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Epigenetic memory and parliamentary privilege combine to evoke discussions on inheritance

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Summary

Understanding the basis of epigenetic memory is a fast-moving challenge in modern biology. At a recent Company of Biologists Workshop held at Steyning's historic Wiston House, thirty researchers led by John Gurdon interrogated three central questions: how are cell type-specific programs generated, what mechanisms duplicate this programmatic information as cells divide, and how does epigenetics contribute to trans-generational inheritance? We report some of the emerging themes arising from this debate.

Key words: Chromatin, Mitosis, Epigenetics, Gene expression

Introduction

For more than a century it has been known that 'heritable traits', or gene expression patterns, are faithfully transmitted from mother to daughter cells as they divide. For much of this time, we have also known that multicellular organisms generally develop from a single fertilised egg without significant change to, or loss of, the underlying genetic material. The fact that the DNA polymer genome within each cell is identical, despite the cells having discrete functions, has two important implications: first, that development has an indisputable epigenetic component that allows subsets of the genome's repertoire to be harnessed by different tissues; and second, that individual terminally differentiated cells can be repurposed to generate an entire organism if complete epigenetic reprogramming is achieved. Since much of our current thinking relies upon these important early reprogramming experiments, it was fortunate that John Gurdon, a pioneer of nuclear transfer in *Xenopus*, and Helen Blau, a leading exponent of cell fusion-based reprogramming, teamed up to convene this interesting workshop on epigenetic memory, with help from Steven Henikoff and Wolf Reik. The meeting was based at Wiston House, Steyning, UK, the ancestral home of Sir Thomas Shirley (1542-1612), a member of parliament for Sussex, persistent debtor and occasional jailbird, who famously evoked parliamentary privilege in order to avoid arrest – a showcase in Elizabethan times that still has significant ramifications centuries afterwards (House of Commons Journal, 1604).

John Gurdon (University of Cambridge, UK) introduced the meeting by reminding those assembled of the importance of distinguishing between simple models for the parental distribution of cellular components at division and epigenetic processes that either 'continuously reinstate' gene expression programs at successive rounds of cell division or that propagate cell memory by some intrinsic 'self-templating' mechanism (Fig. 1). Many different epigenetic processes are now known to be important for maintaining gene activity states through DNA synthesis and mitotic division. These include DNA methylation templated by the DNA methyltransferase DNMT1 and its associated tethering complex, which marks DNA synthesised during S phase, and Polycomb/Trithorax-mediated covalent modifications of histone tails that then solicit the binding of appropriate chromatin reader and templating complexes. Bookmarking factors, as exemplified by the double bromodomain-containing factor Brd4 that recognises acetylated chromatin, can also remain bound to mitotic chromatin and hence persist within postmitotic daughter cells (Zhao et al., 2011), so that similar patterns of gene expression can be re-established in the daughters.

Tackling the big questions

During an informal discussion session partway through the meeting, Anna Philpott (University of Cambridge, UK) bravely collected the participants' views on the major questions that face the epigenetics community. As the responses spanned three broad areas, Anna challenged the delegates to identify the most compelling question by contributing to a light-hearted (but none-the-less competitive) debate to rank their importance. As anticipated, unravelling the epigenetic basis of mitotic templating (Fig. 2A) received much support. This was closely followed by the need to understand how sequential gene expression is programmed during development and then erased by in vivo or experimental reprogramming (Fig. 2B). The third area to receive rigorous support was epigenetics in trans-generational inheritance, a subject that has been significantly advanced by recent studies in plants, worms and flies, but has remained stubbornly intractable in vertebrates (Fig. 2C). Reassuringly, most talks at the meeting tackled at least one of these questions!

The centromere as a model for mitotic memory

That both genetic (sequence-driven) and epigenetic (self-templating) mechanisms contribute to mitotic heritability is illustrated by studies on the centromere in different model organisms. The centromere is defined by the presence of a specialised nucleosome(s) incorporating the centromeric histone H3 variant Cse4/CenH3/CENPA. The architecture of the single centromeric nucleosome in budding yeast is uncertain and Steven Henikoff (Fred Hutchinson Cancer Research Center, Seattle, USA) and Carl Wu (Janelia Farm, Ashburn, USA) discussed data supporting opposing models: a hemisome comprising Cse4, H4, H2A and H2B and a tetrasome comprising two Cse4-H4 dimers. Although further experiments might be needed to resolve this key issue, the mechanism for positioning the centromeric nucleosome in budding yeast is in no doubt, being genetically defined via recruitment of the sequence-specific binding factors CBF1 and CBF3 to the CDE sequence element. By tagging Cse4 with a photoconvertible fluorochrome, Wu demonstrated that the

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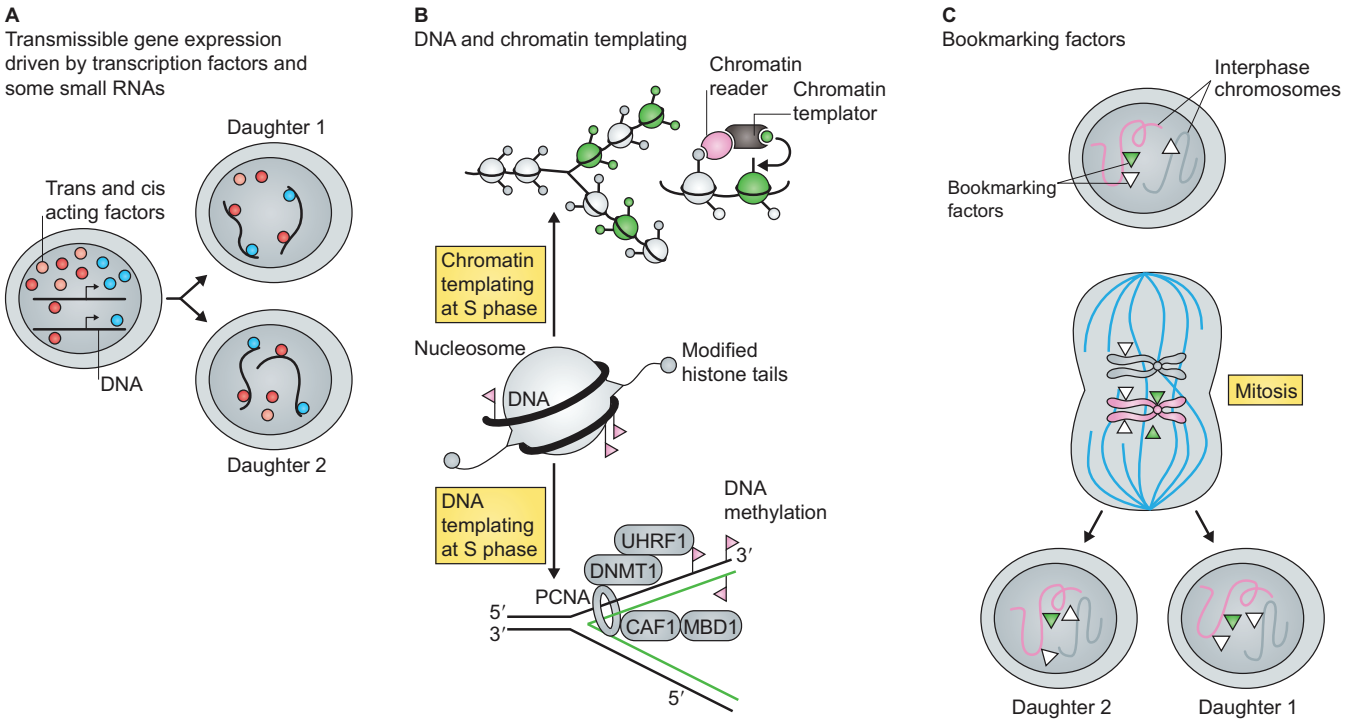

Fig. 1. Possible mechanisms for sustaining gene expression through cell division. (**A**) Gene expression in daughter cells is similar to that of the parent as cytosol containing transcriptional regulators and some small RNAs (red, orange and blue circles) is symmetrically partitioned during cell division. (**B**) Bipartite information contained within the nucleosome (middle panel) is copied during the DNA synthesis (S) phase of the cell cycle. Histone modifications (top panel) may be copied between adjacent nucleosomes using a chromatin reader-writer system (pink and black, respectively, top panel inset). New histones (green) and old histones (white) are incorporated and redistributed as the replication fork progresses (direction right to left). Elements crucial for templating DNA methylation at S phase are shown in the bottom panel. PCNA (grey ring) recruits chromatin-remodelling factors to the DNA replication fork (DNA polymerase, not shown), including chromatin assembly factor (CAF1) and methyl-CpG binding factor (MBD1). In the presence of DNA methylation (pink flags), the tethering factor UHRF1 and DNMT1 are recruited to the PCNA complex and methylate hemi-methylated CpG sites on daughter strands. (**C**) Chromatin adapter proteins (white and green triangles) that recognise features such as acetylated histones and remain bound to mitotic chromosomes, act as a 'genomic bookmarks' in division and accelerate postmitotic gene reactivation in daughter cells.

centromeric nucleosome is replaced in early S phase and then persists through the remainder of the cell cycle.

Sequential replacement of the budding yeast centromeric nucleosome is governed by the sequence-specific factors that bind to CDE elements and inheritance of the centromere is therefore genetically defined. In higher eukaryotes, studies on the formation and inheritance of neocentromeres have demonstrated that centromere location is determined primarily by epigenetic mechanisms rather than the underlying DNA sequence. Patrick Heun (Max Planck Institute, Freiburg, Germany) discussed work in *Drosophila* demonstrating that overexpression of the *Drosophila* centromeric H3 variant cenH3 in tissue culture cells leads to formation of heritable neocentromeres comprising centromeric nucleosome domains of ~200 kb that are usually located in silent intergenic chromatin (Olszak et al., 2011). In further experiments, LacO tethering of cenH3 was used to target neocentromeres to defined sites and it was found that LacO-cenH3-nucleated centromeres could incorporate cenH3 lacking the LacO binding domain, indicating that the neocentromeres were being autonomously maintained by epigenetic mechanisms (Mendiburo et al., 2011). These findings were further verified using a *Drosophila* artificial chromosome (DAC) system in which induction of a neocentromere by tethered cenH3 led to kinetochore formation and stable propagation of the DAC. The DAC was maintained by endogenous cenH3 following depletion of the tethering cenH3, confirming that this histone variant is sufficient for the epigenetic memory of centromeres.

Copying the template through mitosis – classic models with new twists

Kate Alexander from Maria Garcia-Garcia's laboratory (Cornell University, Ithaca, USA) updated the meeting on recent studies showing the importance of the transcriptional repressor TRIM28 for imprinted gene expression, work that was undertaken in collaboration with Mathieu Boulard and Timothy Bestor (Columbia University, New York, USA). Maternal TRIM28 had previously been shown to promote epigenetic stability during the mouse oocyte-to-embryo transition (Messerschmidt et al., 2012). However, using *chatwo*, a hypomorphic allele of *Trim28* (Shibata et al., 2011), this group has identified a novel molecular mechanism by which TRIM28 maintains genomic imprinting at later embryonic stages.

Howard Cedar (The Hebrew University of Jerusalem, Israel) described studies that aimed to understand how CpG islands are protected from DNA methylation at early stages of development (Straussman et al., 2009), a property that is somehow lost from somatic cells and ES cells following differentiation. In an ES cell model in which cells were transfected with a target CpG island that was either unmethylated or methylated, the unmethylated island was protected through multiple rounds of division. Cedar proposed

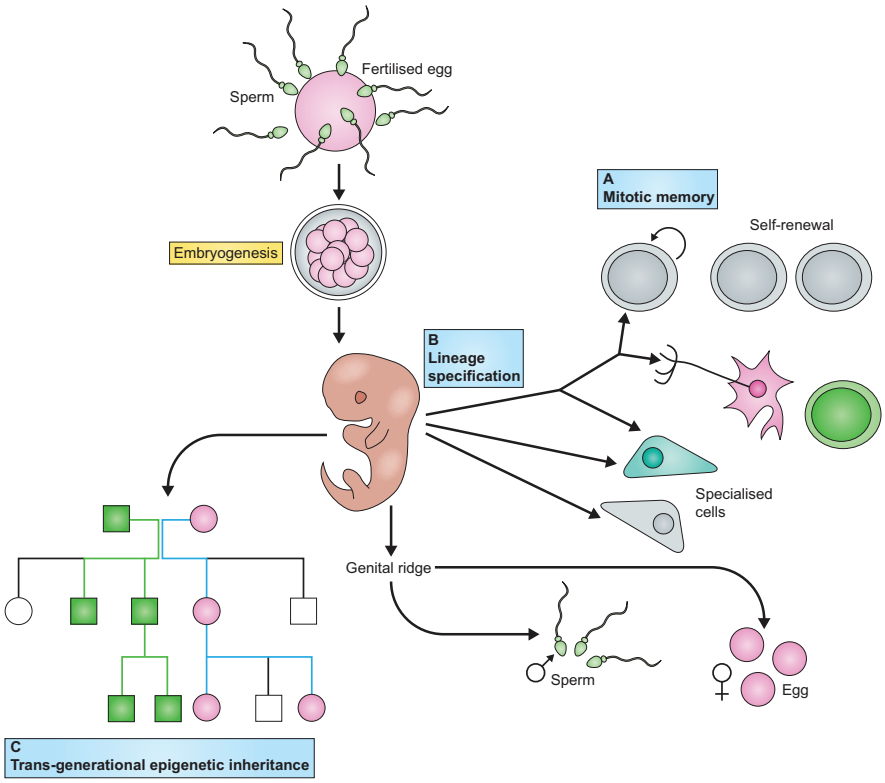

Fig. 2. Major questions in epigenetic memory. As well as understanding the mechanistic basis for 'mitotic memory', in which cellular identity is propagated through stem cell self renewal and somatic cell proliferation (**A**), meeting participants highlighted two other important epigenetic phenomena. The specification of different cell types (and transcriptional programs) from a fertilised egg (**B**) remains a classical epigenetic process in which the same genomic sequence is retained by the 200 or so different specialised cell types of the organism, and cell function is susceptible to experimental reprogramming. Specialised mammalian germ cells that originate from the genital ridge in developing embryos give rise to haploid sperm and egg. Understanding how epigenetics contributes to trans-generational epigenetic inheritance – in which parental experience is transmitted to successive generations of offspring (**C**), presumably through mechanisms that impact on their germ cells – remains a less well understood but important area of epigenetic research.

that this was dependent on the presence of a functioning transcriptional start site at which transcription factors and RNA polymerase II bound, creating a region rich in the histone modification H3K4me3 that prevented DNA methylation by allosteric inhibition of DNMT3 activity (Li et al., 2011; Zhang et al., 2010). Tethering experiments in which a reporter gene linked to Gal4 binding sites was used to recruit TATA-binding protein (TBP) support this model, as TBP recruitment reduced overall DNA methylation levels and enhanced protection of the transgene.

The Polycomb system is a key mediator of heritable gene silencing in multicellular organisms. The two major complexes PRC1 and PRC2 exert their effects at least in part by catalysing H2AK119 mono-ubiquitylation and H3K27 methylation, respectively. Renato Paro (ETH Zurich, Switzerland) had mapped PRC1 binding sites in *Drosophila* S2 cells and found that most correlated with the transcriptional start sites of coding genes. Moreover, the datasets revealed that PRC1 regulates and represses microRNA expression (Enderle et al., 2011). By mapping the binding sites of the molecular chaperone Heat shock protein 90 (Hsp90), Paro also showed Hsp90 binding near to the promoter of coding and non-coding genes, where it pauses RNA polymerase II by stabilising the negative elongation factor complex (NELF) (Sawarkar et al., 2012). As pausing is implicated in Polycomb-mediated gene regulation, these observations might be key for understanding the molecular basis of environmental stress responses that influence the outcome of future encounters.

Targeting of Polycomb repressors to defined loci in *Drosophila* depends at least in part on sequence-specific binding factors. In vertebrates, mechanisms for Polycomb recruitment remain relatively poorly understood. Polycomb occupancy has been found to map to unmethylated CpG islands at target loci and both Cedar and Rick Young (Whitehead Institute, Cambridge, USA) reported that Polycomb target loci in ES cells can be accurately predicted based on CpG island characteristics (AT richness/CpG island modulation). However, in common with earlier studies, it was not possible to identify specific sequence motifs. Picking up on this theme, Neil Brockdorff (University of Oxford, UK) discussed recent evidence demonstrating parallel H3K27me3-dependent and -independent pathways for PRC1 recruitment in vertebrates, highlighting that both pathways show a significant overlap in target sites, including the inactive X chromosome (Tavares et al., 2012). Based on this, he suggested that a single universal recruitment mechanism might operate for both pathways, and further speculated that Polycomb complexes could recognise a specific chromatin configuration at target sites, as determined by ZF-CXXC domain chromatin-modifying factors that bind to unmethylated CpGs (CpG islands) and by the absence of the transcriptional machinery.

Steven Henikoff described a novel method to tag histones and assess nucleosome dynamics at different sites in the genome. Fast dynamics were observed at Trithorax target loci (active promoter regions) and also at Polycomb targets. The fast turnover rates appear to rule out stability of histone modifications as responsible for epigenetic heritability at such loci, highlighting the need to develop new models to explain the stable propagation of histone modification signatures in dividing cells.

Single-molecule approaches and live cell imaging have been widely applied to document transcription, revealing that this is a largely discontinuous process in which transcripts are generated in bursts or pulses (Raj and van Oudenaarden, 2008; Muramoto et al., 2012). Using this approach, Jonathan Chubb (University College London, UK) monitored transcriptional events in individual *Dictyostelium* cells to find out whether transcriptional activity was inherited through mitosis. His data showed a strong correlation in the frequency of transcriptional firing between mother and daughter cells that persisted through multiple cell cycles. This 'memory' of

transcription pulse length and rate was disrupted by mutation of the Set1 H3K4 methylase and Ash2, another component of the methylase complex, and by targeted point mutation of an H3 variant genomic locus (Muramoto et al., 2010; Hsu et al., 2012). Chubb suggested that the loss of correlation in these mutants might be driven by an increase in transcriptional noise, rather than reflecting a loss of specific bookmarking processes.

Mitotic memory was also tackled by Ken Zaret (University of Pennsylvania, Philadelphia, USA), who showed examples of factors that remained bound to mitotic chromosomes (such as the Forkhead transcription factor FoxA), as well as those excluded from mitotic chromosomes (such as c-Myc and NF1) or subject to turnover during mitosis and partial binding (such as GATA4 and HMG1). FoxA1 can bind to both nucleosomes and DNA directly, and is therefore able to remain on the chromosome during mitosis. This allows FoxA to both 'bookmark' the genome and exert 'pioneer activity' in exposing the nucleosome and recruiting RNA polymerase later in telophase (Zaret and Carroll, 2011).

An alternative mechanism for mitotic heritability, the bookmarking of active genes via recruitment of SMC family chromosomal protein complexes (condensin and cohesin), was presented by Rick Young. A screen for factors important for maintaining pluripotency in ES cell cultures identified both condensin and cohesin, along with known players such as the transcription factor Oct4 (Pou5f1). Unexpectedly, two major condensin complexes, condensin I and II, which function in M phase and interphase, respectively, were found to occupy the promoters and enhancers of active genes and to be depleted in heterochromatin. Condensin II, like cohesin, is recruited by the loading factor Nipbl, which interacts with the mediator complex, a key modulator of RNA polymerase II activity. Young speculated that the SMC family proteins could play an important role in the heritability of active gene expression programs through DNA synthesis and mitosis.

Epigenetics in development and disease

The process of X chromosome inactivation has provided an important model for exploring epigenetic mechanisms that control gene silencing and reactivation (Gendrel and Heard, 2011). Edith Heard (Institut Curie, Paris, France) explained how undifferentiated female ES cells (which have two active X chromosomes) undergo a stepwise series of changes at a single X chromosome resulting in heritable silencing. These included Xist RNA binding, an early loss of RNA polymerase recruitment and euchromatic histone modifications, Polycomb repressor complex binding, locus repositioning into the Xist RNA domain and enhanced H3K9me2 and H4K20me1 histone methyltransferase (HMTase) activity, followed by macroH2A binding and increased DNA methylation. As removal of any one of these features, including loss of Xist from differentiated cells, does not normally result in escape from X chromosome inactivation, this implies multiple levels of epigenetic memory that are to some extent redundant. However, some X-linked genes can escape from chromosome-wide silencing and Heard demonstrated that this appears to be developmentally controlled, occurring only in certain lineages for specific genes. Cells of the embryo proper display very little escape from X-inactivation, whereas in some extra-embryonic tissues up to 40% of X-linked genes escape. Since DNA methylation is generally low at all gene promoter regions in these cells, the mechanism of their reactivation remains a puzzle. Heard also pointed to ongoing work on X-reactivation in cancer cells that might culminate in epigenetic biomarkers and the development of 'epi-drugs' capable of rerouting epigenetic silencing. This theme was taken up by Andy Bannister (University of Cambridge, UK), who described the establishment of a therapeutically important group of drugs that target the double bromodomain proteins Brd2, Brd3 and Brd4 (the so-called BET inhibitors) (Dawson et al., 2011), and showed new chromatin immunoprecipitation studies to map Jak2-mediated hyperphosphorylation of histone H3Y41 in human erythroleukaemia cells.

Epigenetic reprogramming – principles governing memory loss

Epigenetic reprogramming, whether induced in the course of normal development or artificially, has become an expanding area of interest within the scientific community. It was therefore not surprising that many of those attending this meeting were concerned with DNA demethylation mechanisms and the plethora of factors and mechanisms that could relieve DNA methylation.

The role of the growth arrest and DNA damage 45 (Gadd45) protein in DNA repair, unscheduled synthesis and its contribution in removing 5-methylcytosine residues from DNA was discussed by Christof Niehrs (Institute of Molecular Biology, Mainz, Germany). Niehrs explained that Gadd45 requires a co-factor to bind DNA and this protein has a PHD domain that recognises and binds H3K4me3. Gadd45 recruits nucleotide and/or base excision repair factors to gene-specific loci during cell differentiation and the stress response and provides a nexus between epigenetics and DNA repair (Niehrs and Schäfer, 2012). Helen Blau (Stanford University, USA) provided a historical perspective on the application of heterokaryon systems for understanding reprogramming, including evidence for activation of mammalian gene expression without DNA replication, before discussing her recent work on the role of the deaminase AID in DNA demethylation during reprogramming (Bhutani et al., 2010). Using an ES-based cell fusion strategy, Amanda Fisher (MRC Clinical Sciences Centre, London, UK) showed that the capacity of ES cells to reprogram somatic cells varied according to their cell cycle stage. Fisher presented evidence that DNA synthesis by somatic cells was required for reprogramming, contrasting with conclusions from prior work from the Blau laboratory. The reasons underlying this significant discrepancy remain to be determined. Wolf Reik (The Babraham Institute, Cambridge, UK) reported on genome-scale studies that showed that ES cells and most somatic cells have high levels of CpG DNA methylation (70-80% of CpGs were methylated), whereas levels in primordial germ cells (PGCs) were substantially lower (5%). This genome-wide erasure in PGCs occurred in multiple steps, with different genes 'losing' DNA methylation at different times in the developmental timecourse. Late demethylation characterised imprinted genes and some other classes of sequences. Interestingly, orphan CpG islands located close to intracisternal A-particle retrotransposons remained highly methylated throughout, and this resistance, Reik suggested, might provide an important pointer for understanding how trans-generational epigenetic inheritance operates.

Francesco Cambuli from Myriam Hemberger's laboratory (The Babraham Institute, Cambridge, UK) presented a comparative analysis of models of ES cell reprogramming to trophoblast stem (TS)-like cells – something that has proved a challenge in the field. This reprogramming can be initiated by activation of the RAS/ERK/Cdx2 pathway and repression of the transcription factor Oct4 (Lu et al., 2008). Cambuli described several experimental models in which Oct4, Cdx2 and RAS/ERK signalling can be

manipulated, and showed that reprogramming toward TS-like cells occurs in all models, albeit incompletely. Reprogramming is associated with activation of the TS cell lineage 'gatekeeper' Elf5 (Ng et al., 2008).

Atsuo Ogura (RIKEN BioResource Center, Tsukuba, Japan) discussed how ectopic activation of the *Xist* gene, the master regulator of X chromosome inactivation, makes a significant contribution to the inefficiency of reproductive cloning in mice. The single *Xist* allele is activated in XY donor somatic nuclei and both *Xist* alleles are activated in XX donor somatic nuclei, indicating that the host oocyte overcomes Xist repression in the somatic nucleus. Thus, the use of donor cells in which *Xist* is deleted results in dramatically improved cloning efficiency (Inoue et al., 2010).

Resistance to transcriptional reprogramming was also a theme in work discussed by Richard Halley-Stott from John Gurdon's laboratory (University of Cambridge, UK) using a system in which mouse nuclei are introduced into the germinal vesicle of *Xenopus laevis* oocytes. In this system, erasure of somatic cell epigenetic memory was inefficient at specific loci and the transcriptional memory of the donor mouse nuclei was not reversed by the transcriptional apparatus of the *Xenopus* oocyte. Furthermore, different genes seem to have different requirements. DNA methylation makes a relatively minor contribution to the observed resistance. Prior extraction of factors from the donor nuclei with high salt leads to improved reprogramming, suggesting that chromatin-binding factors mediate the resistance. Systematic analysis is now being performed to delineate the key factors underpinning resistance to reprogramming.

An epigenetic component in trans-generational inheritance

The mechanisms and players that mediate trans-generational epigenetic inheritance in mammals remain poorly understood, but recent studies in plants and worms are beginning to provide clues as to how they might operate. Alyson Ashe from Eric Miska's laboratory (University of Cambridge, UK) showed studies in *C. elegans*, an organism that lacks DNA methylation and is reliant on small RNA pathways to regulate its development and fertility (Bagijn et al., 2012). Through an EMS screen and candidate gene approach to identify factors necessary for trans-generational inheritance, Ashe and colleagues have shown that Piwi-interacting RNA (piRNA) can induce multi-generational silencing and that this is dependent on a core set of nuclear RNAi and chromatin factors: a germline-specific nuclear argonaute (HRDE1/WAGO-9), an HP1 orthologue (HPL-2) and two putative HMTases (SET-25 and SET-32) (Ashe et al., 2012).

Manoj Kumar from Phil Wigge's laboratory (Sainsbury Laboratory, Cambridge, UK) is interested in understanding how temperature is sensed and how this information is integrated in *Arabidopsis* development and flowering, a classic model of trans-generational inheritance. Previously, using a forward genetic screen the Wigge laboratory had shown that nucleosomes containing the alternative histone H2A.Z are important for accurate perception of ambient temperature, such that genotypes that do not incorporate H2A.Z constitutively express a 'warm' transcriptome and phenocopy warm-grown plants (Kumar and Wigge, 2010). Nucleosomes containing H2A.Z showed altered DNA/nucleosome interactions suggesting that temperature might be 'perceived' by the chromatin-remodelling apparatus or by chromatin. Manoj Kumar now plans to apply high-throughput sequencing to identify mutations in a series of candidate lines with perturbed temperature sensing in order to pinpoint the components of this interesting thermosensory activation pathway (Kumar et al., 2012). Although other speakers – notably Wolf Reik and Renato Paro – did touch on the question of trans-generational inheritance, it is apparent that there is much still to be learned about the degree to which this occurs in different organisms, as well as its underlying mechanisms.

Conclusions

One of the highlights of this extremely enjoyable meeting was a perception that we need to move away from traditional 'non-genetic' definitions of epigenetics to those that take account of the impact of the underlying DNA sequence of the genome. Although epigenetic changes are clearly distinct from DNA mutation, DNA bases are the substrate on which chromatin is built and on which they depend, and where, at a certain level, specificity resides. Alternative definitions based around epigenetics being 'self-templating mechanisms that reinforce heritable gene expression patterns' will need to be developed if we are to allow the rapid and imaginative progress being made in this area to evolve without artificial boundaries being erected between the genetics and epigenetics communities.

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Competing interests statement

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