Lashings of DNA methylation, forkfuls of chromatin remodeling

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Epigenetic control of gene expression in animals and plants is often correlated with changes in cytosine methylation at specific chromosomal loci. This leads to the irreversible promoter silencing of many genes, transposons, and endogenous retroviruses (Bestor 2000; Martienssen and Colot 2001). How DNA methylation patterns are set up during development, especially in the context of chromatin, is not well understood. Cytosine methyltransferases catalyze the transfer of a methyl group onto the C-5 position of cytosine from the universal cofactor SAM (Bestor 2000). Two main types of methyltransferase activity exist in mammals: a de novo activity and a maintenance activity. Dnmt3a and Dnmt3b have been identified as de novo methyltransferases, which methylate cytosine at CpG dinucleotides on both strands with little sequence specificity (Okano et al. 1998; Lyko et al. 1999). Dnmt1 is predominantly a post-replicative maintenance methyltransferase, which recognizes hemimethylated substrates, and acts to restore methylated cytosines at CpGs on the newly duplicated strand (Lyko et al. 1999, Bestor 2000). Mutation studies in mice have shown that the methyltransferase genes are necessary for embryonic (Dnmt1 and Dnmt3b) and postnatal (Dnmt3a) development (Lei et al. 1996; Okano et al. 1999). All three enzymes have been shown to methylate naked DNA substrates in vitro. However, complicating the issue is the in vivo evidence suggesting that patterns of DNA methylation are established and maintained within a nucleosomal infrastructure (Bird and Wolffe 1999). As yet we know little about the mechanistic basis of Dnmt3a and Dnmt3b activity in vivo, but in the case of Dnmt1 it has been shown that this maintenance methyltransferase enzyme can be targeted to PCNA, an auxiliary component of the DNA replication complex (Chuang et al. 1997). It is probable that chromatin assembly precedes DNA methylation behind the replication fork. Assembly of DNA into nucleosomes can prevent access by methyltransferases to their in vivo substrates (Kladde and Simpson 1994). Yet in the genome, methylated CpGs are distributed with equal probability either in the DNA wrapped around the nucleosome or in the linker region (Reik et al. 2001).

Is chromatin remodeling necessary for DNA methylation in vivo?

Somatic methylation patterns undergo dramatic remodeling during gametogenesis with further changes after fertilization and during the cleavage stages of the preimplantation mouse blastocyst (Reik et al. 2001). This results in a genome-wide loss of methylation during the preimplantation period, after which new patterns of DNA methylation are imposed through the combined action of the de novo and, subsequently, maintenance methyltransferases. Dnmt3a and Dnmt3b show nonoverlapping functions in mouse development, with Dnmt3b specifically required for de novo methylation of centromeric minor satellite repeats (Okano et al. 1999). In view of the lack of any evidence for sequence specificity by de novo methyltransferase enzymes in vitro, it is tempting to speculate that in vivo, the targeting of Dnmt3a and Dnmt3b to specific loci may be regulated at the level of chromatin.

So what might determine the accessibility of these enzymes to their chromatin templates? A potential clue came from a genetic screen aiming to identify genes that are required for maintenance of normal cytosine methylation patterns in the flowering plant Arabidopsis thaliana. Mutation in a gene named DDM1 (decrease in DNA methylation) causes a 70% reduction of genomic cytosine methylation, mainly at repeated sequences (Vongs et al. 1993). The DNA within the centromeres of Arabidopsis has a structural role, but also encodes several genes (Tabata et al. 2000). It is possible that the phenotypic defect of ddm1 mutants is caused by activation of cryptic heterochromatic genes and transposons induced by loss of DNA methylation (Jeddloeh et al. 1999). There is a progressive effect of ddm1 on low copy sequences leading to loss of cytosine methylation over multiple generations, which suggests that ddm1 mutations impair the efficiency of DNA methylation after replication (Kakutani et al. 1996). DDM1 is not a methyltransferase. Instead, it encodes a member of the SNF2-like helicase subfamily, many members of which are
able to disrupt histone–DNA interactions [Jeddeloh et al. 1999]. Another putative family member, ATRX, is localized to pericentromeric heterochromatin in human and mouse [McDowell et al. 1999]. Mutations in ATRX give rise to developmental abnormalities and α-thalassaemia, which are accompanied by changes in the pattern of methylation of several highly repeated sequences including the rDNA arrays, a Y-specific satellite, and subtelomeric repeats. This circumstantial evidence suggests that ATRX, like DDM1, may act as a transcriptional regulator through an effect on chromatin configuration and DNA methylation.

Further support for this type of mechanism comes from a recent paper by Dennis and colleagues [Dennis et al. 2001], which describes a new regulator of global DNA methylation levels in mice, lymphoid specific helicase (Lsh). Previous work showed that Lsh, also known as Proliferation-Associated SNF2-like Gene [PAGS], is expressed ubiquitously in fetal mouse tissues and is linked with cell proliferation [Raabe et al. 2001], as well as being essential in postnatal murine development [Geiman and Muegge 2000; Geiman et al. 2001]. Lsh−/− mice die within a few hours after birth with reduced body weight and a pathology suggesting that renal failure may be the direct cause of mortality. On the basis of the high level of identity between Lsh and DDM1 [see Fig. 1], the authors examined global methylation levels in fetal and newborn mice. Lsh−/− mice turned out to have reduced levels of global cytosine methylation [50–60% of wild-type levels] in the absence of any changes in the levels or activity of Dnmt1, Dnmt3a, and Dnmt3b. In particular, the authors found that methylation was much reduced at both satellite DNA and many dispersed repetitive sequences. The effect of the Lsh mutation on the methylation status of low copy sequences was more variable and stage-specific. The imprinted H19 gene, but not the Igf2r locus, was hypomethylated compared to control embryos. β-globin, Pkg1, and Pkg2 genes were hypomethylated in day 13.5 Lsh−/− embryos but not in newborn Lsh−/− mice. It is possible that Lsh, like ATRX, is targeted to specific regions during development. In this respect, it will be of interest to determine whether there are associated changes in transcription from sequences that are normally repressed by DNA methylation in the Lsh−/− mutants. As the authors point out, neither loss of methylation at repetitive sequences nor at the imprinted genes observed in Lsh−/− mice seems to be essential for prenatal development.

This raises the question, what causes the early embryonic lethality of both Dnmt1- and Dnmt3b-deficient mice? One possibility is that Lsh-deficient mice show hypomethylation at a later stage of development than mice lacking maintenance and de novo methyltransferases. It is also worth noting that loss of Dmnt1 function in mice and toad blastocysts affects the ability of embryonic cells to differentiate properly, which can result in programmed cell death [Jackson-Grusby et al. 2001; Stancheva et al. 2001]. The apoptotic phenotype is critically dependent on the level of hypomethylation, as toad blastocysts that have a 50% depletion in DNA methylation levels appear to develop normally [Stancheva and Meehan 2000]. A reduction in DNA methylation below this threshold results in premature gene activation and phenotypic abnormalities. It is possible that the degree of hypomethylation occurring in Dmnt1 and Lsh mutants distinguishes their different phenotypes.

Is Lsh involved in remodeling heterochromatin?

That the loss of a single protein should have such a dramatic impact on DNA methylation levels is all the more remarkable because in Arabidopsis and mice, DNA methyltransferase activity in itself is unaffected in ddm1/Lsh mutant extracts [Kakutani et al. 1995; Dennis et al. 2001]. In addition, the primary amino acid sequence of Lsh/DDM1 does not offer any indication of an association [either by homology or conserved domains] with the methyltransferase activity. This points to an indirect effect of Lsh on DNA methylation.

Based on amino acid sequence, Lsh is [like DDM1] most closely related in its seven conserved ATPase/helicase motifs to the SNF2 subfamily [Geiman et al. 2001]. Most members of the SNF2 family of proteins appear to have the capacity to alter chromatin structure. Central to this activity is their DNA-dependent ATPase domain [for review, see Kingston and Narlikar 1999; Flaus and
None of the SNF2 proteins have been shown to function as helicases in vitro, but these domains are capable of inducing superhelical torsion in DNA or chromatin (Havas et al. 2000; Flaus and Owen-Hughes 2001). Chromatin remodeling can be targeted to promoters or other specific regions of the genome (Kingston and Narlikar 1999; Varga-Weisz 2001). Targeted chromatin disruption at promoters can occur on a background of more long-range effects in the flanking nucleosomal array (Fleming and Pennings 2001).

The first SNF2 ATPases to be characterized, such as SWI2/SNF2, were identified as transcriptional coactivators, which enhanced access to transcription-factor-binding sites in nucleosomal DNA. This association with active gene promoters would be difficult to reconcile with the proposed role of Lsh in regulating methylation of DNA at repressed loci. However, more recent genome-wide studies have revealed that several SNF2 family members can function both as positive and negative regulators of gene expression (Varga-Weisz 2001; Flaus and Owen-Hughes 2001). Furthermore, nucleosome remodeling activities can be expected to enhance the ability of a wide range of proteins involved in DNA metabolism to recognize their substrates within the context of chromatin (Flanagan and Peterson 1999). Therefore, it is plausible to conclude that Lsh may provide access for the cytosine methyltransferases to nucleosomal DNA.

SNF2-like proteins are classified into three subfamilies according to the similarity of their ATPase catalytic domain to either yeast SWI2/SNF2, mammalian Mi-2/CHD, or Drosophila ISWI (Kingston and Narlikar 1999; Varga-Weisz 2001). Close inspection of the homologs of Lsh and DDM1 found in the database suggests that these novel SNF2-like proteins may be most related to the ISWI subfamily of chromatin remodeling ATPases (Fig. 1). This may be functionally relevant because there are differences in catalytic activities between the ATPase subfamilies and their associated complexes. However, Lsh and DDM1 do not have the C-terminal SANT domain typical for the ISWI family (Varga-Weisz 2001). The recently isolated human proliferation factor PASC fits the same pattern. Lsh-homologous genes appear to be present in most vertebrates and also in yeast (Geiman et al. 1998). One candidate is the as-yet uncharacterized yeast ORF YFR038w, which is more similar to Lsh/DDM1 than to ISWI ATPases (Fig. 1). Therefore, although Lsh and DDM1 (but not ATRX) are related to ISWI within specific portions of the putative helicase region, they appear to be part of a subgroup that is more similar throughout and outside of this region.

The SNF2-like ATPases have catalytic activities that can function independently in vitro, but in vivo they are mostly found associated with large multisubunit complexes (for review, see Kingston and Narlikar 1999). For example, biochemical analysis has shown that Drosophila ISWI protein is found in the NURF, ACF, and CHRAC complexes, whereas the yeast ISWI and ISW2 ATPases each form separate assemblies (Tsukiyama et al. 1999; Varga-Weisz 2001). Unlike the SWI2/SNF2 ATPases, which are stimulated by either free DNA or nucleosomal DNA, ISWI and CHD are strictly nucleosome-stimulated, and ISWI additionally requires the presence of histone tails for its ATPase activity. The ISWI activity can disrupt nucleosomes, promote access to enzymes, as well as space nucleosomal arrays in vitro. These capacities may vary among specific complexes. For instance, NURF has no nucleosome spacing activity, whereas the ISW2 complex does not disrupt nucleosomes. In vitro, NURF and ACF were initially found to promote access of transcription factors to chromatin (Kingston and Narlikar 1999). Nevertheless, some complexes of the ISWI subfamily are involved in repression in vivo. This role in transcriptional repression became obvious as more genes under ISWI control were identified (Goldmark et al. 2000, Fazzio et al. 2001). Also, ISWI protein does not colocalize with RNA Pol II on Drosophila polytene chromosomes (Deuring et al. 2000), suggesting that in its most abundant complex forms, it is associated with inactive chromatin. These ISWI complexes may have a global role, either in reverting epigenetic marks by remodeling chromosomes, or in resetting the chromatin structure to a repressed ground state (Varga-Weisz 2001). In addition, ACF and CHRAC are targeted to heterochromatin and are proposed to have a role in setting up its very regular nucleosomal spacing, which is linked to its silencing capacity (Sun et al. 2001). Mutations in ISWI cause alterations in the structure of the Drosophila male X chromosome, which is further evidence of its role in higher-order chromatin formation. Viewed in all its complex formations, ISWI is concentrated in heterochromatin but is also present on the euchromatic arms of mitotic chromosomes (Deuring et al. 2000). This leaves open a role for alternative ISWI complexes in transcription activation. ISWI complexes are active at a very low complex to nucleosome ratio and are unlikely to be a structural component of higher-order chromatin (Tsukiyama et al. 1999). In Drosophila, ISWI is essential for either cell viability or division during development (Deuring et al. 2000).

Lsh was originally found to be expressed only in lymphoid tissue in adult mice [Jarvis et al. 1996]. This may have been indicative of the proliferating nature of lymphoid cells rather than tissue specificity, as expression is nearly ubiquitous in the developing mouse embryo (Geiman et al. 2001; Raabe et al. 2001). Therefore, Lsh may be a proliferation factor like its highly homologous human counterpart PASC, which is associated with dividing cells. In addition, Lsh is primarily expressed in the S-phase of the cell cycle (Geiman and Muegge 2000), when the histones are synthesized for chromatin replication. The relatively undisrupted development of Lsh−/− embryos suggests that Lsh is not essential for chromatin replication itself. This raises the possibility that Lsh could be involved in spacing the newly replicated and assembled nucleosomes in an ISWI-like way [Demeret et al. 2001]. As a consequence, Lsh could provide a means of enabling cytosine methyltransferases access to nucleosomal DNA, coupling methylation to replication. The reduced levels of DNA methylation in Lsh−/− mutants indicate that methylation must be either coin-
cident with or downstream of Lsh action. Consistent with this, the levels of cytosine methylation observed in replicated and genomic DNA are equivalent, which suggests that DNA replication and methylation are simultaneous events [Araujo et al. 1998].

The nucleosome spacing activity of ISWI-like chromatin remodeling proteins depends on an ATP-dependent mobilization of nucleosomes on DNA [Flaus and Owen-Hughes 2001]. Enhancement of nucleosome mobility promotes a state of chromatin fluidity, which, at least in vitro, facilitates access of enzymes to DNA [Kingston and Narlikar 1999]. This suggests possible dual roles for the chromatin-remodeling activities of Lsh/DDM1: to space newly replicated nucleosomes into the regular arrays that promote the heterochromatin structure, while giving access to the DNA methyltransferases to produce the methylated DNA signature of repressed chromatin.

Does remodeling by Lsh lead to histone modification?

Obviously, it is important to know the dynamics of methylation changes during development of Lsh−/− mice in comparison to wild-type animals. In addition, it remains to be determined whether de novo methylation of invading viral sequences is impaired as in [Dnmt3a+/−, Dnmt3b−/−] double-mutant embryonic stem cells. It is worth noting that Dnmt1 has been shown to interact with the histone deacetylases HDAC 1 and HDAC 2. During late S-phase, HDAC2 colocalizes with Dnmt1 at heterochromatin, which may provide a link between histone deacetylation and chromatin remodeling following replication [Rountree et al. 2000]. Dnmt3a and Dnmt3b can repress transcription in a methylation-independent manner via a plant-like, cystein-rich, homeodomain-like motif that is shared with the ATRX protein but is not present in Dnmt1 [Bachman et al. 2001; Fuks et al. 2001]. This repression by Dnmt3a and Dnmt3b is also partially dependent on HDAC activity. Because Lsh protein expression correlates with DNA synthesis, it may facilitate the access of DNA methyltransferase complexes to newly synthesized hemimethylated or unmethylated DNA [Fig. 2]. All three methyltransferases show distinct localization differences within the nucleus and the nuclear complexes they associate with during the cell cycle [Leonhardt et al. 1992; Bachman et al. 2001]. Dnmt1 targets only to DNA replication foci, consistent with its coupling of DNA synthesis with the maintenance of DNA methylation and chromatin states. Depending on the cell type, Dnmt3a and Dnmt3b can be found in foci containing heterochromatin. It may be that these proteins, in conjunction with methyl-CpG repressor proteins (MeCP1, etc.) and other heterochromatin-associated proteins help to maintain pericentromeric heterochromatin silencing throughout different cell and developmental stages [Bird and Wolffe 1999]. In essence, a picture is emerging whereby chromatin-silencing mechanisms may be paramount and required to facilitate additional silencing provided by DNA methylation via methyl-CpG repressor proteins. It may be difficult to disentangle the mutual reinforcement of repression provided by chromatin and DNA modification especially because the MeCP1 repressor complex, which contains

**Figure 2.** A speculative model for Lsh function in animal nuclei. In wild-type mice (Lsh+/+), the remodeling activity of Lsh is hypothesized to be necessary for de novo methylation of acetylated (yellow dots on histone octamers) chromatin after DNA replication. This allows access by the DNA methyltransferases (Dnmt) and their associated histone deacetylase activities (HDAC) and leads to the formation of deacetylated chromatin wrapped up in methylated (red dots on DNA) DNA (heterochromatin). In mutant mice (Lsh−/−), in the absence of Lsh, the de novo (and perhaps maintenance) methyltransferase activities are unable to methylate CpGs on DNA that is assembled into nucleosomes. This gives rise to hypomethylated DNA and hyperacetylated chromatin.
Mi-2, can preferentially bind, remodel, and deacetylate nucleosomes containing methylated DNA (Feng and Zhang 2001). The histones themselves are subject to different modifications including acetylation, phosphorylation, and methylation of N-terminal tails (Jenuwein and Allis 2001). Because methylation of lysine 9 on histone H3 is associated with the formation of repressive chromatin structures (via an interaction with HP1 proteins), it is possible that there are further links between DNA and histone methylation. As HDACs have been linked to the DNA methyltransferases, it would also be of interest to determine whether histone acetylation and methylation levels are altered on a global level and at specific chromatin regions in Lsh−/− mice. In this context, an investigation of the putative Lsh protein complex may be a productive pastime.

**Conclusion**

In summary, these recent exciting findings in plants and animals imply that the levels of DNA methylation can be regulated and altered via changes in chromatin conformation. They also raise a possibility that chromatin-remodeling proteins may be important components of the so long sought DNA demethylation activities, provided that their access to DNA is regulated in a developmental or in a cell-type-specific context (Kress et al. 2001). Perhaps Lsh, as well as other yet unidentified proteins of the same family, are necessary for the maintenance of stable epigenetic states in mammalian cells over many cell generations. However, a number of additional issues arise, in particular whether SNF2/ISWI family members are required for the action of only de novo or both types of DNA methyltransferases and whether their function is essential primarily for maintenance of heterochromatin stability at repeated genomic sequences. Although the loci analyzed by Dennis and colleagues (2001) suggest that both Dnmt1 and Dnmt3 methyltransferase activities may be affected by loss of Lsh, further experiments are required to address these questions in vivo and in vitro.

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