Meeting report An epigenetic role for noncoding RNAs and intragenic DNA methylation James M Flanagan and Laurence Wild

Address: Cancer Research UK Viral Oncology Group, Wolfson Institute for Biomedical Research, Gower Street, University College London, London WC1E 6BT, UK.

Correspondence: James M Flanagan. Email: j.flanagan@ucl.ac.uk

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A report on the Keystone Symposium 'Epigenetics: Regulation of Chromatin Structure in Development and Disease', Breckenridge, USA, 11-16 April 2007.

Epigenetics, which is the study of the information carried by the genome that is not coded by DNA, is a rapidly expanding field of study. A recent Keystone symposium on the regulation of chromatin structure in development covered chromatin structure, epigenetic memory mediated by the binding of Polycomb group proteins and the hot topic of the role of noncoding RNAs (ncRNAs) in heterochromatin formation, DNA methylation targeting and gene silencing. Here we focus on presentations that discussed the role of ncRNAs in epigenetic regulation and the importance of intragenic methylation in regulating transcription.

Noncoding RNAs in chromatin formation

One of the biggest questions in chromatin biology is how the functional state of chromatin is established and maintained, and there is now evidence that ncRNAs may be involved. The role of an RNA interference (RNAi)-type mechanism in heterochromatin formation was discussed by Danesh Moazed (Harvard Medical School, Boston, USA), who showed that known components of RNAi participate directly in heterochromatin formation in the yeast Schizosaccharomyces pombe. He showed that transcripts from a transgene inserted into a centromeric repeat gets processed into small interfering RNAs (siRNAs) and bind to the argonaute protein Ago1 and that this is a requirement for efficient heterochromatic silencing. He proposed that the nascent RNA transcripts from centromeric repeats may act as a platform for heterochromatin assembly and these heterochromatic transcripts are targeted to the exosome and the RNAi pathway for degradation, in contrast to euchromatic transcripts, which are translated into proteins. Working with *Tetrahymena*, Yifan Liu (Rockefeller University, New York, USA) showed that methylation of lysine 27 on histone H₃ (H₃K₂₇), a mark of repressive heterochromatin, is dependent on the RNAi machinery, as H₃K₂₇ methylation is abolished in deletion mutants of the RNA processors dicer-like 1 (*DCL1*) and the piwi homolog (*TWI1*) or the histone methyltransferase *EZL1*. This indicates that ncRNAs may mediate the heritability of histone modifications and heterochromatin formation, although how they achieve this is still unknown.

The Xist ncRNA is thought to be instrumental in the cascade of events that silence genes on one randomly chosen X chromosome in female mammals. Sundeep Kalantry (University of North Carolina, Chapel Hill, USA) described studies analyzing the requirement for Xist in the initiation of imprinted X-chromosome inactivation, which occurs during pre-implantation development in mice. Using a GFP transgene on the paternal X chromosome Kalantry showed that Xist mutant embryos could still initiate stable imprinted X inactivation and that the polycomb group protein Eed did not accumulate on the mutant chromosome, suggesting that X inactivation is initiated via an Xist-RNA independent mechanism. The choice of X chromosome for inactivation may not be as random as previously thought, according to Barbara Panning (University of California, San Francisco, USA), who suggested that the choice has already been made in the embryonic stem cells (the cells of the inner cell mass) and that the future inactive X may be marked by Polycomb group proteins such as Eed. Panning used fluorescence in situ hybridization to visualize the X-inactivation centre (Xic) in paraformaldehyde-fixed female stem cells to show that a high proportion of cells showed a single hybridization at one allele and a double hybridization at the other allele (SD).

Upon differentiation, the 'single' allele cohybridized with the Xist ncRNA and it was the switching between these single and double states that underlies the random X inactivation. It seems reasonable to assume that other ncRNAs may act like Xist, in this case binding to autosomes to inhibit transcription. Indeed, Takashi Nagano (Babraham Institute, Cambridge, UK) showed that the Air ncRNA, which is an antisense transcript from the imprinted Iqf2r locus, interacts with specific regions within the imprinted cluster comprising the Igf2r, Slc22a2 and Slc22a3 genes. He showed that Air RNA interacts with the paternally imprinted Slc22a3 promoter in the placenta of embryonic mice at 11.5 days gestation, which correlates with the silencing of this gene, while at 15.5 days this interaction decreases, concomitant with gene activation. The mechanism by which Air achieves silencing is unclear, but it may involve restricting the nucleosomal mobility of the locus, thus preventing contact with transcription factors, or it may recruit other chromatin-modifying repressors.

John Rinn (Stanford University, Stanford, USA) presented his recent work with the catch phrase "a fibroblast is not just a fibroblast". When he and his colleagues investigated the expression of the HOX gene cluster in fibroblasts isolated from different parts of the human body they discovered that each fibroblast has a 'postcode' written into it by the expression pattern of the HOX cluster ncRNAs. Rinn can confidently describe the anatomical location of a fibroblast on the basis of the expression pattern of as few as four HOX genes, suggesting that adult fibroblasts do systematically retain the embryonic gene-expression patterns that relate to different positions along the embryonic developmental axis. Interestingly, the diametrically opposite domains of heterochromatin and euchromatin within the Hox gene cluster are bordered by the gene for an ncRNA, called HOTAIR, which physically interacts with the Polycomb group protein complex PRC2 and is involved in suppressing transcription of the *HOXD* locus. It is not yet clear whether the differences in HOX gene expression between fibroblasts reflect the microenvironment in which the cells reside, or if they result in phenotypic differences; however, the implications are that other cells in the body may also show similar anatomical differences in gene expression.

Robert Kingston (Massachusetts General Hospital, Boston, USA) addressed the significant question of what makes gene-repressive states heritable. Kingston has identified around 85,000 small RNAs (29-30 bp) from rat testis that associate with the Argonaute protein RIWI (the rat homolog of *Drosophila* PIWI) and have thus been designated PIWIassociated RNAs (piRNAs). The genes encoding piRNAs are typically located in conserved clusters and are expressed as large transcripts from bidirectional promoters. As piRNAs are not expressed in any cells other than the male germline, they are thought to play a role in heritable gene silencing in the male germline, possibly by guiding the cleavage of repetitive transposon transcripts and inhibiting transposition. Sarah Elgin (Washington University, St Louis, USA) reported work investigating the role of ncRNAs in *Drosophila* by looking at position-effect variegation. She and her colleagues have found that mutations in the RNA processing proteins piwi, aubergine and homeless all result in a loss of silencing. Elgin reported that PIWI binds specifically to the heterochromatin protein HP1a in a yeast two-hybrid assay, and that a mutant transgene disrupting this interaction fails to support gene silencing.

It is apparent that ncRNAs and RNAi components play a significant role in gene silencing, but key questions remain. Do the findings in model organisms hold true for humans? Almost 50% of the human genome is composed of transposon sequences and ncRNAs transcribed from these elements might have a significant role in gene regulation. *Xist* can cover the entire inactive X and *Air* ncRNA induces silencing of a 500 kb imprinted locus. Thus, if ncRNAs can silence chromatin locally, how far along a chromosome can they extend and can other gene regions be silenced in a similar manner? Answers to these questions may shed light on how ubiquitous RNAi-mediated gene silencing really is.

Intragenic DNA methylation

While the effects of promoter methylation on chromatin configuration and gene transcription have been well documented, several groups have recently turned their attention to DNA methylation over the rest of the gene (gene-body methylation). Both Steve Jacobsen (Howard Hughes Medical Institute, University of California, Los Angeles, USA) and Steven Henikoff (Fred Hutchinson Cancer Research Center, Seattle, USA) reported chromatin immunoprecipitation and DNA microarray (ChIP-on-chip) studies of Arabidopsis showing that DNA methylation tends to lie within the body of the gene and not at the 5' or 3' ends. Rob Martienssen (Cold Spring Harbor Laboratory, Cold Spring Harbor, USA) noted a similar trend, adding that transposable elements are more often methylated throughout. The role of RNAi components in heterochromatin formation may also be linked to gene-body methylation, as Martienssen shows that the RNAi components are also essential for DNA methylation of transposons in Arabidopsis. In humans, the role of RNAi components in heterochromatin formation may also be linked to gene body methylation as human genes contain transposons within intronic sequences.

There is a strong relationship between gene methylation and transcription. From gene-expression analysis of *met1* DNA methyltransferase mutants, Henikoff concluded that genebody methylation impedes transcriptional elongation and is most deleterious in short genes, where methylation extends further into the 5' and 3' regions. Interestingly, moderately expressed genes showed the highest levels of gene methylation. Henikoff proposes a mechanism whereby intragenic

methylation is caused by siRNAs aberrantly transcribed from cryptic start sites that are exposed in the gene body as chromatin structure is disrupted by the passage of RNA polymerase II. Methylation is less pronounced in highly expressed genes because the frequent passage of the polymerase disrupts the production of these aberrant transcripts. Conversely, in weakly expressed genes, methylation is less pronounced as the chromatin is too condensed to allow initiation from cryptic start sites. This may provide an interesting mechanism for how a small change in gene transcription could instigate a feedback loop leading to further methylation and gene silencing.

An interesting experiment was described by Matthew Lorincz (University of British Columbia, Vancouver, Canada) who investigated the effects of intragenic DNA methylation on transcription. He targeted reporter constructs to a specific intergenic locus in murine erythroleukemia cells. The reporters were either unmethylated or in vitro 'patch' methylated to 1 kb downstream of the transcription start sites of the reporter genes. Whereas the unmethylated reporters were consistently highly transcribed, a subset of clones of the methylated reporters in which the methylation had spread to within 300 bp of the transcription start site showed drastically reduced transcription. Furthermore, enrichment of H3K9 methylation in downstream methylated regions, and altered upstream nucleosome positioning and decreased H3K9/K14 acetylation in the promoters of the methylated cassettes were observed.

Jeff Jeddeloh (Orion Genomics, St Louis, USA) concluded from his methylation profiling of breast cancer that there are at least two epigenetic types (epitypes) of this disease. One, representing about 90% of breast cancer cases, was detectable with Orion's biomarkers, which pick up changes in the methylation status of particular genes; however, none of their biomarkers detected the remaining 10% of cases. Many of the changes specific to breast cancer were hypermethylation (increased methylation) occurring not only in promoters but also within the gene body. Surprisingly, regions of the genome that did not include CpG islands had substantial clinical power as DNA methylation biomarkers. In the case of both lung and ovarian cancers, biomarkers representing hypomethylation (a decrease in methylation compared to normal) had considerable discriminatory ability. There was very little overlap in the biomarker lists (before or after validation) for these three cancers, and so it is likely that each has disease-specific epigenetic pathways.

In humans, gene-body methylation may be associated with the presence of repetitive transposon DNA sequences within introns. Loss of methylation in these regions is likely to permit transcription of the transposon sequences and possibly allow the expression of cryptic transcripts or antisense transcripts that might promote RNAi-mediated gene silencing. It is possible that a loss of gene-body methylation could be more common than hypermethylation in cancers, given that there is a known overwhelming loss of methylcytosine in cancer cell DNA. This will be an interesting avenue for future research, and may also challenge the paradigm that hypomethylation in cancer is only associated with the activation of oncogenes. It is clear that gene-body DNA methylation and ncRNA-mediated silencing are closely related and it will be important to explore the silencing role of gene-body-methylation further.

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