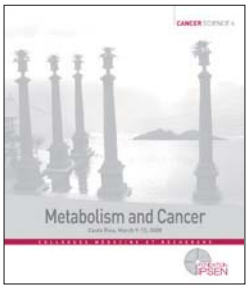

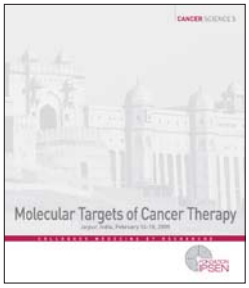

MOLECULAR TARGETS OF CANCER THERAPY • Jaipur, February 14-18, 2009

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STEM CELLS AND CANCER • Bariloche, March 6-10, 2010

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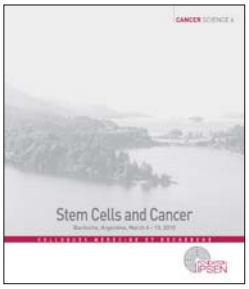

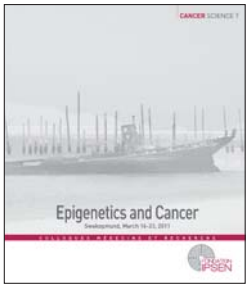

EPIGENETICS AND CANCER • Swakopmund, March 19-23, 2011

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Centro In preparation : Mouse Models of Human Cancer : Are They Relevant ?

Ouro Preto (Brazil), March 10 – 14, 2012

Fondation IPSEN

The Fondation IPSEN, created in 1983 under the auspices of the *Fondation de France*, has two objectives: the distribution of knowledge and encouraging the exploration of emerging areas of research.

Contributing to the development and distribution of knowledge

One mission of the foundation is to promote interaction between researchers and clinicians by creating 'crossroads' and forums for fruitful exchanges. Today, with the extreme specialization of knowledge and the increasing mass of information that many find difficult to decipher, such exchanges are indispensable. For this to be effective, the foundation has focused on some of the crucial biomedical themes of our time: the spectacular developments in neuroscience and the scientific study of cognitive mechanisms, the challenges of neurodegenerative pathologies, the omnipresence of genetics and molecular biology, the growing field of endocrine interactions, and the problems of aging populations and theories of longevity. More recently, activities have expanded into an area that is exciting for both its medical and fundamental challenges and that is currently in a phase of rapid development: cancer science.

Another goal of the Fondation IPSEN is to initiate, in partnership with the specialists and institutions involved, discussions and exchanges on the major scientific challenges of the future. Rather than trying to provide definitive knowledge, or to replace the work of large research organizations, the aim of these discussions is to emphasise multidisciplinary approaches at the boundaries of several disciplines, an approach that is essential for understanding the complexity and originality of human beings and their pathologies.

To fulfil these commitments, the foundation organises several series of international *Colloques Médecine et Recherche*, as well as several series of annual meetings in collaboration with scientific journals and institutions. Also, the Fondation IPSEN is funding awards to encourage research and publishing reports on its meetings. For each of these activities, the foundation brings together partners from the scientific and clinical world, who can independently report on the current state of knowledge and discuss the main issues in the areas on which the foundation has chosen to focus.

Over the past 28 years, the Fondation IPSEN has established its place in the scientific and medical landscape and intends to continue to be at the forefront in forming links, initiating multidisciplinary exchanges and contributing to the spread of knowledge, with time, intelligence, good will and above all, the collaboration of leaders in current biomedical research.

The Colloques Médecine et Recherche series

The *Colloques Médecine et Recherche* were created in 1987, with the first series dedicated to Alzheimer's disease. Its success stimulated the establishment of other several dedicated series: neurosciences, longevity, endocrinology, the vascular tree and more recently cancer. Meetings in each series are held annually, bringing leading international specialists together to present their most recent work, sometimes even before publication. Through these meetings, the *Fondation IPSEN* has over the years developed a large, international network of experts.

By focusing on emerging fields of knowledge, the meetings have supported the development of many new topics and have impacted on scientific advances in areas such as gene therapy and stem cells in the central nervous system, the role of cerebral amyloidosis in neurodegeneration, the contribution of genetic factors in resistance to disease, the benefits of neuronal grafts, biological markers of Alzheimer's disease, apolipoprotein E, brain-somatic cross-talk, relationships between brain and longevity, hormonal control of cell cycle to name a selection.

The series are organized around topics where active research is having or is likely to have a major impact on our knowledge:

• **Neurosciences** – Started in 1990, this series of conferences has both enabled the identification of the major themes to emerge in this area and has supported not only the remarkable expansion of the neurosciences in the past fifteen years but also the effort to integrate its subdisciplines, from molecular mechanisms to human cognition.

• **Alzheimer's disease** – Since 1987, this topic has been explored at annual meetings that have followed or even anticipated the development of the new field of 'alzheimerology', which has gone beyond histology and neurochemistry to establish the underlying pathological mechanisms.

• **Cancer Science** – Annual experts meetings are organized in collaboration with Inder Verma (Salk Institute, La Jolla, USA) and the participation of remarkable leading opinion makers in the field. Challenging topics (Can Cancer be Treated as a Chronic Disease? in 2005, Are inflammation and Cancer Linked? in 2006, Metastasis and Invasion in 2007, Metabolism and Cancer in 2008, Molecular Targets of Cancer Therapy in 2009, Stem Cells and Cancer in 2010, Epigenetics and Cancer en 2011, and, in 2012 Mouse Models of Human Cancer: Are They Relevant?) have generated outstanding discussions among the participants.

• **Endocrinology** – Established in 2002, this series examines the involvement of the endocrine system in the integration of all bodily functions. One example is the recent discovery of many hormones important in the control of metabolism, such as leptin and ghrelin. As aspects of brain-somatic crosstalk, such topics have impacts far beyond studies of hormones and the endocrine organs.

• **Longevity** – Launched in 1996, this series examines the challenges and paradoxes of medicine by focusing on a positive aspect, cases of exceptional resistance to the effects of aging, rather than on disease. The evolution of research dedicated to *aging* into research dedicated to *longevity* represents a remarkable development in this field.

• **Vascular Tree** – This new series, begun in 2004, aims to examine the various steps that lead to development of the vascular system, its growth in harmony with that of other organs, its degeneration, death and the possibilities for its regeneration. A new vision is emerging of blood vessels not as simple 'pipes' but as living, complex organs with interactions throughout the body.

Partnerships

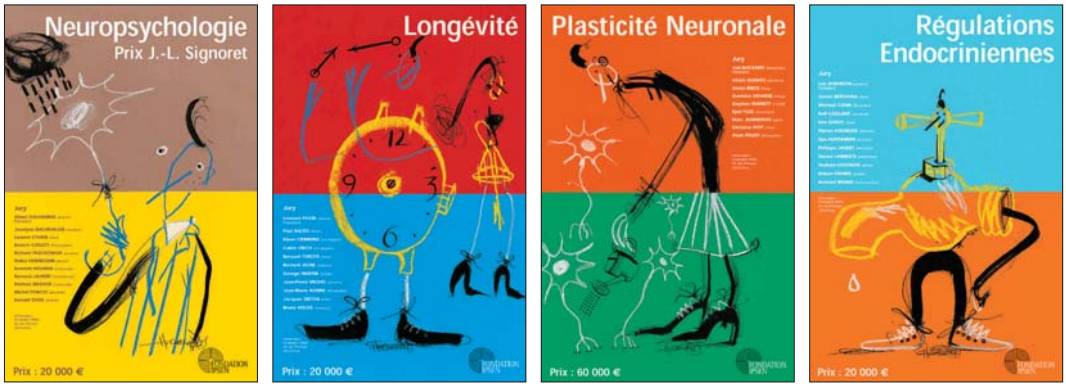
Long ago, the *Fondation IPSEN* has developed partnerships with international institutions and organisations, to encourage cooperation between experts in various disciplines. These partners include: the World Health Organisation (WHO), the *Fondation Nationale de Gérontologie* (FNG) and Harvard University.

Additional series of meetings and partnerships have been implemented since 2007:

- **Biological Complexity** series (*The Salk Institute for Biological Studies, Nature Publishing Group, and Fondation IPSEN*): Transcription Diseases (*La Jolla, 2007*), Genes, Circuits and Behavior (*La Jolla, 2008*), Processes of Aging (*La Jolla, 2009*), Sensory Systems (*La Jolla, 2010*), Future Concepts and Trends (*La Jolla, 2010*), Immunity and Inflammation (*La Jolla, 2012, in preparation*).
- **Emergence and Convergence** series (*Nature Publishing Group* and *Fondation IPSEN*): Small RNAs in Development, Immunology and Cancer (*New York, 2007*), Genome Variation (*2007*), Epigenetics and Behavior (*Houston, 2008*), Multiple Sclerosis: From Pathogenesis to Therapy (*Paris,2009*), Mitochondrial Dysfunction in Neurological Diseases (*Durham, 2008*), Epigenetic Dynamics in the Immune System (*San Antonio, 2010*).
- **Exciting Biologies** series
  - *Cell Press, Massachusetts General Hospital* and *Fondation IPSEN*:
    Biology in Motion (*Evian, 2007*), Biology of Cognition (*Chantilly, 2008*), Biology in Balance (*Buenos Aires, 2009*), Biology of Recognition (*Singapore, 2010*).
  - *Cell Press, The Riken Institute, DMMGF,* and *Fondation IPSEN:*
    Cellular Development: Biology at the Interface (*Kobe, 2011*).
- **Days of Molecular Medicine series** (*AAA Science, Karolinska Institute, Hong Kong University, DMMGF, and Fondation IPSEN*): Tissue Engineering and Stem Cells: Driving Regenerative Medicine Forward (*Hong Kong, 2011*)

Awards to Encourage Research

The *Fondation IPSEN* awards prizes to researchers who publish remarkable, pioneering studies. Currently, four awards are given annually:



Posters advertising the Fondation Ipsen prizes.

- **The Neuronal Plasticity Award** has been given each year since 1990 to three researchers working on the same theme: Albert Aguayo, Anders Björklund and Fred Gage; Ursula Bellugi, Wolf Singer and Torsten Wiesel; Philippe Ascher, Kjell Fuxe and Terje Lomo; Per Andersen, Masao Ito and Constantino Sotelo; Mariano Barbacid, Yves Barde and Hans Thoenen; Jacques Melher, Brenda Milner and Mortimer Mishkin; Friedrich Bonhoeffer, Cory Goodman and Marc Tessier-Lavigne; Antonio Damasio, Richard Frackowiak and Michael Merzenich; Heinrich Betz, Gerald Fischbach and Uel McMahan; Masakazu Konishi, Peter Marler and Fernando Nottebohm; Tomas Hökfelt, Lars Olson and Lars Terenius; Albert Galaburda, John Morton and Elizabeth Spelke; Arturo Alvarez-Buylla, Ron McKay and Sam Weiss; François Clarac, Sten Grillner and Serge Rossignol; James Gusella, Jean-Louis Mandel and Huda Zoghbi; Ann Graybiel, Trevor Robbins and Wolfram Schultz; Mary Kennedy, Morgan Sheng and Eckart Gundelfinger; Nikos Logothetis, Keiji Tanaka and Giacomo Rizzolatti; Jean-Pierre Changeux, Peter Kalivas and Eric Nestler; Alim-Louis Benabid, Apostolos P. Georgopoulos, Miguel A. L. Nicolelis; Thomas Insel, Bruce McEwen and Donald Pfaff; Helen Neville, Isabelle Peretz and Robert Zatorre.

• **The Endocrinology Award**, first given in 2002, has been received by Wylie Vale, Robert Lefkowitz, Pierre Chambon, Tomas Hökfelt, Roger Cone, William Crowley, Ron Evans, Gilbert Vassart, Shlomo Melmed and Paolo Sassone Corsi.

• **The Jean-Louis Signoret Neuropsychology Award**: since 1992, the recipients have been Eric Kandel, Jacques Paillard, Rodolfo Llinas, Steven Kosslyn, Alfonso Caramazza, Jean-Pierre Changeux, Emilio Bisiach, Joseph LeDoux, Joaquim Fuster, Stanislas Dehaene, Deepak Pandya, Utah Frith, Antonio and Hanna Damasio, Marc Jeannerod, Faraneh Vargha-Khadem, Alvaro Pascual-Leone, Elizabeth Warrington, Pierre Maquet, Giacomo Rizzolatti and Patricia Kuhl.

• **The Award for Longevity**, created in 1996, has been bestowed on: Caleb Finch, Vainno Kannisto, Roy L. Walford, John Morley, Paul and Margret Baltes, Justin Congdon, George Martin, James Vaupel, Linda Partridge, Sir Michael Marmot, Cynthia Kenyon, David Barker, Gerald McClearn, Jacques Vallin, Judith Campisi and Tom Kirkwood.

International Publications

Books summarizing of the conferences organised by the Fondation IPSEN are published in English and distributed by international publishers:

- Research and Perspectives in Alzheimer's Disease (Springer, 24 titles)
- Research and Perspectives in Neurosciences (Springer, 17 titles)
- Research and Perspectives in Longevity (Springer, 5 titles)
- Research and Perspectives in Endocrinology (Springer, 8 titles)
- WHO/Ipsen Foundation series (Springer, 7 titles)
- Brain and Mind Collection

Books and brochures recently published by the *Fondation Ipsen.*

In addition, since 1986 the Fondation IPSEN has published over 210 issues of *Alzheimer Actualités*, a newsletter dedicated to Alzheimer's disease; in 1993, a bi-annual journal, the *Bulletin du Cercle de Neurologie Comportementale* was started; and in 2005, the first of two series of annual reports on the conference dedicated to *Cancer Science* and the *Vascular Tree* appeared. The foundation also has widely distributed information in various forms to the medical professions and families of patients, as well as produced teaching films that have received awards from specialized festivals.

FONDATION IPSEN

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